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Editorial

Preface

On behalf of the local organizing committee, it is my pleasure to welcome you to the Ninth International Equine Infectious Disease Conference. This international conference is one in a series focusing on equine infectious diseases which began in 1966 in Stresa, Italy. Other previous meetings included Paris, France - 1969 and 1972; Lyon, France - 1976; Lexington, Ky. - 1987; Cambridge, United Kingdom - 1991; Tokyo, Japan - 1994 and Dubai, United Arab Emirates - 1998. Since the last conference, we have seen the re-emergence of infectious diseases and parasitism as major health concerns for equine populations. We have also seen the emergence of new equine infectious diseases both here in the United States and abroad. This conference will provide a unique opportunity for equine scientists and veterinarians from around the world to meet and discuss recent advances and ongoing challenges in this field. In addition to recognizing the many authors that have contributed to this meeting, I would also like to thank our many sponsors. The Platinum sponsor for the conference is Pfizer Animal Health. Gold sponsors are Boehringer Ingelheim Vetmedica Inc., Coolmore America, Grayson-Jockey Club Research Foundation, IDEXX Laboratories, International Racehorse Transport, Merck Animal Health, Merial and VMRD, Inc. Silver sponsors are the American Association of Equine Practitioners Foundation, American Quarter Horse Association, Bayer HealthCare Animal Health, Butler Schein Animal Health, Darley, Equine Diagnostic Solutions LLC, Hagyard Equine Medical Institute, Juddmonte Farms, Mersant International Limited, Peden Bloodstock, Platinum Performance, Rood and Riddle Equine Hospital and Siena Farm LLC. Bronze sponsors are Castleton Lyons, Centaur, Equine Medical Associates, Equine Diagnostic Solutions LLC, Milburn Equine, Neogen Corporation, North American Equine Ranching Information Council, Platinum Performance, Inc and Virbac Animal Health. Other supporters include Gainesway Farm, Kentucky Thoroughbred Farm Managers' Club, Kentucky Thoroughbred Association/Kentucky Thoroughbred Owners and Breeders and WinStar Farm, LLC.

David W. Horohov, Chair
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Gluck Equine Research Center
Department of Veterinary Science
University of Kentucky
Lexington, KY

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9th ICEID Abstracts

9th International Conference on Equine Infectious Diseases

Emerging Diseases

A study of horses for methicillin-resistant *Staphylococcus aureus* after cleared wound infections

K. Bergström [1,2], B. Bengtsson [2], A. Nyman [2], and U. Grönlund Andersson [2]
[1] Faculty of Veterinary Medicine and Animal Husbandry, Swedish University of Agricultural Sciences, SE-750 07 Uppsala, Sweden, [2] Department of Animal Health and Antimicrobial Strategies, SVA, SE-751 89 Uppsala, Sweden

An outbreak of methicillin-resistant *Staphylococcus aureus* (MRSA) infection in horses in Sweden raised questions concerning the risk posed by horses to their surroundings outside hospital after MRSA-infected wounds have cleared. This initiated a longitudinal study of the effect of time on natural decolonization and sensitivity of different sampling sites for detection of MRSA. MRSA is notifiable in animals (Swedish legislation SJVFS 2007:90) and rare in horses within Sweden. The study, approved by the Ethical Committee on Animal Experiments, Uppsala, Sweden (C 309/8), continuously enrolled all horses notified with MRSA-infected wounds after the horse owner's informed consent was obtained (n=10). Study design: Repeated sampling at five anatomical sites (nostrils, corner of mouth, pastern, perineum, and previous infection site) six times at approximately monthly intervals and a seventh 6 to 12 months after the sixth sampling occasion, starting in October 2008. Day zero relative to the sampling was the date when each horse infection was detected. MRSA-specific enrichment broth and agar was used for culture. PCR-verified MRSA isolates (*mec*A- and *nuc* gene) were *spa*-typed. The effect of time on detecting MRSA was assessed by a multilevel mixed-effect logistic regression model. Sensitivity of sampling sites was calculated. Eight of 10 horses completed the sampling period and two were sampled two and six times, respectively. MRSA were detected in 16/333 (4.8%) samples and on 13/67 (19%) sampling occasions. Four horses tested negative throughout the study, two tested positive once, three twice and one five times. The probability of finding positive MRSA samples significantly decreased over time ($p < 0.01$), with the last finding 205 days after the infection had been diagnosed. The odds of testing positive after 250 days were 0.003 (95% CI 0.00004-0.21). The most sensitive sampling site was the nostrils; sensitivity 0.92 (95% CI: 0.64-1.00). All other sampling sites had a sensitivity of 0.08. The *spa*-types of the isolates corresponded to *spa*-types previously found in the infections of each case, t011 and t064.To our knowledge, this is the first study to examine the effect of time on natural decolonization of MRSA in horses after wound infections have cleared. Few previously infected horses were available, but the odds of testing positive were significantly declined over time and shows that MRSA most likely disappears naturally (or become undetectable). This and the fact that the nostrils were the most sensitive site for MRSA sampling is valuable data in risk assessments of previously MRSA- infected horses. The results should be generally applicable to horses in Sweden, or other low prevalence areas, infected with the MRSA *spa*-types present in the study. Studies in countries with higher prevalence might produce different results.

Methicillin-Resistant *Staphylococcus aureus* (MRSA) Surveillance in Horses at a Veterinary Teaching Hospital

J.W. Stull [1], D. Slavić [2], and J.S. Weese [1]
[1] Ontario Veterinary College, Guelph, Ontario, Canada, [2] Animal Health Laboratory, Guelph, Ontario, Canada

MRSA is an important equine and zoonotic pathogen, and transmission can occur within equine hospitals. Active MRSA surveillance is a critical component of MRSA control in human healthcare and can also be used in horses. MRSA screening of nasal swabs using selective enrichment culture is performed in horses admitted to the Ontario Veterinary College (OVC) on admission, every 7 days in hospital, and on discharge. Data from June 1, 2009 to April 30, 2011 were analysed. Cases were classified as community-associated (CA), community onset-hospital associated (CO-HA), hospital-associated (HA) or indeterminate (IN) using standard definitions. A subset of isolates was typed using *spa*

typing. During the study period there were 2235 admissions (1705 different horses), with 2783 MRSA surveillance samples obtained. Samples were collected from 1239/1277 (97%) of horses that were hospitalized for a duration of at least one day; 728 horses (59%) were screened according to protocol and 511 (41%) were screened, but less frequently than protocol. MRSA was isolated from 80 samples during 50 visits by 40 horses (first positive visits: CA–19; CO-HA–3; HA–17; IN–1; range 0-7 per month). Overall, 3.0% of horses were positive (monthly range 0-8.6%). The prevalence at admission (CA and CO-HA combined) was 1.7% (monthly range 0-7.1%). Three horses had MRSA infections at admission and all were also colonized. No HA clinical MRSA infections were identified. Sixteen (89%) isolates were *spa* type t064, an ST8 strain commonly found in horses. *spa* types t002 and t1779 were also identified. Results for first-time positive animals were available for only 7 (18%) prior to discharge. However 11 (28%) colonized horses were later re-admitted, and 7 were still colonized. Within 6 months of the first positive result, 5 owners of MRSA-positive horses brought 14 additional horses to the OVC (range 1-4). Three horses from one of these owners were also colonized. Compliance with MRSA surveillance was good. The prevalence of MRSA colonization varied throughout the study period; however, the 1.7% admission prevalence is similar to previous reports from this facility. The time required for culture hampers the effectiveness of screening as most horses were discharged by the time results were known. However, screening can identify subsequent high risk cases (e.g. previously positive horse, horse from a farm with a colonized horse) and enable the use of enhanced infection control practices while awaiting test results. Screening also allows for detection of changes in MRSA risk posed by the referral population and early identification of within hospital transmission. Ultimately, rapid testing is required to optimize MRSA screening in horses. At defined periods, MRSA colonization was relatively common among equine patients. Screening can be an effective tool to identify high risk cases and reduce transmission, especially if risk analysis incorporates positive results from previous visits and other horses on the farm.

Distribution of Equine Encephalosis Virus in Israel

K. Aharonson-Raz [1], E. Klement [1], V. Bumbarov [2], and A. Steinman [1]
[1] Koret School of Veterinary Medicine, The Hebrew University of Jerusalem, Rehovot, Israel, [2] Kimron Veterinary Institute, Bet Dagan, Israel

Equine encephalosis virus (EEV) was diagnosed in Israel for the first time in 2009 as a cause of febrile disease outbreak in horses throughout Israel. Until 2009, this Orbivirus was thought to be confined to South Africa. Phylogenetic analysis of segment 10 of the Israeli strain showed that it formed a new cluster and analysis of segment 2 showed 92% sequence identity to the South African serotype EEV-3, the reference isolate [1]. These findings raised an intriguing dilemma whether a recent invasion of a new strain of EEV into Israel has occurred, or whether this virus has long existed and evolved in this region, but was undiagnosed. Following the identification of EEV in Israel, our aim was to determine the factors associated with its distribution. A study was conducted between summer 2010 and spring 2011 to determine the prevalence and force of infection of EEV in the Israeli horse population. Serum neutralization assay (SN) was conducted on samples collected in summer 2010 from 220 horses located in 14 sentinel farms representing the distribution of horses throughout Israel. To evaluate incidence of exposure, serum samples were collected again from 149 out of the 220 horses during spring 2011 and seroconversion between the first and second collection was evaluated using the SN method. The results indicate that among the horses tested, most (141/220, 64%) had an initial positive antibody titer. Out of the horses that were naïve to the virus in the first collection (n=47) 14 have seroconverted in between the two sampling periods, demonstrating a high force of infection of 30%. In summer 2010, horses geographically distributed throughout Israel were shown to have previously encountered EEV, with 90/112 (80%) seroprevalence in the center of Israel and 33/73 (45%) and 19/35 (54%) in the northern and southern farms, respectively. Interestingly, there was no significant difference in the force of infection documented in the three regions. Mean age of EEV serologically positive horses was significantly higher than that of serologically negative horses (11.4yr Vr. 7.5yr, respectively). The findings of this study point to a wide geographical distribution of EEV through the north, center and even in the arid-south region of Israel. In addition, these results may suggest that a dynamic spread of the virus still exists in Israel. Both African horse sickness virus (AHSV) and EEV are orbiviruses transmitted by *Culicoides* spp. and prevalent in South Africa. The close relationship of EEV to AHSV, which has much more devastating effect on the horse population, warrants further investigation of risk factors associated with arrival, outbreak and distribution of such an orbivirus.

Reference

[1] Aharonson-Raz K, Steinman A, Bumbarov V, Maan S, Maan NS, Nomikou K, Batten C, Potgieter C, Gottlieb Y, Mertens P, Klement E. The isolation and characterization of Equine Encephalosis Virus in Israel. Emerging Infectious Diseases 2011;17:1883-6.

Increasing frequency of Methicillin-Resistant *Staphylococcus aureus* in horses infections

A. Léon [1], A.C. Appourchaux [1], N. Bourdon [2], K. Maillard [1], F. Guérin [3], and R. Leclercq [2]
[1] Frank Duncombe Laboratory, 14053 CAEN, France,
[2] Microbiology Laboratory, CHU Caen, 14033 CAEN, France,
[3] Microbiology Laboratory, Centre Hospitalier Mémorial de Saint-Lô, 50009 St Lô, France

Initially reported as a cause of nosocomial infection in humans, methicillin-resistant *Staphylococcus aureus* (MRSA) is increasingly reported in animals. Their role in MRSA infection in humans and the potential to become a reservoir of MRSA are now admitted. These resistance is due to the acquisition of the *mecA* gene, that encodes a protein

designated PBP2a, which present a very low affinity for beta-lactam antibiotics. The *mecA* gene is located on a mobile genetic element, named staphylococcal cassette chromosome *mec* (SCC*mec*) inserted in the *S. aureus* chromosome. In addition, MRSA are frequently resistant to a wide range of additional antimicrobial classes. From January 2007 to June 2011, 912 *S. aureus* isolates were obtained from various clinical samples. Identification of MRSA was confirmed by amplification of the *nuc* (specific of *S. aureus*) and *mecA* genes. MRSA strains were typed by pulsed field gel electrophoresis (PFGE) and by Multilocus Sequence Typing (MLST). SCC*mec* elements (types I to VI) and genes encoding virulence factors Panton-Valentine Leukocidin (PVL), Toxic Shock Staphylococcal Toxin (TSST1) and Enterotoxin A were characterized by using a PCR approach. Susceptibility testing of MRSA isolates was performed using the agar diffusion method and following drugs were tested: erythromycin (ERY), tetracycline (TET), streptomycin (STR), gentamicin (GEN), rifampin (RIF), cotrimoxazole (TMP-SULF), chloramphenicol (CHL) and ofloxacin (OFL). Genes conferring resistance to macrolides [*erm*(A), *erm*(B), *erm*(C)], gentamicin [*aac(6')-aph(2'')*], and tetracyclines [*tet*(M)] were amplified by PCR. Among the 912 *S. aureus* isolates between 2007 and 2011, 19 strains were characterized as MRSA. Their prevalence increased until 6% in 2010 and 4% in the first six months of 2011. 9 types could be distinguished by PFGE. MLST revealed that MRSA isolated belonged to two sequence types, ST8 and ST398. All ST398 MRSA (n=7) were detected in 2010 including two isolates originating from the same farm. These results suggested possible cross-transmission of MRSA. The isolates, except two, possessed SCC*mec* IV and did not harbor the genes for the virulence factors PVL, TSST1, and enterotoxin A. All isolates were multiply resistant to other antimicrobials. PCR experiments showed that resistance to quinolone was active efflux, while to gentamicin and tetracycline was due to the presence of the *aac(6')-aph(2'')* and *tet*(M)genes, respectively. The increase in the prevalence of MRSA in horse infections is related to the emergence and spread of the ST398 type. This type is known to cause outbreaks in horses, to be responsible of cross-colonization and eventually infect humans. Farm hygiene and antimicrobial use contribute to MRSA occurrence in animals. Hygiene measures should, therefore, be maintained or reinforced to limit the transmission of *S. aureus* between horses and also between horses and personnel.

Use of antimicrobials in Equine Practice across North America: a case-based survey

F. Sampieri [1], P.M. Dowling [1], C.R. Clark [2], K.R. Ball [1], J.M. Dhillon [1,2], and S. Gow [2,3]
[1] Department of Veterinary Biomedical Sciences, Western College of Veterinary Medicine, University of Saskatchewan, Saskatoon, SK, Canada, [2] Department of Large Animal Clinical Sciences, Western College of Veterinary Medicine, University of Saskatchewan, Saskatoon, SK, Canada, [3] Public Health Agency of Canada

In equine practice the initial choice of antimicrobial (AM) therapy is often empirical, as delaying treatment, while awaiting culture and susceptibility results, may result in severe consequences. Veterinarians base empirical choices upon published literature and "within the practice" traditions. While patterns of antimicrobial use (AMU) have been surveyed in other animal species, the general patterns of AMU by equine practitioners have never been formally investigated. We surveyed equine practitioners about their AMU preferences for the treatment of common equine clinical conditions. The survey consisted of demographic questions (e.g. current position, specialty board certification) and 23 clinical case-scenarios. For each question, the respondents could indicate no AM therapy or select from a list of 30 AM choices, either as single drug treatment or as a drug combination ($\geq$ 2 AM). Practitioners were to choose therapy without the aid of culture and susceptibility results. The survey was administered 1) through an online system in 2010, and 2) in a "paper-based" form, during the American Association of Equine Practitioners 2011 Convention, in San Antonio, Texas. Due to the poor response rate (9%) to the online survey, it was revised as a "paper-based" version, with a 37% response rate. The individual case responses, from the online and paper-based surveys were combined, compared by descriptive statistics and, where applicable, a one-way-ANOVA with Bonferroni *post hoc* test was performed (α= 0.05). Demographic analysis showed that practitioners of every level participated: interns (1%), residents (3%) generalist practitioners (60%) and specialists (36%), with surgeons and internists comprising the majority of the specialists. Of the participants, 30% were employed in academic specialty practice, 20% in private specialty-referral practice, 47% in general practice and 3% in other types of practice. Ceftiofur sodium, potassium/sodium penicillin G and procaine penicillin G (PPG) were the more commonly used monotherapies among beta-lactams. Significantly ($P < 0.0001$) more respondents selected either a single AM or a 2-drug combination therapy rather than combinations of $\geq$ 3 AM. Antimicrobial selection was more definitive in the cases where the main pathogen was easily recognizable (e.g. *R. equi* case), versus cases where less defined clinical scenarios were presented. An obvious concern raised by the survey was AMU without justification (e.g., treatment of recurrent airway obstruction) and more complicated cases wherein the approach to AM therapy was diverse, but with little consensus on the ideal therapy, and with a few selections not supported by clinical pharmacology principles. In conclusion, the results support the hypothesis that AMU in equine practice frequently deviates from published recommendations, however evidence-based AMU guidelines are lacking for several common clinical diseases of horses.

Staphylococcus delphini and Methicillin-Resistant *Staphylococcus pseudintermedius* in Horses at a Veterinary Teaching Hospital

J.W. Stull [1], D. Slavić [2], J. Rousseau [1], and J.S. Weese [1]
[1] Ontario Veterinary College, Guelph, Ontario, Canada, [2] Animal Health Laboratory, Guelph, Ontario, Canada

Staphylococcus aureus is a well-known pathogen in horses, yet the role of other coagulase-positive

staphylococcal species is unclear. Non-*aureus* staphylococcal species, including *Staphylococcus pseudintermedius* and *Staphylococcus* delphini, are important pathogens in some species, can be multidrug resistant and could be a concern in horses. Methicillin-resistant or unusual staphylococci isolated at the Ontario Veterinary College routinely undergo further characterization. During 2011, six staphylococci isolates from horses that were not methicillin-resistant *S. aureus* (MRSA) were tested. Isolates were identified through MALDI-TOF, staphylococcal species-specific PCR and/or *sod*A sequence analysis. Isolates were further characterized, as indicated, by *dru* typing, PFGE, *mecA* PCR, penicillin-binding protein 2a (PBP2a) latex agglutination test (LAT), and broth microdilution or disc diffusion. Six isolates were evaluated. Two were identified as methicillin-resistant *S. pseudintermedius* (MRSP). These isolates, from the frontal sinus (1-yr filly with sinusitis) and urine (16-yr mare with urolith) were dt11a, a predominant MRSP clone in dogs. In addition to beta-lactams, both isolates were resistant to chloramphenicol, ciprofloxacin, clindamycin, erythromycin, gentamicin, levofloxacin, moxifloxacin, tetracycline and trimethoprim/sulfamethoxazole. Four isolates were initially identified biochemically as *S. pseudintermedius,* but ultimately determined to be *S. delphini* by *sodA* sequence analysis and *S. delphini* PCR. Three were classified as *S. delphini* group B and one Group A. The group B isolates were from a 7-yr mare (chronic otitis externa) and 4-yr and 5-yr mares (incidental findings from *Streptococcus equi* nasopharyngeal wash surveillance cultures). The Group A isolate was from a 5-yr mare (*S. equi* surveillance culture). All were methicillin-susceptible. One Group B isolate was resistant to erythromycin; the remaining isolates were susceptible to all tested antimicrobials. Two of the Group B isolates were closely related by PFGE (3 band difference) while the other two were unrelated to each other and the two related isolates. MRSP is an important emerging pathogen in dogs and cats, yet has been rarely identified in horses, with few cases reported worldwide. In one patient, *Streptococcus zooepidemicus* and *Actinobacillus* spp were also isolated so the clinical relevance of MRSP is unclear. However, the authors are aware of anecdotal reports of MRSP infection in horses in the province, so MRSP may be a mis-identified (mistaken for MRSA or MR-coagulase negative staphylococci) or emerging equine pathogen. Given the rapid expansion of this pathogen in dogs and its highly resistant nature, ongoing surveillance is needed. S. delphini has rarely (if ever) been identified in horses, but it may be misidentified with conventional methods. Although in these cases colonization/contamination appeared most likely, these findings suggest this opportunist can be found in horses and might be pathogenic in certain situations. Ongoing surveillance for the emergence of S. delphini and *S. pseudintermedius in horses* is indicated to determine whether they are of clinical relevance.

A laboratory survey of viral diseases occurred in horses in Argentina from 2007 to 2012

M.A. Vissani [1], C. Olguín Perglione [1], M.S. Tordoya [1], L. Becerra [1], S. Miño [1], F. Darqui [1], S. Russo [2], and M.E. Barrandeguy [1]
[1] Instituto de Virología, CICVyA, INTA Castelar, [2] SENASA, DILAB; Buenos Aires, Argentina

Viral infections represent a threat as a sanitary problem for the equine industry worldwide. The aim of this work was to investigate the viral etiology in clinical syndromes occurring in horses in the last five years in Argentina (2007-2012). During this period, the following types of samples were analyzed: samples of abortions (n:623) from 263 breeding farms; nasopharyngeal swabs from 84 cases of respiratory disease in foals (n:82) and horses in training (n:2); stools from 208 cases of diarrhea in foals; semen (n:27) from Equine Arteritis virus (EAV) seropositive stallions; samples of central nervous system (CNS) from 76 cases of neurological disease; and genital swabs (n:73) of mares and stallions showing typical lesions of equine coital exanthema (ECE). Equid herpesvirus 1 (EHV-1) was detected in 6% (37/623) and EAV in 0,3% (2/623) of abortion samples. From EHV-1 isolated, 16% (6/37) corresponded to the neuropathogenic (G_{2254}) variant, and 5 of the 6 were associated to an abortion outbreak with concomitant neurological disease. Five outbreaks of abortion, with multiple fetal losses, were registered due to EHV-1 (n:1) and *Leptospira spp* (n:1) in 2007, EAV (n:1) in 2010 and *Salmonella abortus equi* (n:2) in 2011. Equid herpesvirus 4 was registered in 12% of the cases of respiratory disease in foals (n:9) and in two-year-old training horses (n:1). Equine Influenza virus (EIV) had not been detected in horses in Argentina since 2005; however, EIV was recently the cause of a huge outbreak of respiratory disease in training horses in Uruguay. Diarrhea in young foals due to Rotavirus infection was detected in 21% (43/208) of the cases. EAV was detected in 22% (6/27) of the semen samples, of which 4 belonged to stallions which became infected during the 2010 EAV outbreak, and 2 came from imported semen (of these, one was responsible for the re-introduction of EVA virus in 2010, and the other one was detected during the importation quarantine). In all cases, EAV isolated clusters in European-1 genotype. Rabies virus was the cause of 3% (2/76) of cases of neurological disease and deaths in horses which take place in an endemic area of vampire bats (*Desmodus rotundus*). The virus was characterized as antigenic variant 3 (vampire). West Nile virus was the cause of neurological disease and death in 2 six-month-old foals in Córdoba province in April 2010. EHV-1 was not isolated from CNS samples; however, it was associated with neurological disease during the outbreak of EHV-1 abortions in 2007. EHV-3 was detected in 56% (41/73) of the samples submitted from suspicious cases of ECE. The data presented shows the horse population in Argentina could be exposed to numerous infectious diseases, and emphasizes the importance of preventive and control measures as well as the benefits of surveillance programs.

Respiratory Diseases

Outbreak of equine influenza among Thoroughbred horses in Maroñas, Montevideo (Uruguay) during March and April 2012

R. Acosta [1], A. Rimondi [4], S. Miño [3], M.S. Tordoya [4], A. Fernandez [2], G. Betancor [1], H. Larrauri [2], A. Nuñez [3], and M. Barrandeguy [4]
[1] Private veterinarian, Facultad de Veterinaria, [2] Ministerio de Ganadería, Agricultura y Pesca, [3] CEDIVE. Montevideo, Uruguay, [4] Instituto de Virología, CICVyA, INTA Castelar, Buenos Aires, Argentina

Equine Influenza (EI) is regarded as the most economically important respiratory disease of horses. The aim of this work is to report the epidemiological picture along with the phylogenetic pattern of the virus detected during an EI outbreak occurred among Thoroughbred horses housed in Maroñas racing and training facilities, in Montevideo, Uruguay, during March and April 2012. Even though vaccination is not mandatory in Uruguay, racetrack internal regulations require a vaccination certificate for racing; therefore, all the affected horses had been vaccinated at least once in their lifetimes. The first suspicious cases were observed around March 10th. The disease affected approximately 40% of a total population of 1700 horses, and was more severe in the two to three year-old horses. The duration of pyrexia and other clinical signs was variable amongst individuals, yet it was, in average, seven days. Some horses were more severely affected, and two deaths occurred presumably due to pulmonary complications. On March 28th the Animal Health Authority intervened, and carried out a clinical surveillance (to determine the scope of the outbreak), sampling of acutely affected horses and laboratory diagnosis. Equine influenza virus was detected by real time PCR in eight out of ten nasopharyngeal swabs obtained. All the horses had haemagglutination inhibition antibodies at the time of sampling, and the titres ranged from 8 to 256. On March 30th, the World Organisation for Animal Health (OIE) was notified. The outbreak resulted in the withdrawal of several horses from races, temporary cancellation of race meetings, and the ban on the movement of Thoroughbred horses both nationally and internationally. Massive booster vaccination of the horse population at risk was made compulsory. No new cases were observed by April 20th. The origin of the virus has not been precisely identified; nevertheless, the press had reported a similar respiratory syndrome in Porto Alegre and Curitiba (Brazil) racetracks, and horses from these regions had been moved to Maroñas for racing purposes. Phylogenetic analysis of the haemagglutinin (HA) gene revealed this EIV is closely related to Clade 1 of the Florida sublineage within the American lineage. HA amino acid alignment shows the Uruguay virus is identical to those identified in Kentucky and New York in 2011, and differs from those causing the outbreak in Japan and Australia in 2007, which have serine (S) instead of proline (P) in the position 176 and lysine (K) instead of glutamine (Q) in the position 203. Recent outbreaks of Clade 1 EIV in the United States and Uruguay indicate its predominance in America. Nowadays, EIV inactivated vaccines currently marketed in Uruguay are under revision and updating following the OIE recommendations.

Clinical relevance of low virulent or subclinical viral respiratory infections in Swedish trotters

H. Back [1], J. Penell [2], J.F. Valarcher [1], L. Treiberg-Berndtsson [1], N. Ronéus [3], K. Ståhl [4], and J. Pringle [5]
[1] Department of Virology, Immunology and Parasitology, the National Veterinary Institute, Uppsala, Sweden, [2] Institute of Environmental Medicine, Karolinska Institutet, Stockholm, Sweden, [3] Equine Clinic Solvalla, Bromma, Sweden, [4] Department of Disease Control and Epidemiology, the National Veterinary Institute, Uppsala, Sweden, [5] Department of Clinical Sciences, Swedish University of Agricultural Sciences, Uppsala, Sweden

Poor performance in trotters is a major issue for the horse industry, where lameness and respiratory diseases are the most common medical reasons for not performing as well as expected. Regarding respiratory diseases, the role of subclinical and low virulent virus infection contributing to poor performance is unclear. Further, ready diagnosis of such infections is still a challenge. The aims of this longitudinal study were to investigate the possible role of subclinical virus infections in the poor performance syndrome in trotters who are competing at high level. The study evaluated potential methods of detecting subclinical infections with different diagnostic methods such as serology and PCR-assay from nasal swabs. Further, the usefulness of the acute phase protein Serum Amyloid A (SAA) in identifying subclinical virus infections in horses was examined. A total of 66 high performing Swedish trotters from four different trainers were included in a cohort study from August 2010 to August 2011. Once a month nasal swabs and blood samples were taken, whereas additional samples were obtained at other times in the event of clinical signs of respiratory disease or “poor performance”. The body temperature and the presence of any clinical respiratory signs were recorded at the different sampling occasions. The nasal swabs were analyzed for Equine Influenza Virus (EIV), Equine Herpesvirus (EHV)1 and 4, Equine Rhinitisvirus (ErhV) B and Equine Arteritis Virus (EAV). The blood samples were examined for

antibodies against EHV 1, 4 and ErhV A, B, and levels for the acute phase protein Serum Amyloid A (SAA). To be able to relate the results of the analysis to performance, a standardized workload in a cross over design was done after each sampling. The horses remained in the training yards during the whole period and competed according to their normal schedule. A total 629 blood samples and the same number nasal swabs were taken during the study period. There was a difference in the levels of antibody titers for ErhV A,B between the stables and it appeared that some horses have remaining high titers for a longer period. No cases of EIV or strangles were observed whereas EHV-4 and ErhVB were detected by PCR assay in some clinical healthy horses. Virus infection was identified in horses with "poor performance" on the racetrack where no other clinical reasons could be found. Increases in SAA appeared so far related to horses with fever or soft tissue injury. Data from the questionnaires, workloads and the results from assays performed will be further analyzed and presented.

Streptococcus zooepidemicus: more than just an opportunist?

S. Björnsdóttir [1], M.T.G. Holden [2], V. Svansson [3], S.R. Harris [2], K. Webb [4], C. Robinson [4], K.F. Steward [4], N. Wright [4], R. Paillot [4], J.R. Newton [4], E. Gunnarsson [3], and A.S. Waller [4]
[1] Icelandic Food and Veterinary Authority, [2] The Wellcome Trust Sanger Institute, Cambridge, UK, [3] Institute of Experimental Pathology, Reykjavik, Iceland, [4] The Animal Health Trust, Newmarket, UK

In April 2010 an epidemic of respiratory disease, typified by coughing, swept through the Icelandic horse population, affecting a large proportion of the national herd of 80,000 animals by the following November. Export of horses was stopped with significant economic consequences. Clinical samples from affected horses were shown to be negative for all known viral agents and the only consistent finding was the presence of *Streptococcus zooepidemicus*, a common opportunistic pathogen of horses that is often regarded as a commensal organism. We used high throughput DNA sequencing to discriminate different strains of *S. zooepidemicus* and identify the cause of this National epidemic. A detailed epidemiological and microbiological analysis of the Icelandic horse population was conducted to identify horse movements, transmission points and potential causative agents. The genomes of two hundred and eighty eight isolates of *S. zooepidemicus* recovered from Icelandic horses pre- and during the 2010 epidemic and the AHT's strain collection were sampled by high throughput Illumina sequencing. Significant population diversity was identified across the *S. zooepidemicus* population recovered from Icelandic horses. Sampling the core genome of sequenced strains enabled their separation into distinct sub-groups including four large clades. Strains of clade 4 were only recovered from the Icelandic horses at the Keldur Veterinary Institute, Reykjavik and indicated an isolated closely related bacterial population that diversified over time. Strains of clade 2 and clade 3 were identified from several farms across Iceland and clustered according to their geographical origins. These clades had significantly greater genetic diversity, suggesting that they had persisted in Icelandic horses for a longer period of time. However, clade 1 (ST-209) strains, which were the most frequently identified, were closely related despite being recovered from almost every affected farm sampled, suggesting rapid, near-simultaneous transmission from a point source. Network analysis of affected farms identified a single common training yard and highlighted the probable incursion of the ST-209 strain between the 5th and 10th February 2010. The use of new sequencing technologies permits fine mapping of disease outbreaks at the genetic level that can provide new insights into the transmission and virulence of pathogens. The ST-209 strain demonstrates that some strains of *S. zooepidemicus* have significant potential to cause disease in naïve horse populations.

Emergence of widespread macrolide and rifampin resistance in *Rhodococcus equi* isolates from a horse breeding farm

A.J. Burton [1], S. Giguère [1], T.L. Sturgill [1], L.J. Berghaus [1], N.M. Slovis [2], J. Whitman [3], K.R. Kuskie [4], and N.D. Cohen [4]
[1] University of Georgia, Athens, GA, USA, [2] Hagyard Equine Medical Institute, Lexington, KY, USA, [3] Equine Medical Associates, Lexington, KY, USA, [4] Texas A & M University, College Station, TX, USA

The combination macrolide-rifampin has been the mainstay of therapy in foals infected with *Rhodococcus equi* for nearly 30 years, with only rare isolated reports of resistance. In recent years, mass antimicrobial treatment of subclinically affected foals has become common practice on endemic farms in an attempt to prevent mortality. The objective of the present study was to describe the emergence of macrolide and rifampin resistance on a farm where widespread use of these drugs was instituted. In 2010, 45 of 138 (33%) foals born on the farm were treated for subclinical ultrasonographic pulmonary lesions attributed to infection caused by *R. equi*. Culture of a tracheobronchial aspirate (TBA) was performed on 28 foals and yielded macrolide- and rifampin-resistant *R. equi* in 9 of 22 (41%) foals from which *R. equi* was cultured. Subsequently, air samples were collected from multiple sites on the farm. In 2011, TBAs were collected from each foal with subclinical lesions prior to initiation of therapy and 2 weeks later. *R. equi* isolates were tested for susceptibility to macrolides and rifampin, and genotyped by rep-PCR. Two of 83 (2%) *R. equi* isolates from air samples were resistant. Resistant isolates were recovered from pre-treatment TBA in 7 of 21 (33%) foals from which *R. equi* was cultured. The proportion of resistant isolates from the post-treatment TBA was 7/12 (58%). Resistant isolates formed 2 distinct genotypic clusters whereas there was considerable genotypic heterogeneity among susceptible isolates. These results underscore the potential risk of widespread use of macrolides and rifampin.

Rattles epidemiology: the role of the foal

C. Chicken [1], G.F. Browning [1], G. Muscatello [2], and J.R. Gilkerson [1]
[1] Centre for Equine Infectious Disease, The University of Melbourne, Victoria 3010, Australia, [2] Faculty of Veterinary Science, The University of Sydney, NSW 2067, Australia

Rhodococcus equi causes pyogranulomatous bronchopneumonia (rattles) and gastrointestinal abscessation of foals. Early diagnosis is essential for minimising the impact of this disease on the horse industry. Current best clinical practice involves ultrasonographic (U/S) thoracic screening to detect small abscesses and initiate treatment before serious clinical abnormalities are obvious. Ecological and epidemiological studies in Australia have demonstrated that foals shed virulent *R. equi* from the respiratory tract and that high airborne levels of virulent *R. equi* are associated with high disease prevalence. The role of the foal as a contributor to environmental contamination remains an important practical area of *R. equi* research. Two studies were conducted. Samples from the breathing zone were collected from 53 foals as neonates and again at 6 weeks of age. These samples were cultured on selective bacteriological media. Samples were also collected from 80 mares and foals. Serum samples were collected from foals at 12 hours, 3 weeks and 6 weeks of age. Faecal and breath samples were collected at 3 weeks and 6 weeks of age. Colostrum samples were collected from mares at parturition. Serum and colostrum samples were tested using a VapA ELISA that detects antibody to the major *R. equi* virulence factor (the VapA protein). Faecal samples were cultured on selective media. There was strong correlation between anti-VapA IgG level in serum samples from newborn foals and maternal colostrum. However, foal VapA antibody concentration at 12 hours was not related to the likelihood of subsequent *R. equi* abscessation. Virulent *R. equi* was detected in breathing zone samples from 19% of neonatal foals and from 45% of 6 week old foals. The concentration of virulent *R. equi* in these samples was greater in the older foals. Antibody to VapA declined from 12 hours to 3 weeks of age as maternal antibody waned, but increased significantly between 3 and 6 weeks of age. Foals with ultrasonographic evidence of rattles had higher levels of antibody than non-diseased foals. Although >90% of 6 week old foals had virulent *R. equi* in faeces, foals with U/S evidence of rattles had a greater proportion. However, the concentration of virulent *R. equi* in faeces was not statistically greater than in foals with no U/S lesions. Six week old foals are a significant source of environmental contamination with virulent *R. equi*. These foals shed higher amounts of virulent *R. equi* from both faeces and breath compared to younger foals. This has significant implications for management and reinforces the current practice of separation of mares and foals based on age. These results also support collection of faeces from high traffic areas such as yards. Importantly, neither samples from the breathing zone, nor maternally derived antibody levels, were useful predictors of subsequent development of rattles.

Perinatal airborne exposure to *Rhodococcus equi* is associated with increased risk of pneumonia caused by *R equi* in foals

N.D. Cohen [1], M.K. Chaffin [1], K.R. Kuskie [1], M. Syndergaard [2], S. Takai [3], and G.P. Blodgett [2]
[1] Texas A&M University, College Station, TX, USA, [2] 6666 Ranch, Guthrie, TX, USA, [3] Kitasato University, Towada, Aomori, Japan

The objective of this study was to determine whether the concentration of airborne virulent *Rhodococcus equi* in stalls housing foals during the first 2 weeks of life was associated with development of pneumonia caused by *R. equi*. Air samples were collected from January through May, 2011 on days 1, 4, 7, and 14 of life from stalls and pens where foals were housed during the perinatal period at a breeding farm in Texas. Virulent concentrations of airborne *R equi* were determined using culture and immunoblotting, and the association between development of disease and *R equi* counts was analyzed using random-effects Poisson regression. A total of 471 air samples were collected from stalls of 121 foals; 19% (90/471) of air samples were positive for virulent *R equi*. Twenty-four of 121 (20%) foals developed *R equi* pneumonia. The concentrations of virulent *R equi* in air samples from stalls or pens of foals that developed *R equi* pneumonia were significantly ($P < 0.05$) higher than those for foals that did not develop pneumonia. Accounting for effects of disease, concentrations of virulent *R equi* in air did not differ significantly by sampling age or month of birth. Exposure of foals to airborne virulent *R equi* during the first 2 weeks of life was significantly associated with subsequent development of disease. These findings suggest exposure to airborne *R. equi* during early life may be causally associated with disease.

Performance of Strangvac, a multicomponent recombinant vaccine against strangles

M. Flock [1], L. Frykberg [2], B. Guss [2], C. Robinson [3], A. Waller [3], R. Bergman [4,5], and J.-I. Flock [1,4]
[1] Department of Microbiology, Tumor and Cellbiology, Karolinska Institutet, Stockholm, [2] Department of Microbiology, Swedish University of Agricultural Sciences, Uppsala, Sweden, [3] Department of Bacteriology, Animal Health Trust, Newmarket, UK, [4] Intervacc AB, Stockholm, Sweden, [5] Nordvacc AB, Stockholm, Sweden

Strangles in the horse is caused by Stretococcus equi subsp. equi and is a severe and highly contagious infection. An improved vaccine against strangles is needed on the market since present vaccines have problems with efficacy and safety. We have previously used recombinant proteins derived from *S. equi* to vaccinate ponies against strangles with encouraging results. The cost to produce a multicomponent vaccine is high. Therefore several proteins were fused and tested in the current study. The objectives with the current studies were to assess the efficacy of

various combinations of recombinant fusion proteins as vaccines against strangles. Ponies were vaccinated by a combination of intranasal and subcutaneous route with different sets of recombinant fusion proteins. Ponies were then subjected to experimental infection with *S. equi* to induce early stages of strangles. The ponies were followed clinically and were pathologically analysed at post mortem. ELISA was used to follow antibody response. The best combination of fusion proteins, Strangvac was used on 16 ponies. Of these, 15 did not become pyrexic or had any clinical signs of infection. Nine had no pathological signs on post mortem analysis and 5 had reduced signs. All non-vaccinated control ponies (n=16) became infected and with clear pathological scoring on post mortem. Antibodies were obtained against all subcomponents in the fusions. Large scale and cost effective production of Strangvac could be obtained by optimizing various conditions during fermentation and downstream processing. The stability of Strangvac appears, so far as the study has gone on, to be good. One day after immunization, pyrexia was observed as the only adverse reaction. We therefore conclude that Strangvac is a safe and efficient vaccine against strangles and will now be taken through additional clinical trials for a registration.

Investigation of the innate immunity in the lower respiratory tract in horses

L. Frellstedt [1], P. Gosset [2], C. Desmet [3], D. Pirottin [3], F. Bureau [3], P. Lekeux [1], and T. Art [1]
[1] Equine Sports Medicine, University of Liège, Liège, Belgium, [2] Pasteur Institute Lille, Center for Infection and Immunity, Lille, France, [3] Laboratory of Cellular and Molecular Physiology, Interdisciplinary Cluster for Applied Genoproteomics (GIGA-Research), University of Liège, Liège, Belgium

Respiratory diseases, including inflammatory airway disease (IAD), viral and bacterial infections, are common problems in exercising horses. The etiology, diagnosis and treatment of these diseases have been investigated but the underlying innate and acquired immune mechanisms remain poorly documented. In humans, exercise has been associated with down-regulated expression of Toll-like receptors (TLRs), costimulatory and antigen-presenting molecules on monocytes. The airway epithelium constitutes the first line of defense and is an important part of the innate immune response. This first barrier determines the downstream cascade for elimination of the imposed pathogen. The objectives of this study are 1) to examine the expression of TLRs in equine bronchial epithelial cells (EBEC) and peripheral blood monocytes at rest and after exercise; 2) to stimulate EBEC and monocytes *in vitro* with specific TLR ligands, in order to resemble bacterial/viral infections; 3) to describe the cytokine expression of EBEC and monocytes at rest and after exercise. Bronchial biopsies were taken from 8 horses during lower airway endoscopy at rest and 24 hours after exercise. Bronchial epithelial cells were grown *in vitro* and activated with TLR ligands. Blood was collected at rest and after exercise. Monocytes were isolated via density gradient centrifugation and adherence. RNA was extracted from EBEC and monocytes. The TLR and cytokine expression were evaluated via real-time PCR. This is the first study to report a cell culture model for EBEC in live horses thereby allowing the investigation of immune responses under natural conditions. The expression of TLRs in EBEC differs from that in monocytes. Stimulation with TLR ligands allowed the identification of functional TLRs in EBEC and monocytes. TLR3 is the most important TLR in EBEC and stimulation with Poly(I:C), an analog of viral dsRNA, triggered a strong inflammatory response demonstrated by significant up-regulation of IFN-beta production. In equine monocytes, TLR2 and TLR4 are the predominant TLRs and stimulation with synthetic lipoprotein (FSL) and bacterial lipopolysaccharide (LPS) resulted in increased production of TNF-alpha. We propose that EBEC are one of the cell types responsible for an anti-viral response in the lower airway. Their unresponsiveness to LPS may suggest a defense mechanism that protects EBEC from 'overstimulation' due to constant bacterial endotoxin exposure. In contrary, monocytes are extremely sensitive to bacterial endotoxin and a strong immune response is induced.

Equine Influenza Vaccination – Which product? What regime?

S. Gildea [1], S. Arkins [2], C. Walsh [3], and A. Cullinane [1]
[1] Virology Unit, The Irish Equine Centre, Johnstown, Naas, Co. Kildare, Ireland, [2] Department of Life Sciences, University of Limerick, Limerick, Ireland, [3] Department of Statistics, Trinity College, Dublin, Ireland

The correlation between antibodies against equine influenza virus haemagglutinin following vaccination and protection against disease has been well established. In Ireland the Turf Club has a mandatory vaccination policy for equine influenza (EI). It requires a primary vaccination course be administered between 21 and 92 days apart followed by a third vaccination 150 to 215 days later. Thereafter annual boosters must be administered. The objective of this study was to compare the antibody responses of Thoroughbred horses to the whole inactivated, subunit and canary pox recombinant EI vaccines available in Ireland. The antibody response of 66 Thoroughbred weanlings and 44 National Hunt horses was monitored for six months post primary (V1, V2, V3) or booster vaccination respectively by single radial haemolysis (SRH). The antibody response of the Thoroughbred weanlings vaccinated with the whole virus vaccine Duvaxyn IET Plus was significantly higher than that of the horses vaccinated with any of the other products. In this field study 43% of the weanlings failed to seroconvert after V1. The poor responders were observed in all vaccine groups except those vaccinated with Duvaxyn IET Plus. Post V2 the incidence of poor responders was reduced to 7% but a rapid decline in antibody level was observed within three months with all products. All weanlings responded to V3. Forty one per cent of National Hunt horses did not demonstrate a significant rise in antibody level following

booster vaccination. There was a significant correlation between the SRH level at the time of vaccination and the antibody response. There was no significant difference between antibody responses induced by any of the vaccines. Antibody levels peaked between two and four weeks post vaccination. Peak antibody response to the canarypox recombinant vaccine was delayed in comparison to the other products. Antibody levels decreased significantly by three months post vaccination and declined to their original levels by six months post vaccination. Although analysis of the mean antibody levels suggested that the horses were protected post booster vaccination, analysis of the individual responses suggested that there was potential for vaccination breakdown. The study demonstrates that independent evaluation of influenza vaccine performance in the field is critical to add to the body of knowledge gained from experiments carried out for regulatory or marketing purposes. The high incidence of poor responders observed in this study has not been reported in previous experimental studies relating to these products. The presumption that annual revaccination is the minimum necessary to protect all horses against EI needs to be systematically evaluated. It has been demonstrated that shorter intervals are required for optimum protection of young horses and it may be that longer vaccination intervals are sufficient for horses with several years of vaccination history.

Equine herpesvirus-1 and -4 serological survey of South African Thoroughbred yearlings

J.R. Gilkerson [1], J. Crafford [2], C.A. Hartley [1], N.P. Ficorilli [1], and A.J. Guthrie [3]
[1] Equine Infectious Disease Laboratory, Faculty of Veterinary Science, The University of Melbourne, Victoria 3010, Australia, [2] Department of Veterinary Tropical Diseases, Faculty of Veterinary Science, University of Pretoria, Onderstepoort 0110, Republic of South Africa, [3] Equine Research Centre, Faculty of Veterinary Science, University of Pretoria, Onderstepoort 0110, Republic of South Africa

Equine herpesviruses 1 and 4 (EHV-1 and EHV-4) are important endemic pathogens of horses worldwide. Epidemiological studies in Australia demonstrated that both EHV-1 and EHV-4 circulate within equine populations in a silent cycle of infection, with a small proportion of foals being infected prior to weaning, most likely following reactivation of a previously latent maternal infection. Although EHV-1 and EHV-4 have been detected in South Africa there have been no previous large scale epidemiological studies of these viruses in South African horses. Five hundred and thirty one thoroughbred foals were sampled (3 - 6 months of age) in January 2008, and again at the South African National Yearling Sales (NYS) in March 2009. Blood samples were collected and the serum stored at -20°C until it was tested using an EHV-1/EHV-4 type specific ELISA. Of the 531 horses in the study population, 292 were male (55.0%) and 239 (45.0%) were female. The total number of EHV-1 antibody positive foal samples was 13 (2.4%: 95% confidence interval 1.3 – 4.2). The total number of EHV-4 antibody positive foal samples was 412 (77.6%: 95% confidence interval 73.8 – 81.1). While there was no significant difference in the seroprevalence of EHV-1 between commercial or private farms, nor between different sized farms, foals on commercial farms were significantly more likely to be EHV-4 antibody positive than foals on private farms (λ^2=8.65, p=0.003). Of the 531 samples from yearlings, the total number of EHV-1 antibody positive yearlings was 13 (2.4%: 95% confidence interval 1.3 – 4.2). Only 4 of these antibody positive yearlings were also positive as foals. The total number of EHV-4 antibody positive yearlings was 518 (97.85%, 95% confidence interval: 95.85 – 98.69). The relative risk of a yearling from a commercial farm being EHV-4 antibody positive was 5.36 times that of a yearling consigned by a private farm (95% confidence interval: 1.50 – 19.19). This study confirmed circulation of both EHV-1 and EHV-4 in a silent cycle of infection in the foal and yearling populations sampled. Interestingly, the seroprevalence of both these viruses in the foals sampled was substantially lower than that reported in similar studies in Australia and may be due to the more dispersed location of the Thoroughbred industry in South Africa. The significant increase in EHV-4 seroprevalence between foals and yearlings indicates that this virus circulates readily through the majority of horses in the herd, although often in the absence of overt significant clinical disease. Although EHV-1 was also circulating in these horses, it was a much less common infection.

Serological evidence of circulation of Equine H3N8 Influenza Virus in Algeria and its molecular characterization

A. Hans [1], F. Laabassi [2], G. Amelot [1], L. Legrand [3], D. Gaudaire [1], C. Laugier [1], S. Pronost [3], and S. Zientara [4]
[1] Anses, Laboratory for equine diseases, France, [2] Département Biomédecine Vétérinaire, Institut des Sciences Vétérinaires, University Ibn-Khaldoun, Tiaret, ALGERIA, [3] Frank Duncombe laboratory, EA 4655 U2RM Caen University Lower Normandy, France, [4] Anses, Laboratory for Animal Health, UMR 1161 ANSES/INRA/ENVA, France

Equine Influenza Virus (EIV) belongs to the Orthomyxoviridae family and is a major cause of respiratory diseases in Horses. EIV can spread very rapidly in a susceptible population and may lead to important economical losses to horse industry worldwide. Clinical signs associated to EIV infection are coughing, nasal discharge and high fever. Two EIV subtypes have been isolated from horses. The equine H7N7 viruses have been isolated in 1956 and have not been isolated in horses since 1979. The equine H3N8 viruses have been isolated in 1963 after an important outbreak in USA. This virus called A/Eq/Miami/63 is the prototype of equine flu H3N8 subtype and continues to circulate nowadays. In 1972 the first equine influenza virus isolated in Algeria has been characterized, since then no evidence of equine flu circulation in this country has been published.

The objectives of this work were to evaluate seroprevalence of the disease in Algeria, knowing that vaccination against equine flu is not mandatory in this country and to characterize new viral isolates from nasal swabs collected during 2010 and 2011. 397 sera from distinct horses have been collected, 297 sera were collected in 2009/2010 and 100 were collected during 2011. Serological analysis of equine sera was conducted using haemagglutination inhibition assays (HI), using H7N7/Prague/1956 and H3N8 equine influenza viruses (H3N8/Miami/1963; H3N8/Russie/1983; H3N8/Grosbois/2009). Nasal swabs were taken from horses showing signs of acute respiratory disease. Genotyping was performed by amplification and sequencing of the haemagglutinin gene (HA1) using RNA extracted directly from nasal swabs. Around 59% from 297 sera collected during 2009/2010 showed antibodies anti-H3N8 subtypes, this percentage rises to 81% for sera collected in 2011. Moreover, antibodies anti-H7N7 were found in 6 sera, those horses were imported from Europe and have been certainly vaccinated against equine influenza viruses with a vaccine containing H7N7 antigens. In the meantime, nasal swabs were collected from 7 sick horses showing fever and nasal discharge. Nasal swabs were found positive using a quantitative RT-PCR assay. Phylogenetic analysis using these HA1 sequences and some reference strains and others from GenBank grouped the viruses in different clusters: the Predivergent and American lineages with the Florida sublineage clades 1 and 2. Viruses from Algeria are members of the Florida sublineage Clade 2. In conclusion, the seroepidemiology study performed shows that equine influenza viruses from H3N8 subtypes are circulating in horse population from Algeria. Moreover, the virus characterized in this paper, named A/equine/H3N8/Tiaret/2011, is the first one to be characterized in Algeria since 1972.

Diversity, microevolution and within-host niche adaptation of *Streptococcus equi*

S.R. Harris [1], K. Webb [2], D.M. Aanensen [3], C. Robinson [2], K.F. Steward [2], J.F. Timoney [4], B. Guss [5], A. Efstratiou [6], M.T.G. Holden [1], and A.S. Waller [2]
[1] The Wellcome Trust Sanger Institute, Cambridge, UK, [2] The Animal Health Trust, Newmarket, UK, [3] Imperial College, St. Mary's Hospital Campus, London, UK, [4] Gluck Equine Research Center, Lexington, USA, [5] Swedish University of Agricultural Sciences, Uppsala, Sweden, [6] Health Protection Agency, London, UK

The microevolution of bacterial pathogens to better fit their pathogenic niche is a key process, which determines the success or failure of different lineages. *Streptococcus equi* is the causative agent of equine strangles, the most frequently diagnosed infectious disease of horses worldwide. The global population of *S. equi* was thought to be genetically and immunologically identical. However, new methodologies currently identify 114 different strains of *S. equi* on the basis of the 5' sequence of the SeM gene. This single locus sequencing approach has proven useful, but does not provide sufficient resolution to discriminate closely related strains. In this study we employed a high-throughput genomics approach to define global population diversity, within-outbreak and within-host microevolution of this important host-restricted pathogen. The genomes of two hundred and forty isolates recovered between 1955 and 2011 from the UK, USA, Canada, Ireland, Sweden, Saudi Arabia, Belgium, New Zealand and Australia were sampled by high throughput Illumina sequencing. This global collection of *S. equi* included the 4047 reference strain (*Se*4047), which has been sequenced to completion, and the Pinnacle IN and Equilis StrepE vaccine strains. The collection also permitted the in depth analysis of strains recovered from the same UK outbreaks over time. A total of 3,111 genome variable sites were identified, illustrating a hitherto unknown level of population diversity. 'Accessory' sequences comprising mobile genetic elements, primarily phage that have the potential for horizontal transfer between isolates were omitted from analysis of the core genome. A maximum likelihood tree that was constructed based on the core genome tree shares a striking resemblance to that constructed using the SeM variable region, but could differentiate strains sharing the same SeM allele. We report genome decay in certain isolates, predominantly those recovered from persistent infections of the guttural pouch, which may render affected strains incapable of causing acute disease. Interestingly, comparison of accessory sequences identified examples of within-outbreak exchange and loss of mobile genetic elements. Sampling the genomes of this diverse collection of *S. equi* has revealed a dynamic and flexible genome that continues to decay, acquire and exchange new mobile genetic elements. Examples of multiple incursions of *Streptococcus equi* and pre-existing carriers in individual outbreaks shed new light on the epidemiology of this global equine disease.

Reasons for the age-dependent susceptibility of young foals to *Rhodococcus equi*

D.W. Horohov, M. Sanz, L. Sun, A. Betancourt, and A. Page
Maxwell Gluck Equine Research Center, University of Kentucky, Lexington, KY

The age-related susceptibility of young foals to infection with *Rhodococcus equi* is widely appreciated with very young foals being at greatest risk for infection. However, the reasons for this unique susceptibility remain unknown. Overall, neonates have reduced expression of Th1 cytokines which are required for resistance to *R. equi*. While older foals have increased expression of interferon-gamma (IFN-g) and increased resistance to infection, the mechanism whereby they acquire this capability is unknown. Given the ubiquitous occurrence of *R. equi* on equine farms, it is reasonable to presume that all foals are exposed to this bacterium shortly after birth and that this exposure primes their immune system leading to increased expression of Th1 cytokines and resistance to *R. equi* infection. Foals of different ages (3 to 42 days) were exposed to various doses (10^3 to 10^7) of *R. equi* to determine their susceptibility to infection. Necropsies were performed on all foals following challenge. Foals were screened prior to challenge for exposure to *R. equi* using both

a VapA-specific ELISA and *R. equi*-specific IFN-g expression following *in vitro* stimulation of PBMC with *R. equi*. We also assessed their IFN-g response to PMA and ionomycin stimulation. While the youngest foals were highly susceptible to infection, the oldest foals were resistant to 1000-fold higher challenge. There was an age-related increase in IFNg production in response to *in vitro* stimulation with PMA and ionomycin, similar to what we have previously reported. We also observed age-related production of IFNg in response to *in vitro* stimulation with *R. equi*. The oldest foals likewise exhibited VapA-specific antibodies prior to the challenge. Our results indicate that very young foals are highly susceptible to low doses of *R. equi* and that the older foals' resistance could be the result of immunological priming following natural exposure to *R. equi*.

Mapping serotype specific epitopes in equine rhinitis B viruses: development of an ELISA to examine erbovirus epidemiology

J.J. Horsington *, J.R. Gilkerson, and C.A. Hartley
Centre for Equine Infectious Disease, Faculty of Veterinary Science, The University of Melbourne, Australia

Erbovirus is a genus of the family Picornaviridae and equine rhinitis B virus (ERBV) is the sole species. Erboviruses infect horses causing acute respiratory disease, sub-clinical and persistent infections. Despite their high seroprevalence and worldwide distribution, the pathogenesis and epidemiology of the three ERBV serotypes (ERBV1, -2 and -3) is poorly understood. The aim of this study was to better understand the antigenic relationship between ERBV serotypes and identify antigens for a serotype-specific ERBV antibody detection ELISA. The ELISA was then used to examine the seroprevalence of ERBV antibodies in a group of 50 weanling horses from farms in New South Wales, Australia. Full length VP1 and VP2 from all 3 serotypes, and proteins representing some of their surface exposed loops were expressed as *E. coli* fusion proteins. These recombinant proteins were used to map linear B cell epitopes. The antigenicity of the fusion proteins was tested in western blot and ELISA using sera obtained from ERBV-immunised rats and naturally infected horses. Antigens showing highest correlation with virus neutralisation titres were used to develop the serotype-specific ELISAs. The seroprevalence of antibodies to the 3 serotypes was examined in 50 weanling thoroughbred horses located on two adjacent farms in the Hunter Valley, New South Wales (Gilkerson et al. 1998). Three serum samples from each horse were used, collected when the animals were 4-6, 7-9 and 11-13 months old. VP1 contained serotype specific epitopes, whereas VP2 was highly cross-reactive across the serotypes. The C-terminus of VP1 accounted for most of the reactivity of full-length VP1 and was also the location of a neutralising site in each serotype. The serotype specific ELISA was developed with these antigens, and showed a high seroprevalence of ERBV in the weanlings tested (74-84%). ERBV3 antibodies were most prevalent (58-62%) and ERBV2 antibodies were least prevalent (10-16%). Many horses were seropositive to two or more serotypes. The antigenic structure of erboviruses strongly parallels that of other picornaviruses. The major neutralisation sites locate to VP1, and the serotype-specific neutralisation epitopes locate to regions of high-level amino acid variation within VP1. The results of this survey with the serotype specific ERBV antibody detection ELISA are consistent with other studies that highlight the ubiquity of ERBV antibodies in populations worldwide. The detection of antibodies to more than one serotype in more than half of the animals, along with the observation that some horses seroconverted simultaneously to 2 ERBV serotypes, is consistent with previous studies that show active co-infection with 2 distinct erbovirus serotypes (Horsington et al., 2011).

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Persistence and chronic urinary shedding of the aphthovirus equine rhinitis A virus

S.E. Lynch [1], J.R. Gilkerson [1], S.J. Symes [1], J. Huang [2], and C.A. Hartley [1]
[1] Centre for Equine Infectious Diseases, Faculty of Veterinary Science, The University of Melbourne,
[2] Veterinary Medicine Research and Development, Pfizer Animal Health

Equine rhinitis A virus (ERAV), is a member of the genus *Aphthovirus* family *Picornaviridae,* classified alongside foot-and-mouth disease virus (FMDV). ERAV and FMDV bear many physical, structural and genotypic similarities. Although, the pathogenesis of FMDV has been extensively studied, the similarities in the pathogenesis of ERAV and FMDV disease are not well documented. This study describes and compares the pathogenesis of ERAV both in the natural host and a small animal model alternative. Two 8-month old horses were infected with ERAV supernatants administered into the nasopharynx via an endoscope guided catheter. Blood, nasopharyngeal and oral swabs were collected on alternate days after infection and at days 21 and 37 post infection (p.i). Urine was collected on 4 occasions up to day 37 p.i. To develop the small animal model, 30 CBA mice were inoculated intranasally with ERAV.393/76. Organs and urine were collected at necropsy on days 1, 3, 5, 7 and 28 days after infection. Virus load was determined by reverse transcription-quantitative PCR with ERAV specific primers. ERAV was detected in nasopharyngeal swabs from sub-clinically infected horses over 37 days p.i., at levels ranging between 5.1 to 7.1 $\log_{10}$ RNA copies/mL, and also detected in oral swabs and plasma within the first 6 days p.i. ERAV was detected in all urine samples at concentrations between 6.1 to 7.9 $\log_{10}$ RNA copies/mL, except for horse 1 on day 4 p.i. Given the relative volumes secreted at each site, the urine represents the highest load

* Currently; School of Molecular Bioscience, The University of Sydney, Australia

of ERAV excretion. The detection of ERAV in 23% of 215 urine samples collected from Thoroughbred horses post racing, is also consistent with this finding. Following infection of mice, the most consistent detection of ERAV RNA was from the lungs where the viral load peaked on days 1 and 3 p.i. and reduced 10-fold at every 48 hour time point until day 7 p.i. Despite the detection of ERAV in lung cells by immunohistochemistry, histological evidence of pulmonary epithelial pathology was not detected, consistent with the subclinical nature of the infection. However, the detection of ERAV in the urine in the absence of ERAV immunohistochemistry antigen positive bladder or kidney cells, suggests that the source of ERAV in the urine may be associated with the transient viraemia detected at day 1 p.i., rather than with infection in these regions of the genitourinary tract. There are distinct parallels in the pathogenesis of the acute infection of aphthoviruses, where infection in the upper respiratory tract precedes shedding virus from the nasopharynx and a transient viraemic phase before dissemination to distal sites. Maintenance of high levels in the urine of infected horses for at least 37 days p.i., however, is a feature unique to ERAV amongst all picornaviruses.

Duration of nasopharyngeal and nasal virus shedding and infectivity in immunised horses after experimental infection with EIV A/eq2/Richmond/1/07

R. Paillot [1], L. Prowse [1], F. Montesso [1], R. Newton [1], and J.R. Gilkerson [2]
[1] Animal Health Trust, Lanwades Park, Newmarket, CB8 7UU, Suffolk, United Kingdom, [2] Centre for Equine infectious Disease, Faculty of Veterinary Science, The University of Melbourne, Victoria 3010, Australia

Equine influenza (EI) is a major respiratory disease of horses. Despite quarantine procedures to reduce the risk of spread, recent outbreaks of EI in countries previously considered EI-free (South Africa, Australia and Japan) have meant that EI is a major focus of quarantine authorities worldwide, and therefore a considerable impediment to ease of movement of horses internationally. This study aimed to improve our understanding of equine influenza virus (EIV) shedding after infection of vaccinated horses, which would inform possible changes to current quarantine requirements. Our first objective was to compare nasal and nasopharyngeal swabs in Welsh mountain ponies experimentally infected with EI. To inform the duration of the pre-export quarantine procedures, our second objective was to evaluate the duration of detectable levels of virus shedding. The EIV NP qRT-PCR presents a high sensitivity of detection, thus, our third objective was to correlate the levels of virus shedding detected by the EIV NP qRT-PCR in vaccinated animals experimentally infected with EIV with actual EIV infection of naïve sentinel ponies after commingling. Two EIV naïve ponies and 5 EI vaccinated ponies that had received the primary course of a commercial EI vaccine (V1 and V2) were experimentally infected with A/eq2/Richmond/1/07 (Florida clade 2). Challenge infection took place 12 weeks after V2. Nasal/nasopharyngeal swabs were taken daily for 14 days and every 2 days for another 2 weeks. The 5 vaccinates were introduced to 2 naïve sentinel ponies for 48 hrs (transmission 1) on day 2 post-challenge. On day 4, the 5 vaccinates were introduced with a second group of 2 naïve sentinels (transmission 2). On day 6, the 5 vaccinates were introduced with a third group of 2 naïve sentinels (transmission 3). The sentinels were monitored for clinical signs of disease and EIV shedding for 14 days after commingling with vaccinates. Presence of EIV was determined by 3 different methods of detection (EIV NP ELISA, EIV NP qRT-PCR and titration in embryonated hens' eggs). Preliminary results indicated that nasopharyngeal swabs were superior to nasal swabs, as both the amount of virus detected and the frequency of positive samples were increased. The average duration of EIV shedding was 6-8 days in naïve animals. All 3 sentinel groups were infected successfully with EIV after commingling with vaccinates, indicating that vaccinated ponies could transmit EI for up to 6 days. In infected sentinels, EIV was detectable up to 12 days after commingling. Vaccinated horses were infectious for at least 6 days following challenge, and were qRT-PCR positive for at least 8 days, suggesting that tests such as qRT-PCR may be a suitable substitute for time spent in pre-export quarantine.

The population diversity of *Streptococcus zooepidemicus*

C. Robinson [1], S.R. Harris [2], K.F. Steward [1], K. Webb [1], A. Efstratiou [3], A. Darby [4], M.T.G. Holden [2], and A.S. Waller [1]
[1] The Animal Health Trust, Newmarket, UK, [2] The Wellcome Trust Sanger Institute, Cambridge, UK, [3] Health Protection Agency, London, UK, [4] University of Liverpool, UK

Streptococcus zooepidemicus is an opportunistic zoonotic bacterium capable of occupying many pathogenic niches. Multilocus sequence typing (MLST) analysis indicates that *S. zooepidemicus* is highly diverse and suggests that some clusters are associated with infections of particular hosts and tissues (http://pubmlst.org/szooepidemicus/). However, this scheme only samples fragments of seven housekeeping genes and does not permit the identification of novel loci that might play a key role in the infectious process. We sampled the genomes of a diverse collection of 26 *S. zooepidemicus* strains at high density to capture the population diversity of this important zoonotic pathogen. 26 *S. zooepidemicus* isolates recovered between 1996 and 2010 from the UK, Iceland and Spain associated with causing upper (n=5) and lower respiratory (n=5) disease, abortion/uterine infection (n=7), nephritis (n=1) and lymph node abscessation in horses (n=1), meningitis/blood poisoning in humans (n=3), mastitis in sheep (n=1) and acute fatal haemorrhagic pneumonia in dogs (n=3) were sequenced using 454 and Illumina technologies. Sequence data was aligned and compared with the *S. zooepidemicus* strain H70, reference genome, which has been sequenced to completion. The *S. zooepidemicus* population is highly recombinogenic and has acquired novel loci with similarity to those found in a plethora of other bacterial species, including oral and vaginal species, soil organisms,

clostridia, streptococci, pneumococci, staphylococci, enterococci and lactococci. In particular, the gene complement encoding cell wall anchored proteins is highly diverse. Antibiotic and metal resistance have also been acquired by some strains. One clone associated with an eight-year-long outbreak of mastitis in sheep and goats in Spain has acquired novel sugar metabolism components that may play an important role in the exploitation of this niche. Our data highlight the diversity of the *S. zooepidemicus* population, which enables this pathogen to exploit an array of hosts and environments. The association between genetic complement and disease is complex, but particularly successful strains may specialise and become host-restricted. Our data suggest that *S. zooepidemicus* is a mixing vessel for cross-species genetic exchange and that the diversity observed may have implications for the design of effective vaccines and diagnostic tools, which may have to be tailored to specific clonal complexes.

Pili of *Streptococcus equi* and *Streptococcus zooepidemicus*

K.F. Steward [1], C. Robinson [1], D.J. Maskell [2], M.T.G. Holden [3], S.R. Harris [3], A.C. Darby [4], and A.S. Waller [1]
[1] Animal Health Trust, Newmarket, UK, [2] Department of Veterinary Medicine, University of Cambridge, Cambridge, UK, [3] Wellcome Trust Sanger Institute, Cambridge, UK, [4] University of Liverpool, Liverpool, UK

Pili are important virulence factors and potential vaccine targets. *S. equi* contains the pilus locus Fim1, also found in *S. zooepidemicus* isolates MGCS10565 (*Sz*MGCS10565) and H70 (*Sz*H70). However, the gene encoding the TetR-like repressor of Fim1 is a pseudogene in *S. equi*. Three other pilus loci are known to be present in the *S. zooepidemicus* genomes: Fim2 and Fim3 in *Sz*MGCS10565 and Fim4 in *Sz*H70. This study examined regulatory loss, gene deletion and over-expression of Fim1 in *S. equi* and analysed pilus variation in *S. zooepidemicus*. *tetR* was repaired using allelic replacement mutagenesis in *S. equi* strain 4047 (*Se*4047) and transcription of the downstream pilus elements under temperature, nutrient and anaerobic stress conditions quantified by RT-qPCR. A repaired copy of *tetR* was inserted into a pseudogene elsewhere in the genome, over-expressed under control of the iron-dependent regulator for *eqbB* and transcription of the downstream pilus elements quantified by RT-qPCR. Fim1 genes were deleted and complemented in *Se*4047 by allelic replacement mutagenesis and adherence quantified using tracheal explants in an air-interface organ culture system. 22 *S. zooepidemicus* genomes were sequenced and putative pilus loci analysed. Fim4 was actively inverting in *S. zooepidemicus* strain 1770 (*Sz*1770). The importance of the SZO08550-like recombinase on Fim4 inversion was determined following its deletion by allelic replacement mutagenesis and quantification of Fim4 orientation by qPCR. 254 diverse *S. equi* and *S. zooepidemicus* isolates were surveyed by qPCR for the presence of Fim1, 2, 3 and 4. *tetR* repair alone showed no statistically significant impact on Fim1 gene expression, but over-expression of the repaired *tetR* did. Over-expression of Fim1 genes significantly increased bacterial adhesion. One strain of *S. zooepidemicus* had a disruptive inversion of the structural pilin genes of Fim4. Inversion of Fim4 was shown to be mediated by the SZO08550-like invertase. All strains surveyed possessed Fim1, all *S. equi* isolates lacked Fim2, 3 and 4, Fim2 was mostly found with Fim3 and Fim4 was generally mutually exclusive to Fim2 and 3. *tetR* regulates Fim1 pilus transcription. Overproduction of Fim1 pilus increases the ability of *S. equi* to adhere to ciliated columnar epithelium. Fim2 occurrence in *S. zooepidemicus* suggests its more recent acquisition. Inversion of the structural genes of the Fim4 pilus is mediated by the SZO08550-like invertase. Ubiquitous occurrence of Fim1 suggests an important role in attachment and colonisation common to *S. equi* and *S. zooepidemicus*. Multiple pilus loci may cause bacteria to adhere too tightly to surfaces, impeding activity and potentially explaining the exclusivity of Fim4.

Major outbreak of rhodococcosis in adult equidae

J. Tapprest [1], B. Bouyer [2], M. Pannequin [3], C. Sévin [1], J. Cauchard [1], J.L. Le Net [4], F. Duquesne [1], S. Petry [1], A. Lefleche-Mateos [5], A. Hans [1], C. Laugier [1], and F. Moutou [6]
[1] Anses, Laboratory for equine diseases, 14430, Goustranville, France, [2] Veterinary practice, 97680, Tsingoni, Mayotte, [3] Epidemiological surveillance system in animal health, 97670, Coconi, Mayotte, [4] Laboratory of veterinary pathology, 37400, Amboise, France, [5] Institut Pasteur, Paris, France, [6] Anses, Laboratory for animal health, 94700, Maisons-Alfort, France

Disease due to *Rhodococcus equi* (*R.equi*) is rare in adult horses and pyogranulomatous pneumonia is the most common clinical manifestation [1]. A major outbreak of rhodococcosis in adult equidae with extrapulmonary clinical forms is described. Since August 2011, several cases of skin disorders and/or pneumonia have been identified in several adult ponies in the riding school of Mayotte (French overseas department, Indian Ocean). In August, the center had six horses and sixteen ponies. Horses come from Madagascar and have been introduced in Mayotte in 2004. Ponies come from France and have been introduced in 2006. Over a period of eight months (August 2011 to March 2012), ten ponies were affected and eight died. Horses were not affected. Various tests were performed to investigate the cause(s) of these conditions. Glanders and melioïdosis have been notably excluded. In contrast, *R. equi* was isolated from abscesses of five ponies. The strains carry the virulence plasmid containing the gene vap A (PCR) that confers pathogenicity to the bacteria. Confirmation of the involvement of *R. equi* was late (January 2012). This has led to the quarantine of sick animals, disinfection of the contaminated premises and administration of a specific treatment to the five ponies still alive (azithromycin-rifampicin). Two of them had already been sick during more than one month before the beginning of the treatment and died despite it. One died from enterotoxemia following 11 days of treatment. The two others were cured. A one-week mission in March 2012 allowed us to collect comprehensive information, to observe sick animals and

achieve an autopsy and numerous samples (on animals and the environment) for bacteriological, mycological, parasitological, histological and serological tests. Clinical information, results of autopsies and of histological examinations, results of other complementary tests and bacteriological culture of virulent *R. equi* strains on numerous samples allowed confirming an outbreak of rhodococcosis in adult equidae. The occurrence of unusual clinical forms of rhodoccoccosis (including multiple subcutaneous abscesses without pulmonary involvement) affecting exclusively the ponies seems to have a multifactorial origin with the intervention of a lack of acclimatization of the ponies to local climatic conditions (tropical humid lowland region) associated with constant hyperthermia even at rest, maybe some failures in the everyday breeding practices and the probable involvement of biting arthropods.

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Mechanism of Influenza A Virus Mediated Inhibition of IL-23 Expression in Macrophage Cells

A. Tiwari, S.E. Reedy, D.W. Horohov, and T.M. Chambers
Maxwell H. Gluck Equine Research Center, University of Kentucky, Lexington, KY-40546

Influenza A virus (IAV) infection has been associated with an increased susceptibility to bacterial pneumonia. Inhibition of the IL-23/IL17 axis of the innate immune response has been implicated in an increased susceptibility to bacterial pneumonia in IAV infected animals. Recently, IAV induced type I interferons have been proposed to mediate the inhibition of IL-23 in dendritic cells (DCs) in the lungs of mice co-infected with IAV and *Staphylococcus aureus*. However, IAVs have been shown to be potent inhibitors of type I interferon induction. Also, in human DCs, IAV lacking NS1 protein of IAV has been observed to induce both IL-23 and IFNβ. Endoplasmic reticulum (ER) stress induced transcription factor C/EBP homologous protein-10 (CHOP) has been shown to be crucial for expression of IL-23 in human DCs. In murine tracheal epithelial cells IAV infection results in induction of ER stress markers activating transcription factor 6 (ATF6) and ER protein p57 (ERp57) but not CHOP. Therefore, we hypothesized that IAV NS1 protein mediated inhibition of CHOP is responsible for inhibition of IL-23 expression in IAV infected cells. In order to test our hypothesis we used the mouse macrophage like cell line (RAW264.7). We used IAV strain PR/8/34 (PR8) and an isogenic IAV lacking NS1 protein (delNS1) to discern the role of NS1. RAW64.7 cells were infected with either PR8 or delNS1 IAV. Quantitative real-time PCR was performed using validated Taqman assays for mouse IL-23p19, IFNβ and CHOP. Indirect immunofluorescence assay using CHOP specific monoclonal antibody was performed on RAW264.7 cells plated in 24 well plates with glass coverslip inserts and infected with either PR8 or delNS1 virus. In delNS1 virus infected cells we observed significantly higher expression of IL-23p19 and IFNβ as compared to the PR8 virus infected cells ($p < 0.05$). Cells infected with delNS1 virus also showed significantly higher expression of CHOP as compared to the PR8 virus infected cells ($p < 0.05$). Indirect immunofluorescence assay also suggests induction of CHOP in delNS1 infected cells but not in PR8 virus infected cells. From these results it appears that IFNβ has no direct inhibitory effect on IL-23 expression in IAV infected macrophage cells. Additionally, IAV NS1 protein appears to mediate the inhibition of IL-23 and CHOP expression. Also, expression of CHOP appears to be positively associated with IL-23p19 expression. Based on these finding we suggest that IAV NS1 protein mediated inhibition of CHOP contributes to the inhibition of IL-23 expression in IAV infected macrophage cells. This could contribute to the pathology of influenza infection by enhancing susceptibility to bacterial pneumonia.

Rhodococcus equi pneumonia and future racing performance of the Thoroughbred

S.K. Treloar, N.K. Dhand, and G. Muscatello
The University of Sydney, Faculty of Veterinary Science, NSW 2006, Australia

Bronchopneumonia caused by virulent *Rhodococcus equi* is one of the most significant respiratory diseases of foals worldwide. The disease typically affects foals less than six months of age with cases often requiring extensive antimicrobial therapy to resolve pathology and clear the pathogen. The long-term effect of *R. equi* pneumonia on the athletic performance of the horse has been evaluated in small scale studies with reference to national standards and the normal horse, with mixed results. This study, aims to determine the effects of *R. equi* pneumonia on the performance and career length of the racing Thoroughbred, by examining various racing performance outcomes, that not only relate the ability to compete but also the ability to excel at the elite level. A retrospective cohort study was performed, involved a total of 491 Thoroughbred horses, comprising of 125 cases, defined as foals diagnosed with *R. equi* pneumonia based on clinical signs of pneumonia combined with a virulent *R. equi* culture from tracheal wash samples, confirmed through PCR diagnoses between 1993-1999. Controls totaled 366, and where randomly selected and then matched for sire, age/year of birth, sex and where possible breeder. The veterinary records of all controls lacked evidence of confirmed *R. equi* pneumonia as foals. Each case had up to 3 controls. A range of racing performance and career longevity data was collected for cases and controls and compared using REML (quantitative outcomes) or GLMM (binary outcomes). Statistical significance was set at $P < 0.05$.With respect to starting in at least one race, age at first race and probability of racing as a 2 year-old there was no difference between cases and

controls. The mean number of starts and career length in horse affected with *R. equi* pneumonia as foals was significant lower than the control horses (P=0.048, P=0.022 respectively). With respect to prize money, cases general had a trend towards earning less over a career and per start than cases (P=0.06, P=0.07 respectively). Cases won less races than controls (P=0.037), and when evaluating the horses performance in group and listed races, cases won statistically fewer elite races than control (P=0.022), with the odds of a foal with *R. equi* pneumonia winning a group or listed race approximately 4.5 times less then control horses (OR=0.22, 95%CI=0.05, 0.97). These results suggest that clinical *R. equi* pneumonia as a foal negatively impacts upon the career longevity and ultimate capacity to perform as an elite Thoroughbred. Interestingly, the residual effects of the disease appeared had no impact on the ability for the horse to race, even as a 2 year-old. Future prospective studies where reasons for retirement can be noted, occurrence of other diseases as adults or foals and training variables could be evaluated may further clarify the role *R. equi* pneumonia as a foal plays in determining the future athletic performance of the Thoroughbred.

Novel immunoreactive proteins of *Streptococcus zooepidemicus* as vaccine candidates

S. Velineni, and J.F. Timoney
Gluck Equine Research Center, University of Kentucky, Lexington, KY, 40546

Streptococcus zooepidemicus (Sz) of Lancefield group C is an important opportunistic pathogen of the equine respiratory and reproductive tracts. A normal tonsillar and mucosal commensal, it becomes invasive under conditions of stress such as concurrent virus infection, weaning, high temperature, prolonged transportation and failure of uterine involution. Moreover, it has recently emerged as a cause of fatal hemorrhagic pneumonia of shelter dogs in the US, and also in horses on a US race track in 2008. Virulence factors and their regulation are poorly understood. We have identified several surface exposed and secreted proteins in an expression gene library of SzNC78 probed with a pool of convalescent equine sera from pneumonia outbreaks. These proteins have predicted involvement in anti-phagocytosis, adhesion, iron acquisition, protease activity, binding of equine plasminogen, cellular metabolism and rapid bacterial proliferation. The aim of this study was to evaluate the potential of a subset of these proteins as vaccine components. Based on putative functions, sub-cellular localization and possible importance as virulence factors, 12 immunoreactive proteins were cloned, expressed and specific antisera produced. Groups of ICR mice (n = 8) were vaccinated subcutaneously with each recombinant antigen and QuilA and later challenged with SzNC78 and W60. Male mice were found in preliminary tests to be susceptible to 10x fewer CFU of SzNC78 than females and so challenge inocula were adjusted for each sex. Mice that became sick (ruffled coat, depression) were euthanized and their heart bloods cultured on CNA blood agar to confirm bacteremia and susceptibility to challenge. Absence of Sz in heart blood of vaccinated mice for 5 days following I/P was considered as protection. Cumulative morbidity/mortality of vaccinated and control mice (n = 10) were compared to determine relative protective efficacies (Table 1).

In separate vaccination trials, SzM, MAP, MAC and ScpC elicited antibody responses that were highly protective in mice. SzM, a homologue of SeM of *S. equi* protected 100% mice. A membrane anchored protein (MAP), a MAC family protein (MAC) and a chemokine protease (ScpC) each protected 5/8 (62.50%) challenged mice. Only 1/10 (10.00%) of control mice survived challenge. Moreover SzM, MAP and ScpC in combination protected mice against a 10 fold higher dosage of SzNC78 than each antigen given separately. Thus, Sz proteins reactive with convalescent horse sera include potential targets for inclusion in experimental vaccines to aid in reduction of opportunistic invasion by Sz.

Cross-neutralizing reactivity of the antibodies elicited by Japanese equine influenza vaccine strains to the epidemic strains

Takashi Yamanaka, Hiroshi Bannai, Manabu Nemoto, Koji Tsujimura, Takashi Kondo, and Tomio Matsumura
Epizootic Research Center, Equine Research Institute, the Japan Racing Association

Table 1
Resistance of mice vaccinated with recombinant SzNC78 proteins to challenge with SzNC78

Antigen	Localization	Incubation periods (Days)					% Resistant	P-value
		1	2	3	4	5		
M - protein (SzM)	Surface	0	0	0	0	0	100.00	0.0001
Membrane anchored protein (MAP)	Surface	0	1	0	2	0	62.50	0.02
MAC family protein (MAC)	Secreted	0	2	0	0	0	62.50	0.02
Chemokine protease (ScpC)	Surface	0	1	0	1	0	62.50	0.02
Internalin A-like domain containing protein	Surface	1	2	0	1	1	50.00	0.05
Enhancin-like mucinase	Secreted	0	2	1	0	1	50.00	0.05
Enolase	surface	0	2	0	2	3	37.50	0.16
Streptokinase	Secreted	0	7	0	0	0	37.50	0.26
Oligopeptide-binding protein	Surface	0	2	1	3	0	25.00	0.40
Fe^{3+} siderophore receptor	Surface	1	1	1	1	1	25.00	0.40
Control	–	0	4	1	2	3	10.00	–

Equine influenza caused by equine influenza virus (H3N8) is considered the most important respiratory disease of horses due to its rapid transmission. Inactivated vaccines are widely used for limiting the spread of disease and reducing the clinical severity in individual level. However, many outbreaks have been reported among vaccinated horses primarily because of the antigenic differences between the vaccine strains and epidemic strains. It is therefore necessary to periodically review the strain composition of vaccines. The hemagglutination inhibition (HI) test has been widely used for assessing the cross-reactive antibody responses induced by vaccines to epidemic strains. Though there is general agreement between results of HI tests and vaccine efficacies, it remains unclear whether cross-antibody titer measured by the HI test correlates with cross-neutralizing reactivity. Here, we assessed the cross-reactivity of the antibodies elicited by the Japanese local vaccine strains to the current epidemic strains, by virus neutralization (VN) test. We made the horse antisera by inhalations of the Japanese vaccine strains [A/equine/La Plata/1993 (American) and A/equine/Ibaraki/1/2007 (Fc1)] [$10^{8.3}$ 50% egg infectious dose (EID50)/horse each] to horses. Two weeks after the inhalations, each serum was taken from each horse. All the antisera were treated with trypsin-heat-potassium metaperiodate to remove non specific inhibitors before VN tests. Then the required final dilution of treated antiserum (1:8) was prepared and absorbed with packed chicken red blood cells. Two-fold serial dilutions of the antiserum were prepared and added to the equal volume of each virus (approx. $10^{4.0}$ EID50/200 μl). After incubation for 60 min at 34°C, 200 μl of the mixture was injected into an embryonated hen's egg (5 eggs per each serum dilution). After three overnights incubation at 34°C, the allantoic fluids were harvested and examined hemagglutination activities. VN titers were expressed as the log (2) of the reciprocals of the dilution of antisera which reduced infectivity to 1 EID50/200 μl. Whereas the horse antiserum raised to A/equine/Ibaraki/1/2007 (Fc1) showed significantly lower VN titer (6.2) to A/equine/Yokohama/aq13/2010 (Fc2) than that to the homologous virus (9.0), the horse antiserum raised to A/equine/La Plata/93 neutralized A/equine/Yokohama/aq13/2010 well at the similar VN titer (9.3) to the homologous VN titer (9.2). The World Organization for Animal Health (OIE) annually reports the antigenic traits of circulating viruses in the world. It is recommended that vaccines for the international market should contain both Fc1 and Fc2 viruses. Our results however showed that the inoculation of A/equine/La Plata/93, which is one of the Japanese local vaccine strains and not genetically classified into Fc2, can elicit the antibody cross-neutralizing the Fc2 viruses well in horse serum.

Seroprevalence of Equine Rhinitis Virus in Louisiana Horses

F.M. Andrews [1], C. Durussel [2], F. Garza, Jr. [1], P. Loftin [1], S. Zaccarato [2], M.L. Keowen [1], and R. Keene [3]
[1] Equine Health Studies Program, Louisiana State University School of Veterinary Medicine, Baton Rouge, LA, [2] Ecole Nationale Veterinaire De Toulouse, Toulouse, France, [3] Boehringer Ingelheim Vetmedica, Inc. St. Joseph, MO

Respiratory disease is common in race horses and infectious respiratory disease was ranked second, only to colic, among equine practitioners as the most important medical disease concern. The economic impact of viral induced infectious respiratory disease, such as Equine Influenza virus (EIV) and Equine Herpes virus (EHV), on the horse industry is substantial. However, little is known about Equine Rhinitis Virus (serotypes ERV-1 and ERV-2) in racehorses at race tracks and training facilities in Louisiana. The purpose of this study was to determine the seroprevalence of ERV in horses at a racetrack, training center, and a university farm in Southern Louisiana. Blood samples were collected from horses housed at a Louisiana racetrack (n = 77), a Louisiana Thoroughbred training facility (n= 44) and at the Louisiana State University Equine Health Studies Program Thoroughbred and Quarter Horse research and teaching farm (n=55). Samples were allowed to clot and centrifuged at 2000 x g for 15 minutes. Serum from each horse was harvested and placed into 2.0 ml cryogenic vials and stored at -80oC until serum titer determinations for ERV-1 and ERV-2 were made. All samples were shipped by overnight carrier for analysis. Serum neutralization (SN) titers were done using a standard cytopathologic assay performed by the federally accredited lab at the Cornell Animal Health Diagnostic Center in Ithaca, NY. Titers were reported as inverse of the ratio. Serum neutralizing antibodies to ERV-1 and ERV-2 were present in 84.1% and 94.9 %, respectively, of horses at these facilities. Mean SN titers were relatively low for ERV-2 (57.05 +/- 9.49) in horses at these facilities, while SN titers to ERV-1 were significantly higher, ($p < 0.05$), in horses at the racetrack (392.7 +/- 45.9) and in horse at the university farm (535.8 +/- 91.6), when compared to horses at the training center (42.6 +/- 2.3). We concluded that ERV-1 and ERV-2 SN antibodies were present in horses at a racetrack, training center, and a university farm in Louisiana. Also, SN titers to ERV-1 were higher in horses housed at the race track and university farm, compared to horses at the training facility. The difference in SN titers at these facilities was not apparent but may be due to age, as horses at the racetrack and university farm were older allowing more time for virus exposure and antibody production. ERV appears to be prevalent in horses in Louisiana and warrants further epidemiologic investigation to determine its impact on racing and training and the need for vaccine development to reduce impact.

Rhodococcus equi's Extreme Hydrogen Peroxide Resistance is Mainly Conferred by One of its Four Catalase Genes

P. Bidaud [1], L. Hébert [1], C. Barbey [1], Anne-Cécile Appourchaux [1], R. Torelli [2], M. Sanguinetti [2], C. Laugier [1], and S. Petry [1]
[1] ANSES, Dozulé Laboratory for Equine Diseases, Unit Bacteriology and Parasitology, Goustranville, France, [2] Catholic University of Sacred Heart, Institute of Microbiology, Rome, Italy

Rhodococcus equi is the agent of rhodococcosis, one of the most important diseases in foals aged from 1 to 6 months.

Rhodococcosis classically caused severe lung affections as suppurative bronchopneumonia and sometimes unusual forms as digestive or bone forms. *R. equi* is able to survive and multiply in macrophages despite unsuitable conditions, caused notably by reactive oxygen metabolites such as hydrogen peroxide (H_2O_2) generated during the respiratory burst of phagocytic cells. It was known that *R. equi* presents a great capacity of resistance to H_2O_2 exposition independently to the presence of the virulence plasmid. Recently, the analysis of *R. equi* 103 genome revealed the presence of four potential catalase genes named *katA* to *katD*. The aim of this study was to determine the implication of each catalase in *R. equi* oxidative stress resistance. For that, we used the *R. equi* 103 plasmid-less strain to construct four catalase deficient mutants, each lacking one catalase gene to obtain Δ*katA*, Δ*katB*, Δ*katC* and Δ*katD*. Survivals of those mutants were analyzed *in vitro* after exposition to oxidative stress by H_2O_2 treatment and *in vivo* by survival assay in mouse peritoneal macrophages. A quantitative RT-PCR analysis of catalase genes expression levels, in *R. equi* 103 plasmid-less WT, after exposition to H_2O_2, was also performed. We observed that, after exposition to H_2O_2 in stationary or exponential phase, survival of Δ*katA* was respectively completely abolished or decreased by two orders of magnitude, compared to the parental strain. Results showed also decrease by three orders of magnitude in Δ*katC* survival after treatment in exponential phase. *In vivo* experiments in mice macrophages showed that Δ*katA* was the most susceptible mutant and was completely eliminated from macrophages 72h post infection (Figure 1). The others mutants were more susceptible to elimination by macrophages than *R. equi* 103 WT, but were not completely eliminated 48h and 72h post infection (Figure 1). Quantitative RT-PCR results showed that external H_2O_2 exposition only increased *katA* gene expression. After treatment in stationary or exponential phase, *katA* was respectively overexpressed 367.9 (± 122.6) times and 3.11 (± 0.59) times, compared to untreated conditions. Taken together, our results revealed the main implication of KatA and in a less manner, KatC, in resistance to external H_2O_2 exposition. The role of the others catalases of *R. equi* 103 in oxidative stress resistance is not clearly demonstrated in our experimental conditions. In conclusion, this study highlights the main role of *katA* in response and resistance to external oxidative stress in *R. equi* 103 plasmid-less starin.

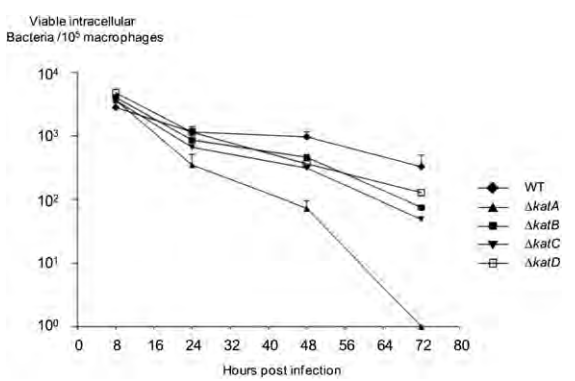

Figure 1. Δ*katA* is the most susceptible to elimination by macrophages.

Risk Factors for high serum SeM titers after natural outbreaks of *Streptococcus equi equi* in horses

A.G. Boyle, M.A. Smith, and R.C. Boston
Department of Clinical Studies New Bolton Center, University of Pennsylvania, School of Veterinary Medicine, Kennett Square, PA 19348

The purpose of this study was to identify the risk factors associated with SeM antibody titers ≥ 1:3200 and the duration which titers remain ≥ 1:3200 after a natural *Streptococcus equi* subsp *equi* outbreak. This prospective cohort study included 57 client–owned horses residing on 3 independent farms convalescing from *S. equi* outbreaks. Blood samples were collected at approximately 6 weeks, and 3, 6, 9, 12 months after the resolution of the last clinical case with sampling extending an additional 6 to 12 months if titer reached ≥ 1:3200. Multivariate logistic regression was used to identify factors associated with having a titer ≥1:3200 and to compare to single sampled titers obtained from a previously studied population of 188 healthy client-owned horses from 26 other farms. 57 client-owned horses (24 clinical, 22 exposed, 11 with no exposure) had a median length of clinical disease was 10 days (IQR 7-21). 3/51 (6%) had titers ≥ 1:3200 at 6 months, 2/49 (4%) had titers ≥ 1:3200 at 9 months, 1/8 (12.5%) had titers ≥ 1:3200 at 20 months, and 0/4 (0%) had titers ≥ 1:3200 at 27 months. Titers peaked at 1:12800. Using multivariate logistic regression to evaluate outbreak horses to healthy horses, the odds of having a titer ≥ 1:3200 is 9.3 times higher if the horse had a history of clinical strangles disease. We recommend not vaccinating for *S equi* within a year of outbreak and testing SeM titers within two years of outbreak prior to vaccinating. Study supported by the Frances Cheney Glover Endowment Fund.

Different strains of *Streptococcus equi* subsp. *equi* isolated from a guttural pouch empyema

C.P. Bustos, A.J. Muñoz, J.A. Picos, E.V. Moras, and N. Guida
Cátedra de Enfermedades Infecciosas, Facultad de Ciencias Veterinarias, Universidad de Buenos Aires, Argentina.
UBACyT 20020100100149

Streptococcus equi subsp. *equi* (*See*) is a gram positive cocoid bacterium, Lancefield group C, ß-haemolytic streptococcus. *See* causes Strangles, a highly contagious infection of the upper respiratory tract and associated lymph nodes. The guttural pouch is commonly infected during the early stages of strangles. A small percentage of animals may continue to harbor infection in the guttural pouch for months after clinical recovery and be a source of contagion for other susceptible horses [1]. It is possible that some guttural pouch empyemas are the result of failure of clearance of *See* following initial infection and are not necessarily derived from retropharyngeal lymph node abscesses that secondarily rupture into the guttural pouch [2].The present study was designed to characterize 3 isolates of *See* phenotypically from a guttural pouch empyema from a horse without another clinical signs. From a guttural pouch empyema was

isolated 3 strains with different colonies sizes on the blood agar plate, UBA8Ch (0.5 mm), UBA8Md (2 mm) and UBA8Gd (4 mm). Expression of capsule was observed by negative staining with India ink solution and with electron microscopy with fosfotungstic acid. Disk diffusion test were used as recommended by Clinical and Laboratory Standards Institute (CLSI). The antibiotic tested were: erithromycin (ERY), penicillin (PEN), ciprofloxacine (CIP), enrofloxacin (ENR), tetracycline (TET) trimethoprim-sulfamethoxazole (TMS), cefotaxime (CTX) and florfenicol (FFC). Results were categorized by using the guidelines recommended by the CLSI 2008, 2010 [3]. The capsule was observed in the 3 strains with optical microscope and with electron microscope. The strains expressed different levels of capsule, UBA8Ch: small sized, UBA8Md: medium sized and UBA8Gd: high sized. The UBA8Ch and UBA8Gd strains were susceptible for all the antimicrobials tested. The UBA8Md strain was intermedia susceptibility for ERY, ENR and TMS. The immune status of the host and the guttural pouch environment could influence in the phenotypic characteristics variation of the strains. It was isolated three strains from a same sample that expressed different levels of capsule, different colonies sizes on the blood agar plate and antibiotic sensibility. This suggests the presence of different clones of *See* in carriers.

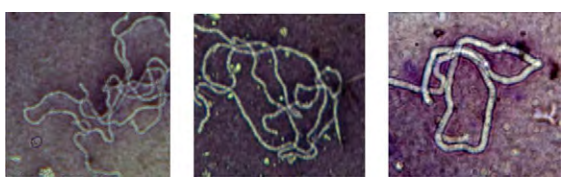

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Figure 1. Capsule by negative staining with India ink solution (100X). UBA8Ch: small sized (left), UBA8M d: medium sized (center) and UBA8Gd: high sized in (right).

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Evaluation of hematologic screening methods for early detection of *Rhodococcus equi* pneumonia in foals

M.K. Chaffin [1], N.D. Cohen [1], G.P. Blodgett [2], and M. Syndergaard [2]
[1] Texas A&M University, College Station, TX, USA, [2] 6666 Ranch, Guthrie, TX, USA

The objective was to estimate the sensitivity and specificity of 3 hematologic screening methods (sequential measurement of white blood cell [WBC], neutrophil [NEUT], and fibrinogen [FIB] concentrations) for early detection of *R. equi* pneumonia. 270 foals were studied at an *R. equi*-endemic farm. Foals were screened every 2 weeks from 3 to 19 weeks of age. Farm personnel were blinded to screening results. Foals were not treated with antimicrobials unless they demonstrated clinical signs of pneumonia. Tracheobronchial aspirates were obtained from all pneumonic foals. 17% of foals developed clinically-apparent *R. equi* pneumonia. Cumulative sensitivities for WBC, NEUT, FIB were 59%, 50% and 59%, respectively. Respective cumulative specificities were 37%, 55%, 33%. Hematologic screening demonstrated limited performance for prediction of subsequent development of clinically-apparent *R. equi* pneumonia.

Lateral thoracotomy for management of recurrent or refractory thoracic abscesses associated with equine pleuropneumonia

M.K. Chaffin, C.E. Arnold, G.K. Carter, T. Norman, and T. Taylor
Texas A&M University, College Station, TX, USA

The objective was to describe case selection, pre-operative assessment, intra-operative methods, findings and complications, post-operative management and long-term outcome and return to athletic function of horses with refractory or recurrent thoracic abscesses (associated with pleuropneumonia) that were treated with thoracotomy. Medical records and all imaging data of such cases at our hospital between 1987 and 2012 were examined retrospectively. Long-term (>2 years) outcome was obtained via recheck examination and/or interviewing owners.27 cases met inclusion criteria. Pre-operative sonographic mapping revealed a compartmentalized abscess in all cases. Surgical approach included partial rib resection in most cases. Bronchial communications were identified in 2/3 of cases, and dead-end tunnels were identified in 1/3. All complications were resolved effectively, and all horses were discharged alive. Long term, thoracic disease was completely resolved in 88% of cases. The others had minor, manageable residual abnormalities. Most horses returned to their previous occupation. Thoracotomy is a valuable method of managing refractory thoracic abscesses. Proper case selection and pre-operative assessment is important for successful outcome. Complications are manageable with proper care. Long-term outcome is good with most horses returning to their previous occupation.

Evaluation of ultrasonographic screening methods for early detection of *Rhodococcus equi* pneumonia in foals

M.K. Chaffin [1], N.D. Cohen [1], G.P. Blodgett [2], and M. Syndergaard [2]
[1] Texas A&M University, College Station, TX, USA, [2] 6666 Ranch, Guthrie, TX, USA

The objective was to estimate the sensitivity and specificity of 3 ultrasonographic screening methods (sequential measurements of pulmonary consolidations including total maximal diameter [TMD], total cross-sectional area [TCSA]

and total number [TNC] of consolidations) for early detection of *R. equi* pneumonia; and 2) to determine the proportion of sonographically-positive foals that progress to clinically-apparent *R. equi* pneumonia. 270 foals were studied at an *R. equi*-endemic farm. Foals were screened every 2 weeks from 3 to 19 weeks of age. Farm personnel were blinded to screening results. Foals were not treated with antimicrobials unless they demonstrated clinical signs of pneumonia. Tracheobronchial aspirates were obtained from all pneumonic foals. 17% of foals developed clinically-apparent *R. equi* pneumonia. Cumulative sensitivities for TMD, TCSA, and TNC were 89%, 83%, and 78%, respectively. Respective cumulative specificities were 62%, 64%, and 64%. 216 (80%) foals developed sonographically-visible pulmonary consolidations, and of those, 21% foals progressed to clinically-apparent *R. equi* pneumonia. Ultrasonographic screening was reasonably sensitive, but had limited specificity for early detection of *R. equi* pneumonia. In 79% of foals with sonographic consolidations, pulmonary lesions resolved without clinically-apparent illness or medical treatment.

A survey of endemic equine herpesviruses in 559 Victorian horses

C.M. El-Hage, C.A. Hartley, and J.R. Gilkerson
Centre for Equine Infectious Disease, Faculty of Veterinary Science, The University of Melbourne, Victoria 3010, Australia

This study aimed to determine the prevalence of EHV-1, -2 and -4 in respiratory samples from a large number of horses both with and without signs of respiratory disease across the state of Victoria, Australia. Nasal swabs were taken from 559 horses during the 2007 equine influenza outbreak in Australia. Criteria for inclusion into the diseased category were horses displaying any or all of the following signs; coughing, fever (temperature $> 38.5\ ^{\circ}C$) or nasal discharge. Horses without apparent respiratory disease were sampled for a variety of regulatory purposes. Nucleic acid was extracted from all swabs prior to quantitative PCR testing for EHV-1, -2 and -4. Correlation between the presence of respiratory disease and detection of herpesvirus was then determined. Of the 559 horses tested, 347 were clinically normal, 151 presented with clinical signs consistent with respiratory disease and 61 horses had no clinical history. Herpesviruses were detected in a minority of the 559 samples collected. EHV-1 was detected in 5 horses (0.9%), EHV-4 in 9 (1.6%) and EHV-2 in 117 horses (20.9%). There was no significant difference (P=0.13) in the proportion of EHV-2 positive samples from horses with (17.9%, 27/151) or without clinical signs of respiratory disease (24.5%, 85/347). At higher EHV-2 loads of $\geq 8.7 \times 10^5$ copies/mL of nasal swab, both diseased and non-diseased groups recorded a prevalence of 7.2 %. Although EHV-2 has been implicated as a predisposing factor to susceptibility to other respiratory diseases [1], infected horses were no more likely to be co-infected with other equine herpesviruses. Two of the nine horses (22.2%) shedding EHV-4 were also excreting detectable EHV-2, and this was comparable to that found in the overall study population (20.9%). Both of these co-infected horses were clinically normal. These findings reinforce the epidemiological significance of the silent cycle of equine herpesvirus infections since approximately 25% of clinically normal horses were EHV-2 positive, as were the majority of EHV-1 and EHV-4 infected horses. Although there was no apparent susceptibility to concurrent EHV infections identified, the clinical significance of EHV-2 infection remains yet to be determined [2-4]. Subclinical infection with any equine herpesvirus has ramifications for prudent horse husbandry, given the potential for disease outbreaks following reactivation of latent infections [4].

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Standardbred foals from large farms predominated among cases of *Rhodococcus equi* pneumonia in Sweden 2010

G. Gröndahl, and E. Mandorf
National Veterinary Institute (SVA), SE-751 89 Uppsala, Sweden

Endemic infection with *Rhodococcus equi* is perceived as mainly a problem of Standardbred farms in Sweden, but the incidence and breed distribution has not been studied before. Data on cases treated for *Rhodococcus equi* pneumonia (REP) in Sweden during 2010 were collected by a prospective questionnaire to 721 Swedish equine practitioners and compared to foal registration statistics from horse breed organizations. Ninety-one REP cases were reported from 147 answering veterinarians. The cumulative incidence of reported REP was 0.8% of the registered foal crop in Sweden in 2010 (n=11,103). The true incidence of REP in foals was higher, as not all veterinarians reported their cases. The distribution of breeds among the cases and in all registered foals in Sweden in 2010 is presented in Figure 1. REP was reported more than 4 times more often in Standardbred foals compared to Swedish Warmblood foals, the two most common horse breeds in Sweden (3,202 and 2,888 foals registered in 2010, respectively).

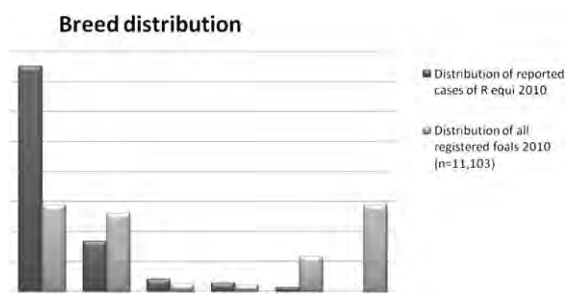

Figure 1. Breed distribution of reported cases of *Rhodococcus equi* pneumonia and of all registered foals in Sweden in year 2010.

Ninety percent of reported REP cases were from large farms (>10 foals). The median farm size for cases was 80 adult horses and 47 foals (no data on yearlings and 2-year-old horses). Ninety percent of the REP cases were born within April to June. Fillies and colts were equally affected. Foals were in median 8 weeks old (1-22 weeks) at diagnosis of REP. The predominance of Standardbred foals among of REP cases in Sweden may be explained by management factors rather than genetic factors. A high number and density of animals, especially young animals, are well-known risk factors for infectious diseases. Standardbred foals are more often kept in large farms compared to foals of other breeds in Sweden. Twenty-one percent of Standardbred foals registered in Sweden in 2010 were from breeders that registered 10 foals or more, compared to only 1% of the Swedish Warmblood foals, and less in other breeds. Despite the limited material in the present study, the observations are in accordance with an epidemiological study in the USA where significant risk factors for *Rhodococcus equi* infections on farm level were farm size ≥50 horses and foals, and ≥15 foals (Cohen *et al*, 2005). Interestingly, the Thoroughbred breed instead of the Standardbred breed was significantly associated to the disease in the American material, and the discrepancy may indicate a lesser impact of breed compared to management. In conclusion, Standardbred foals from large farms dominated the reports of *Rhodococcus equi* infections in Sweden 2010. The large foal/mare groups in many Swedish Standardbred farms are suggested as the main risk factor.

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Reference intervals for equine inflammatory cytokines from bronchoalveolar lavage fluid

S. Hansen [1], J. Fjeldborg [1], K.E. Baptiste [1], and D.W. Horohov [2]
[1] Departments of Large Animal Sciences, Faculty of Life Sciences, University of Copenhagen, Taastrup, Denmark, [2] Gluck Equine Research Center, Department of Veterinary Science, University of Kentucky, Lexington, KY 40546-0099, United States

Airway inflammation is a common problem among sports horses. Equine respiratory diseases represent the second most common reason for lost training days in athletic horses. Airway inflammation can be caused by bacteria, viruses as well as environmental agents such as endotoxin, ammonia, inspired dust and metals. Inflammatory cytokines (e.g. IL-1b, IL-6, IL-8 and TNFα) are secreted primarily from monocytes and macrophages and known to initiate and amplify the inflammatory response. TLR-4 is expressed on alveolar macrophages and the main receptor type responsible for recognition of endotoxin/ LPS. The purpose of this study was to characterize variations in pro-inflammatory cytokines in a homogenous group of clinically healthy horses. Cytokine gene expression was evaluated following three different methods of *in vitro* stimulation. Bronchoalveolar lavages (BAL) were obtained from 53 clinically healthy horses using a three-meter endoscope. In total, three million BAL cells in each well were stimulated with PMA/Ionomycin, LPS and PGN for 24 hours, harvested and transferred to RNA-STAT60 and immediately frozen until processing using the manufacturer's RNA-STAT and reverse transcription protocols. Cytokine gene expression was measured from the cDNA samples using an Applied Biosystems 7900 sequence detection system (Applied Biosystems, Foster City, CA). Cytokines measured were IL-1, IL-6, IL-8 and TNF-α. We also evaluated the expression of TLR-4. Beta-glucoronidase β-Gus was used as the housekeeping gene. The relative quantitation method was used to calculate mRNA expression with the average of the medium control for all horses used as the calibrator for each gene of interest. Statistical comparisons were performed using a two-way ANOVA and multiple comparisons using Tukey's test. Mean age of the horses in the study was 12.1 + 4.75 years, and all were geldings. The mean values for the different pro-inflammatory cytokines are presented in Table 1. Baseline expression (media controls) for each of the genes and for all of the horses were quite similar. As expected, stimulation with the various mitogens resulted in increased expression of the pro-inflammatory cytokines, though with variable effects. Thus, the bacterial products (LPS and PGN) effectively stimulated IL-1 expression, whereas PMA/Ionomycin was a more potent stimulator of TNF-α.

Table 1
Mean values +standard deviation for cytokines expressed as Relative Quantifications values. COV = Coefficient-of-Variation. Within each column, mean values with the same letter are not significantly different. Within each row, mean values with the same symbol are not significantly different.

RQ values/ 53 horses	IL-1β		IL-6		IL-8		TNFα		TLR-4	
	Mean ± S.D	COV	Mean ± S.D	COV	Mean ± S.D	COV	Mean ± S.D	COV	Mean ± S.D	COV
Media	$^{\#}1.37^{C} \pm 1.2$	84.4	$^{\#}1.49^{C} \pm 1.6$	106	$^{\#}1.19^{B} \pm 0.9$	72.6	$^{\#}1.70^{A} \pm 1.5$	85.7	$^{\#}1.20^{B} \pm 0.8$	65.0
PMA/ Iono	$^{\#¤}4.92^{B} \pm 5.1$	103	$^{\#}7.73^{A} \pm 7.1$	91.8	$^{\#¤}3.83^{A} \pm 2.8$	74.0	$^{\#¤}7.75^{B} \pm 7.9$	103	$^{\&}1.75^{A} \pm 1.2$	65.6
LPS	$^{\#}10.60^{A} \pm 5.1$	47.6	$^{¤}3.13^{B} \pm 3.1$	97.5	$^{¤}2.82^{A} \pm 1.4$	49.4	$^{¤}4.30^{A} \pm 3.9$	85.1	$^{\&}1.47^{AB} \pm 1.4$	82.7
PGN	$^{\#}10.58^{A} \pm 5.4$	50.8	$^{¤}3.22^{B} \pm 2.9$	88.6	$^{¤}2.65^{A} \pm 1.1$	41.6	$^{¤}4.46^{A} \pm 4.3$	96.8	$^{\&}1.56^{AB} \pm 1.1$	67.5

Establishing reference intervals for pro-inflammatory cytokines following stimulation with mitogens can be challenging because of the variability (coefficient-of-variation) between horses. This group of horses represents an excellent resource to use for the establishment of reference values because they are homogenous in stabling conditions, daily handling and exercising.

Development of a peptide ELISA for discrimination between serological responses to equine herpesvirus type 1 and 4 (EHV-1 and EHV-4)

A. Lang [1], S. Feines [1], E. Müller [2], N. Osterrieder [1], and A.M. Damiani [1]
[1] Institut für Virologie, Freie Universität Berlin, Philippstraße 13, 10115 Berlin, Germany, [2] LABOKLIN GmbH, Bad Kissingen, Germany

Infections with equine herpesviruses type 1 (EHV-1) and 4 (EHV-4) can result in important economic losses for the horse industry worldwide. Both viruses are common causes of acute respiratory disease, while EHV-1 frequently induces abortion but also perinatal foal mortality and devastating neurological disease. EHV-1 and -4 are closely related alphaherpesviruses. Detection of EHV antibodies in serum is a common tool to determine whether a horse has been exposed to EHV. Despite the differences in their biological characteristics and genomic sequences, both viruses show strong antigenic cross-reactivity in any immunological assay that uses polyclonal serum as the source of antibodies. An effective serological tool capable of discriminating between antibodies responses to EHV-1 or EHV-4 in horses is of importance in disease control. In the present study, we describe the development and application of a type-specific EHV-1/EHV-4 ELISA based on peptide antigens. Seven and four peptides for EHV-1 and EHV-4, respectively, were initially studied with respect to their discriminatory potential in an ELISA setup using sera from experimentally infected foals. In our optimized protocol, plates were coated overnight at 4°C with 100 μl/well streptavidin at 1 μg/ml in 50 mM carbonate/bicarbonate buffer (pH 9.6), washed 3 times with washing buffer (PBS containing 0.1% Tween 20) and coated with 100 μl/well of respective biotinylated peptide at a concentration of 2 μg/ml in carbonate/bicarbonate buffer. After incubation for 2 h at 37°C and extensive washing, wells were blocked with 100 ul/well of blocking buffer (1% goat serum in washing buffer) for 1 h at 37°C. After washing, 100 μl of horse serum diluted 1/400 or 1/1200 in washing buffer was added in triplicate and incubated for 1 h at 37°C. After 3 further washes, plates were incubated with 100 μl/well of HRP goat anti-horse immunoglobulin diluted 1/20,000 in blocking buffer for 1 h at 37°C. Following a final wash, the color reaction was developed for 10 min at room temperature by adding 100 μl/well of a chromogen/substrate mixture of TMB (240 μg/mL 3,3',5,5' tetramethylbenzidine) in Gallati buffer (42 μg/mL citric acid, pH 3.95/0.01% H_2O_2). The reaction was stopped with 100 μl/well of 1 M sulfuric acid and the optical density measured at 450 nm. Results were expressed as absorbance OD values and sera tested in triplicate against the EHV-1 and EHV-4 antigen. The most promising pair of peptides, EHV-1 glycoprotein E and EHV-4 glycoprotein G peptides, was further evaluated using acute and convalescent sera from experimentally and naturally infected horses (Figure 1) as well as a panel of field sera. The results show that our peptide ELISA clearly identifies horses that have been infected with EHV-1 or EHV-4 using acute and convalescent sera and, when applied to a large number of field samples, revealed to be a robust assay for determining the EHV-1 and EHV-4 antibody status. With further validation, the developed EHV-1/EHV-4 peptide ELISA could serve as an effective and cheaper alternative to other current tools for EHV-1 and -4 serodiagnosis.

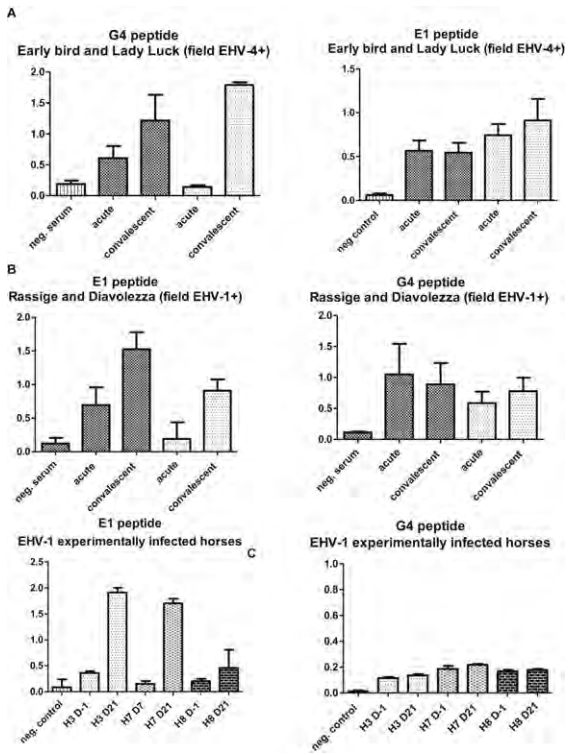

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Figure 1. Peptide-ELISA results using sera from EHV-4 (A) and EHV-1 (B) naturally infected horses. Results from EHV-1 experimentally infected horses are shown in C.

Surveillance of equine influenza viruses through the RESPE network in France from November 2005 to October 2010

L. Legrand [1,2], P.H. Pitel [1], C. Marcillaud-Pitel [1,3], A. Courouce-Malblanc [3,4], A. Cullinane [5], G. Fortier [1,2], F. Freymuth [6], and S. Pronost [1,2]
[1] Frank Duncombe Laboratory, Animal Health Department, 14053 Caen, France, [2] University of Caen Lower Normandy EA 4655 U2RM 14000 CAEN France, [3] Acute Respiratory Syndromes Sub-network, RESPE Association Caen, Normandy, France, [4] Oniris, ENV, Agroalimentaire et de l'alimentation de Nantes Atlantique, Laboratoire de physiologie animale et de pharmacologie fonctionnelle, Atlanpole, Nantes France, [5] Virology Unit, Irish Equine Centre, Johnstown, Naas, County Kildare, Ireland, [6] Laboratory of human and molecular virology, University Hospital, 14033 CAEN France

Outbreaks of equine influenza (EI) in endemic population cause economic loss despite a greater surveillance and vaccination. According to the Federation Equestre Internationale (FEI), all horses attending to their competitions have to be vaccinated in the previous 6 months. Vaccination

reduces symptoms and limits virus propagation, but doesn't completely protect against new infection. Therefore, equine influenza virus (EIV) surveillance is essential as an early warning system to veterinary practitioners, professionals within the equine industry and the concerned institutions. RESPE (French epidemiological network for equine diseases) is the first European network for epidemio-surveillance of major equine diseases based around sentry veterinarians in France. The aim of this present study was to demonstrate how the RESPE had contributed to a more efficient surveillance of EI in France since 2006. 920 naso-pharyngeal swabs sent by RESPE-associated veterinarians were analysed. 582 respiratory samples were sent by non RESPE-associated veterinarians. RNA was extracted from 140 μL of respiratory fluids with the QIAamp Viral RNA minikit. Detection was performed by rRT-PCR amplification targeting the M1 gene. Phylogenetic analysis was performed after a RT-PCR amplification targeting the H1 fragment of hemagglutinin (H3) gene, directly on RNA extracted from nasal swab. Among 920 samples sent by RESPE-associated veterinarians, 121 (13.1%) were positive for EIV divided into 42 premises. Horses infected by EIV were mainly those participating to equestrian manifestations with a majority of French trotters (44.6%), French saddle ponies (23.1%) and ponies (9.9%). Among the 582 samples received from non RESPE-associated veterinarians, 26 (5.1%) EIV positive horses were detected. The repartition of EIV positive cases is clearly related to the localization of the RESPE veterinarians. The last important outbreak was observed between February and May 2009. 70 horses were found positive by rRT-PCR. Fifteen of the 23 premises, including the Grosbois training yard (index premise), were managed by RESPE-associated veterinarians. The HA1 nucleotide sequence was completely determined on 39 strains and partially on 8 others. All strains analyzed in this study belonged to the American lineage, Floridian sublineage, Clade 1 and Clade 2. Clade 1 was identified only during the Grosbois episode. The findings of this study demonstrate that the analysis realized by the RESPE allowed detecting more EIV in France. RESPE also allow characterization of the strains and participate to epidemiological and vaccine efficacy survey. Implementation of this type of network in other countries should reduce the economic losses associated with outbreaks of EI.

A clonal outbreak of upper respiratory disease in horses caused by *Streptococcus equi* subsp. *zooepidemicus*

S. Lindahl [1,2], A. Aspán [1], V. Båverud [1], H. Ljung [1], R. Paillot [3], J. Pringle [2], R. Söderlund [1], N.L. Wright [3], and A.S. Waller [3]
[1] Dept of Bacteriology, National Veterinary Institute, SE-751 89 Uppsala, Sweden, [2] Dept of Clinical Sciences, Swedish University of Agricultural Sciences, SE-750 07 Uppsala, Sweden, [3] Centre for Preventive Medicine, Animal Health Trust, Lanwades Park, Kentford, Newmarket, Suffolk CB8 7UU, United Kingdom

Streptococcus equi subsp. *zooepidemicus* (*S. zooepidemicus*) is considered a commensal of the upper airways in horses and an opportunistic pathogen causing respiratory disease. The most serious and contagious bacterial upper respiratory disease in horses is strangles, caused by *Streptococcus equi* subsp. *equi* (*S. equi*). While *S. equi,* believed to have evolved from *S. zooepidemicus,* has a relatively conserved genome, *S. zooepidemicus* displays wide genetic variation between different strains. The importance of this genetic diversity to the pathogenicity of different strains is currently being investigated, as it appears that strains of *S. zooepidemicus* can differ in virulence. Genetic differentiation of *S. zooepidemicus* strains can be performed by sequencing of the M-like SzP protein gene (*szP*), which has been shown to vary greatly between different strains of *S. zooepidemicus*, and by multi-locus sequence typing (MLST). Questions of major clinical importance are whether certain strains of *S. zooepidemicus* can cause upper respiratory disease as a host-specific pathogen in horses, and if there are certain genogroups of *S. zooepidemicus* that are more virulent than others. Here, we describe an outbreak of upper respiratory disease in a group of horses that could be related to one strain of *S. zooepidemicus*. An outbreak of upper respiratory disease occurred in a herd of 17 Icelandic horses in a loose housing system. Twelve of the 17 horses were selected for bacterial sampling during the outbreak, of which ten horses displayed clinical signs of respiratory disease including swollen submandibular lymph nodes, serous to purulent nasal discharge, fever, anorexia, and depression. Two of the selected horses displayed no signs of respiratory disease. Paired serum samples were collected from all 17 horses. Bacterial sampling was repeated twice after the outbreak had subsided. Bacteriological samples were cultured and analyzed by real-time PCR for *S. equi* and *S. zooepidemicus*. Several colonies were isolated from each horse and analyzed by *szP* sequencing and MLST. Isolated strains were further investigated for mitogenic activity and the presence of *S. zooepidemicus* superantigens SzeF, SzeN and SzeP. Serum samples were analyzed for antibodies against *S. equi* and four common viral respiratory pathogens. All horses were positive for *S. zooepidemicus* in upper airway bacterial samples collected during the outbreak. All horses with clinical signs of disease carried the same strain, while the healthy horses carried other strains of *S. zooepidemicus*. None of the horses were positive for *S. equi.* Bacteriological results and serological analyses support that the isolated *S. zooepidemicus* strain is likely to be responsible for the disease outbreak.

Diversity of superantigens in *Streptococcus equi* and *zooepidemicus* populations

R. Paillot [1], N. Wright [1], R. McLean [1,3], A. Darby [2], E. Anderson [1,3], K. Steward [1], K. Webb [1], S. Priestnall [3], K. Erles [3], K. Smith [3], A. Efstratiou [4], M. Holden [5], C. Robinson [1], and A. Waller [1]
[1] Animal Health Trust, Newmarket, UK, [2] University of Liverpool, Liverpool, UK, [3] Harper Adams University College, Shropshire, UK, [4] Health Protection Agency, London, UK, [5] Wellcome Trust Sanger Institute, Cambridge, UK

The horizontal transfer of mobile genetic elements and their associated cargo genes between streptococcal species can significantly alter the virulence properties of recipient

strains. Gain of prophage elements by *Streptococcus pyogenes (S. pyogenes)* that contain superantigen-encoding genes has been linked with increased morbidity and mortality in humans. Superantigens are powerful toxins that induce uncontrolled lymphocyte proliferation and release of pro-inflammatory mediators. This report reviews current knowledge about *Streptococcus equi* (*S. equi*) and *Streptococcus zooepidemicus* (*S. zooepidemicus*) superantigens. *Streptococcus equi* is a host-restricted equid pathogen that causes a disease characterised by the abscessation of the lymph nodes. *Streptococcus equi* culture supernatant presented the ability to induce equine T lymphocyte proliferation *in vitro*. Mitogens were also detected in retropharyngeal lymph node abscess material and guttural pouch chondroids from horses affected with Strangles. This mitogenic activity is induced by four phage-associated bacterial superantigens (SeeH, SeeI, SeeL and SeeM) produced by *S. equi*. These *S. equi* superantigens share homology with the mitogenic toxins of *S. pyogenes.* Recombinant SeeI, SeeL and SeeM induced proliferative and cytokine responses in equine T cells, while SeeH stimulated asinine T lymphocytes only. This difference of activity illustrates possible differences of equid MHC class II preferences. Allelic replacement mutants of *S. equi* strain 4047 with sequential deletion of the superantigen genes were also generated. Deletion of seeI, seeL and seeM completely abrogated the mitogenic activity of the strain 4047 culture supernatant in equine T cells. In horses, *S. equi* superantigens were natural targets of immunity as specific antibodies were detected after *S. equi* infection. However, their neutralising activity *in vitro* was limited. The acquisition of φSeq3 (containing *seeL*, *seeM*) and φSeq4 (containing *seeH*, *seeI*) by *S. equi* probably represents key steps in the evolution of *S. equi* from an ancestral *S. zooepidemicus* strain. *Streptococcus zooepidemicus* is the most frequently isolated opportunistic pathogen of the horse but also infects a wide range of other animal species including humans. We have recently identified four novel active superantigens (SzeF, SzeN, SzeP and SzeQ) in several strains of *S. zooepidemicus*. Their presence was significantly associated with isolation from cases of non-strangles lymph node abscesses in the horse (p=0.000367). Furthermore, 15 isolates from human cases of *S. zooepidemicus* infection were screened and several contained the novel superantigen genes. We believe that *S. zooepidemicus* superantigens are of importance to both animal and human health.

Investigation of the role of lesser characterized respiratory viruses associated with upper respiratory tract infections in horses

N. Pusterla, S. Mapes, C. Wademan, A. White, and E. Hodzic
School of Veterinary Medicine, University of California, Davis, CA 95616, USA

The aim of this study was to investigate the frequency of lesser characterized respiratory viruses (EHV-2, EHV-5, equine rhinitis A (ERAV) and B (ERBV), equine adenovirus 1 (EAdV1)) in nasal secretions of horses with confirmed causes of IURD (EHV-1/-4, EIV, *Streptococcus equi* subsp. *equi*) and horses with signs of IURD but no detected common respiratory pathogens. Horses with IURD were selected from the diagnostic submissions to the Real-time PCR Research and Diagnostics Core Facility at Davis from January 2009 to December 2011. Case selection included horses with either EHV-1 (40), EHV-4 (40), EIV (40) or *S. equi* subsp. *equi* (44) infection confirmed by real-time PCR testing. Only horses with reported clinical signs of fever and nasal discharge were retained as study cases. Further, an additional 172 horses with fever and nasal discharge but negative results for EHV-1, EHV-4, EIV and *S. equi* subsp. *equi* by real-time PCR served as IURD control group. For each selected study case, nucleic acid was available and stored at -80 °C until used. Nucleic acid from nasal secretions was assayed for the presence of EHV-2, EHV-5, ERAV, ERBV and EAdV1 using previously reported real-time TaqMan PCR assays. Absolute quantitation of EHV-2 and EHV-5 target molecules was performed using standard curves for EHV-2/-5 and eGAPDH and expressed as EHV-2 or EHV-5 gB gene copies per million cells. The most commonly detected virus was EHV-5, followed by EHV-2, EAdV1 and ERBV. ERAV was not detected in the nasal secretions of any of the study horses. Statistical difference in EHV-2 and EAdV1 distributions were determined between EHV-4 and EIV group horses (Pearson's chi-square test, $P = 0.04$) and between *S. equi* subsp. *equi* and IURD group horses ($P = 0.01$), respectively. Significantly higher EHV-2 viral loads were determined in IURD horses compared to horses form the *S. equi* subsp. *equi* group (Wilcoxon-Mann-Whitney tests; $P < 0.01$). EHV-5 viral loads were significantly higher in horses from the EHV-4 group compared to the additional 4 groups ($P < 0.02$). In conclusion, the result of this study showed that EHV-2 and EHV-5 were commonly detected in respiratory secretions of horses with confirmed causes of IURD and horses with signs of IURD but no detected common respiratory pathogens. In view of the study results, it still needs to be determined if the detection of EHV-2 and/or EHV-5 in horses with IURD relates to a primary pathogenic effect, reactivation of latent stage or to modulation of the host immune response predisposing horses to co-infections. The detection of EAdV1 mainly in adult horses represents a likely incidental finding with no direct clinical impact. Although less frequently detected in the study population, ERBV appears to be an important respiratory virus associated with IURD alone or combined with other more common respiratory pathogens.

Induction of Xenophagy in Pulmonary Alveolar Macrophages by Infection with *Rhodoccocus equi*

P.C. Shivalingappa, and B.A. Sponseller
College of Veterinary Medicine, Iowa State University, Ames, IA

It has been well documented across species that the early postnatal period is characterized by an increased susceptibility to infectious diseases, particularly those caused by intracellular pathogens, suggesting a functional deficit in cell-mediated immunity. Similarly, foals frequently succumb to pulmonary pathogens, including *Rhodococcus equi*, an intracellular macrophage-tropic pathogen with potential for causing a fatal pyogranulomatous pneumonia. Within the past decade, autophagy of foreign microorganisms (xenophagy) has become increasingly recognized

as an important innate defense mechanism, particularly with respect to intracellular bacterial pathogens [1]. Recent studies have demonstrated that *specific induction of autophagy can overcome* inhibition of phagosomal maturation and lead to suppression of intracellular *M. tuberculosis* load, indicating promising clinical outcomes by enhancement of innate mechanisms of xenophagy [2]. Following autophagy induction, Beclin-1 and its binding partner class III phosphoinositide 3-kinase (PI3K) are necessary for initiation of autophagosome formation [3], inferred by detection of microtubule-associated protein light chain 3 (LC3), which exists as a cytosolic protein (LC3-I) until induction of autophagy whereupon a lipidated membrane bound protein is formed (LC3-II) [4]. For this study, 1 X 10^6 cells were seeded in T-25 flasks. Cells were infected with *R.equi* (moi 5) for 2, 6, 12, 24 and 48 hours. For each time point, duplicates were considered. After the infection, cells were collected and resuspended in 150 μl homogenization buffer, sonicated and centrifuged at 14,000 g for 20 minutes at 4^0C. Following estimation of proteins through Bradford assay, proteins were separated by 10 - 15% SDS-PAGE. Membranes were incubated with 1:4000 Rabbit polyclonal LC3 (Abcam), 1:1000 Rabbit polyclonal Beclin-1 (Santa Cruz) and Mouse monoclonal 1:5000 β-actin (Sigma) primary antibodies. Later, the membranes were washed with PBS-T and incubated with IRdye 800 conjugated anti-rabbit (1:5000) and Alexa 680 conjugated anti-mouse (1:15,000) secondary antibodies for an hour. Western blot images were captured and analyzed with an Odyssey IR Imaging system (LICOR). Our data suggest the induction of cellular autophagy machinery in macrophages infected with *R.equi*. Understanding signaling pathways involved in the regulation of autophagy is important in developing new strategies to abrogate infection with *R.equi*.

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"Strangles" in less regulated sectors of the Irish horse industry

N. Walshe, J. Johnston, E. MacCarthy, and V.E. Duggan
School of Veterinary Medicine, University College Dublin, Ireland

Thousands of equine events take place in Ireland every year which are not regulated. The potential for contagious disease transmission both at these events, and when horses return from these events to their farm of origin, is immense, particularly for diseases such as "Strangles" *(Streptococcus equi* sub-species *equi* infection), where persistent subclinical carrier status exists and transmission by direct and indirect contact may occur. This disease has major economic and equine welfare implications for the equine industry. Over 2 summers, 31 premises were attended: riding schools, stud farms, pony club events, unregulated horse fairs, Harness racing meetings, Harness racing training yards, competition yards, a youth community horse project and a horse pound. A list of appropriate questions was assembled to gather information about awareness of the disease among owners. Jugular blood samples were collected from 319 randomly selected clinically normal horses on the premises with owner permission and under licensure and animal research ethical approval. Samples were analysed at the Animal Health Trust for antibodies against two antigens unique to *Streptococcus equi* subsp *equi.* Among horse owners in the less regulated sectors of the industry, awareness of the disease and the common clinical signs was apparent. However, knowledge of its epidemiology, treatment, prevention and control was poor. Of the 319 clinically normal horses tested in this study, 133 (42%) tested positive for antibodies to the *Streptococcus equi* subsp *equi* antigens, suggesting exposure to "Strangles" either recently or during the previous 6 months. Among the less regulated sectors of the horse industry, in depth knowledge of "Strangles" infection in horses is lacking, and exposure to the bacteria appears to be widespread even in clinically normal horses.

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Mutations in *S. zooepidemicus* superantigens *szeF* and *szeQ:* a functional characterisation

N. Wright, C. Robinson, A. Waller, and R. Paillot
Animal Health Trust, Lanwades Park, Kentford
Newmarket, Suffolk, United Kingdom

Streptococcus equi subspecies *zooepidemicus* (*S. zooepidemicus*) is the most frequently isolated opportunistic pathogen of the horse, and is able to cause a variety of infections in a broad range of species. Superantigens are bacterial toxins that trigger unspecific T-cell proliferation and overzealous inflammatory cytokine production in the host. Numerous strains of *S. zooepidemicus* have been found to have mitogenic activity *in vitro*. We have recently discovered and characterised *in vitro* 4 novel *S. zooepidemicus* superantigens*: szeF, szeN, szeP,* and *szeQ,* and determined their prevalence in 213 diverse strains. Stimulation of equine PBMC with supernatants from these strains showed that presence of *szeN* and *szeP* was significantly associated with mitogenic activity ($p=<0.000001$). However, presence of *szeF* was not associated with T cell proliferation ($p=0.610$), despite recombinant SzeF being active *in vitro*. One aim of this study was to sequence *szeF* across the population to explain the absence of mitogenic activity measured in most *szeF* positive *S. zooepidemicus* strains. We also aimed to further characterise functionality of the most recently discovered *S. zooepidemicus* superantigen; *szeQ.* 423 isolates representing 213 diverse sequence types (ST's) of *S. zooepidemicus* were screened for the presence of the 4 novel superantigen genes using qPCR. Not all strains

containing *szeF* were mitogenic, so the gene was sequenced where present. A number of mutations were found, and recombinant proteins of these forms were produced in pGEX3X. These were used to stimulate equine PBMC to ascertain whether these mutations affect gene expression and/or functionality. Recombinant SzeQ was produced and its functionality characterised in vitro. The *szeQ*-TCR binding site was characterised by site directed mutagenesis. 56.8% of strains screened contained genes encoding these novel superantigens; 24.4% possess *szeF*, 25.3% *szeN*, 26.7% *szeP* and 1.4% *szeQ.* We have previously shown that presence of *szeF* alone was not associated with mitogenicity on equine PBMC despite activity in a recombinant form. Sequencing revealed a gene encoding the full length SzeF in only 45.1%. The remaining sequences contained a number of single nucleotide polymorphisms, deletions or insertions causing frameshift or non-synonymous mutations. These modifications fell into 4 main groups. Recombinant mutated forms were expressed from each of the 4 groups and used to stimulate equine PBMC, with many exhibiting no/reduced mitogenic activity. These mutations explained the absence of mitogenic activity from the culture supernatants and also tend to indicate that *szeF* is maybe not essential to *S. zooepidemicus*. Recombinant SzeQ is active *in vitro* with a median half-maximal proliferation response (P_{50}) of 130±2 pg/ml. Introduction of mutations into SzeQ highlighted a region of the protein which may be responsible for TCR binding, with 2/5 mutants being completely inactive, and one showing significantly reduced activity. Function was completely restored in a reversion mutant. This study highlights the diversity of superantigen genes present in *S. zooepidemicus* population.

Streptococcus equi infection increases Equine herpesvirus-4 antibody levels *in vivo*

N. Wright, N. Hamzianpour, C. Robinson, N. Butcher, A. Waller, and R. Paillot
Animal Health Trust, Lanwades Park, Kentford Newmarket, CB8 7UU, Suffolk, United Kingdom

Equine herpesviruses type 1 and 4 (EHV-1/4) are the most prevalent equine respiratory pathogens and can cause a variety of symptoms from mild respiratory disease to encephalomyelopathy and abortion storm. EHV establishes latency in several locations including the respiratory lymph nodes. Its reactivation may result in shedding; contributing to the re-emergence and spread of the disease. Factors involved in reactivation of EHV are still largely unknown, but involve stressful events such as transport, castration, weaning etc... *Streptococcus equi (S. equi)* is endemic in most UK horse populations. It is the causative agent of Strangles, an infection resulting in the formation of abscesses in the lymph nodes of the head. Supported by preliminary results, this study aimed to investigate a potential relationship between *S. equi* infection and EHV immunity *in vivo*. The antibody responses specific to EHV types 1 and 4 have been analysed in serum samples from 554 horses of 29 different UK yards involved in natural outbreaks of Strangles. The EHV-1/4-specific glycoprotein G ELISA has been used for this purpose. To investigate the potential relationship between *S. equi* pathogenic factors and EHV infection/reactivation, recombinant *S. equi* superantigen SeeM was used to stimulate equine PBMC. The virus EHV-1 Dgp2 GFP (EHV-1-GFP), which is derived from the plaque-purified strain Ab4/13 with the GFP gene inserted instead of the gene encoding glycoprotein 2, was used to infect equine PBMC *in vitro*. The seropositivity for EHV-1 and EHV-4 was 21.4% and 95.7%, respectively, in the studied population. Interestingly, 20% of horses seroconverting to *S. equi* have simultaneously shown a statistically significant increase in their level of EHV-4 antibody (p-value=0.011) when compared to results obtained in a group of 132 horses free of *S. equi* infection. EHV-4 antibody levels remained stable in later stages of *S. equi* infection and recovery (p-value=0.46). The increased level of EHV-4 antibody observed during the early stage of Strangles could be associated with either EHV-4 re-infection and/or reactivation of latent EHV-4 infection. The record of clinical signs after *S. equi* infection did not reveal an association between EHV-4 seroconversion and increased severity of diseases in natural outbreak of strangles. *In vitro* investigations have also revealed that *S. equi* superantigens increased the frequency of GFP+ PBMC after infection *in vitro* with EHV-1-GFP. This result was consistent with previous reports about the impact of mitogens on cell susceptibility to EHV-1 *in vitro*. This report suggests a relationship between *S. equi* infection *in vivo* and stimulation of EHV-4 immunity. Both *S. equi* and EHV-4 are equine respiratory pathogens. On some occasions, EHV-4 has been found to establish latency in lymph nodes, which are also targeted by *S. equi* infection. Therefore, one or several *S. equi* pathogenic factors may have increased susceptibility to re-infection and/or recrudescence of latent EHV-4 infection/reactivation.

Immunology

Deacylated Polyethyleneimine (PEI) and a Modified Equine IL-15 Expression Construct Enhance Adaptive Immune Responses to DNA Vaccination in Horses

D.L. Even, C.J. Issel, S.J. Cook, D.W. Horohov, and R.F. Cook
Department of Veterinary Science, Gluck Equine Research Center, University of Kentucky

A major limitation of DNA vaccines is that although they can produce strong protective immunity in mice, immune responses in large animals are almost invariably significantly weaker. This is because immunization with unprotected plasmid vector DNA is an extremely inefficient process. Even in the successful mouse model, significant amounts of DNA are detectable in almost all

tissues just one hour after an intramuscular inoculation. Furthermore, in contrast to the relative stability at the muscle injection site where DNA is detectable up to eight weeks, nucleic acids distributed by the circulatory system are rapidly degraded. In horses where blood volumes are at least 20,000 fold higher than in mice losses of vector DNA from the injection site and resultant exposure to nucleases will be significantly greater and almost certainly contribute to the poor immunogenicity of genetic vaccines observed in this species. This problem is further exacerbated by the fact that even if DNA molecules survive to enter a cell, their translocation from the cytoplasm to the nucleus is a "perilous journey" with 50% of all unprotected DNA degraded within two hours. Therefore, it was hypothesized that complex formation of plasmid vector DNA with PEI would improve intracellular uptake plus increase resistance to *in vivo* nucleases and therefore significantly enhance adaptive immune responses to gene-based vaccinations in the horse. Additionally, it was predicted, based on results from different animal species, that immune responses to DNA vaccines in horses will be further augmented by co-administration of expression vectors encoding codon-optimized equine Il-15 (SYNEIL-15). In these experiments a plasmid mammalian expression vector (pCI) containing a codon-optimized synthetically produced (SYN) gene encoding the surface unit (SU) glycoprotein of equine infectious anemia virus (EIAV) was used as a model antigen. Horses (3 per group) were immunized (4 vaccinations at 0, 2, 12, 24 weeks) with pCISYNSU, pCISYNSU+PEI, pCISYNSU+PEI+SYNEIL-15 or pCISYNSU+PEI+a fusion protein between the sushi domain of the EIL-15 α-receptor and SYNEIL-15 (Sushi15). All plasmids were administered at 500μg per dose and PEI was used at an N:P ratio of 10. Biological activity measured by horse lymphocyte proliferative responses was undetectable in cell culture fluids from HEK cells transfected with sequences encoding native EIL-15. However lymphocyte proliferation was stimulated using culture fluids at dilutions or 1:20 and $\geq$1:1280 from HEK transfected with SYNEIL-15 and Sushi15 respectively. Immune responses were statistically significantly enhanced in pCISYNSU+PEI vaccinated horses compared to those receiving pCISYNSU alone with evidence that immune responses could be further augmented by SYNEIL-15. Interestingly, responses in pCISYNSU+PEI+Sushi15 recipients were similar to those immunized with just pCISYNSU. PEI and SYNEIL-15 can enhance immune responses to DNA vaccines in the horse. Apparent suppression of immunity by Sushi15 is consistent with results reported for situations when IL-15 is over-expressed.

Equine Arteritis Virus replication in monocytic cells suppresses differentiation and function of dendritic cells

N.A. Moyo [1,2], D. Westcott [1], and F. Steinbach [1]
[1] Virology Department, Science Division, Animal Health and Veterinary Laboratories Agency, Weybridge, UK,
[2] Dept of Microbial & Cellular Sciences, Faculty of Health and Medical Sciences, University of Surrey, Guildford, UK

Equine viral arteritis (EVA) is an infectious disease of equids caused by equine arteritis virus (EAV), a RNA virus of the family *Arteriviridae*. Dendritic cells (DC) are important modulators of the immune reaction with their ability to present antigen to naïve T cells. DC can be generated *in vitro* from monocytes as MoDC. Little is known of the effect EAV has on host immune cells, particularly DC. An optimized system was established to generate equine monocyte-derived DC (MoDC) *in vitro*. Purified recombinant equine cytokines (GM-CSF & IL-4) were used to differentiate DC and a cocktail of both cytokines and other factors were used for maturation. The phenotype of DC was studied, using flow cytometry and functional assays such as endocytosis/phagocytosis and mixed leukocyte reaction were applied to characterise immature and mature DC. To study the interaction with EAV, MoDC were infected at multiplicity of infection of 5 with EAV strains of different genotypes and pathogenicity. Virus replication was determined through titration and real-time PCR (qPCR). Real-time qPCR and viral titrations revealed that EAV replicates in monocytes and MoDC. The replication was most efficient in mDC, but variable between the strains. Only the virulent strain caused a significant down-regulation of CD14 and CD163 on monocytes and of CD83 on mDC. The replication of the virus resulted in an apoptosis mediated cell death, which inevitably inhibited the differentiation and long-term function of DC. Functional studies carried out early after infection showed that EAV inhibited the endocytic and phagocytic capacity of Mo and mDC with minimal effect on iDC. In line with the detrimental effects and results of the virus replication, particularly infected mDC showed a reduced ability to stimulate T cells. EAV evades the host anti-viral immunity both by inhibition of antigen presentation early after infection and through killing infected DC during replication.

Modulation of type-I IFN response by equine herpesvirus-1

S. Sarkar, U.B.R. Balasuriya, D.W. Horohov, and T.M. Chambers
Department of Veterinary Science, Maxwell H. Gluck Equine Research Center, University of Kentucky, Lexington, KY 40546-0099, USA

Equine herpesvirus-1 (EHV-1) is one of the major viral pathogens causing respiratory disease, abortion and neurologic disease among horses. In recent years there has been an increase in the incidence of the neuropathogenic form of EHV-1 infection in several countries. Type-I interferons (IFNs) act as a first line of defense against many viral infections. In this study we investigated the type-I IFN response against the neuropathogenic T953 strain of EHV-1 in equine endothelial cells (EECs). Confluent monolayers of EECs were infected with T953 strain of EHV-1, Sendai virus (SeV) or both at an m.o.i. of 5 or with polyinosinic acid:-polycytidylic acid (poly I:C). SeV and poly I:C treated EECs served as positive controls. At 3, 6, 12 and 18 h post infection (h.p.i.) IFN-β mRNA production was determined by real-time RT-PCR assay. At the same time points, IFN-β protein

levels in supernatants were also determined by type-I IFN bioassay using vesicular stomatitis virus expressing green fluorescent protein (VSV-GFP). To determine the effect of EHV-1 T953 strain in the type-I IFN mediated signaling pathways, EECs were infected as above and at 12 h.p.i. cells were either treated with recombinant equine IFN-α (rEq IFN-α) or left untreated. Subsequently, western blotting analysis of cell lysate was performed to determine the phosphorylation of STAT-1. The T953 strain of EHV-1 caused an initial induction of IFN-β at 3 h.p.i followed by an inhibition at 12 h.p.i. as compared to the mock infected cells. In contrast, SeV and poly I:C strongly induced IFN-β mRNA as well as protein expression at all the time points. Furthermore, the T953 strain of EHV-1 also inhibited SeV or poly I:C mediated IFN-β induction. The UV inactivated EHV-1 did not show any increase or decrease of IFN-β production suggesting that viral gene expression was necessary for this effect. Interestingly, further studies showed that EHV-1 could neither alter the endogenous level of interferon regulatory factor-3 (IRF3) nor prevent the nuclear translocation of IRF3 at 12 h.p.i. which suggested that the virus was unable to prevent the activation of IRF3. Western immunoblotting analysis showed that EHV-1 could prevent STAT-1 phosphorylation induced by exogenous rEq IFN-α in EECs indicating that EHV-1 also interfered with the type-I IFN signaling pathways. In summary, the T953 strain of EHV-1 interferes with both type-I IFN production and signaling pathways. Results from this study indicate that EHV-1 interferes with the innate immune response by modulating type-I IFN response at multiple levels in EECs.

Methylation of the IFN-γ gene promoter is correlated with IFN-γ expression in foals

L. Sun [1], Z. Gong [2], and D.W. Horohov [1]
[1] Maxwell Gluck Equine Research Center, University of Kentucky, Lexington, KY, [2] Department of Biochemistry, Shanghai University of Traditional Chinese Medicine, China

Interferon-gamma (IFN-γ) plays an important role in protection against intracellular pathogens such as *Rhodococcus equi* and its expression is reduced in neonates. While foals are born with a limited capacity for IFN-γ production, its expression increases with time. The underlying mechanism regulating IFN-γ production remains unknown. Studies from humans and mice have shown that regulation of IFN-γ production involves DNA hypermethylation on *Ifng* promoter region which is induced by DNA methyltransferases (DNMTs). Likewise, demethylation of the promoter region at critical sites results in increased *Ifng* transcription. Therefore, we hypothesize that the *Ifng* promoter region is hypermethylated and this correlates with IFN-γ expression. Peripheral blood mononuclear cells were isolated from neonatal (<7days old, n=9) and adult horses (10yrs old, n=6) and purified $CD4^+$ T cells were obtained by cell sorting. The methylation status of three sites within the equine *Ifng* promoter was examined using methylation-specific PCR. In addition, DNA methyltransferases (DNMTs) activity of CD4+cells was determined using an ELISA method. The DNA on a specific site of the IFN-γ gene promoter was hypermethylated in neonatal foals (Figure 1), compared with adult horses. A decrease in DNA methylation at this site significantly correlated with IFN- γ mRNA expression and age, but not with DNMT activity. To conclude, IFN-γ expression in foals appears to be controlled by DNA methylation at specific sites within the promoter region of equine *Ifng*.

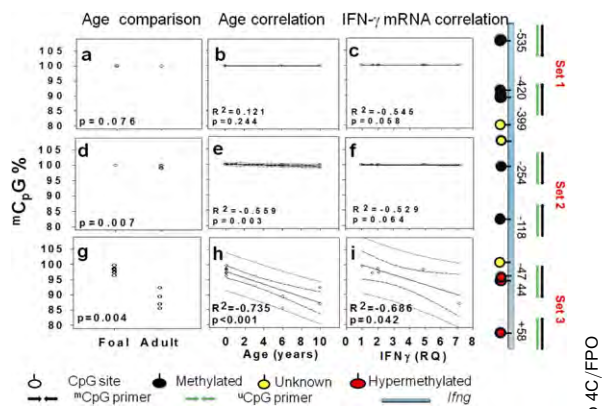

Figure 1. DNA hypermethylation in the promoter region of IFN-g in neonatal foals and its correlation with mRNA expression and age. The DNA methylation in *Ifng* promoter of $CD4^+$ cells from foals and adult horses was compared (a, d, and g) and its relationship with age (b, e, and h) and IFN-g mRNA expression (c, f, and i) was determined.

Gastrointestinal Diseases

Serological diagnosis of *Strongylus vulgaris* infection

U.V. Andersen [1], D.K. Howe [2], S.N. Olsen [1], J. Monrad [3], P. Nejsum [3], E.T. Lyons [2], and M.K. Nielsen [2]
[1] Department of Large Animal Sciences, University of Copenhagen, Taastrup, Denmark, [2] Department of Veterinary Science, University of Kentucky, Lexington, Kentucky, USA, [3] Department of Veterinary Disease Biology, University of Copenhagen, Frederiksberg, Denmark

Strongyle parasites are ubiquitous in grazing horses, with cyathostomins being the most prevalent but the large strongyles having the larger clinical impact. *Strongylus vulgaris* is considered the most pathogenic in horses, causing verminous endarteritis and ischaemic infarction of intestinal segments. Due to developing anthelmintic resistance in equine parasites, less intensive treatment regimens are now recommended to maintain efficacy of current anthelmintics. However, this has been associated with apparent re-emergence of *S. vulgaris*. Currently there are no methods

for antemortem diagnosis of the pathogenic migrating larval stages of *S. vulgaris*. To identify potential diagnostic proteins, a cDNA library was constructed from migrating stages of *S. vulgaris.* The excretory-secretory antigen (ESA) fraction from *S. vulgaris* adult specimens was dialysed against PBS and used to immunise a rat. This hyperimmune serum was used to immunoscreen the cDNA library to identify immunogenic *S. vulgaris* proteins. Immunoreactive clones were rescreened, PCRs were performed and the PCR product was sequenced. An immunoreactive cDNA clone was subcloned into *E.coli* and the plasmid sequenced. The open-reading frame encoding the predicted mature part of the protein was amplified using high fidelity polymerase, and the PCR product was cloned into the pET22b expression vector. The resulting plasmid was transformed into BL21 expression cells, and expression of the recombinant protein was induced using isopropyl β-D-1-thiogalactopyranoside. The His-tagged recombinant protein was purified by affinity chromatography on immobilized cobalt. An indirect enzyme-linked immunosorbent assay (ELISA) was optimized by checkerboard titration using the recombinant protein as antigen. Antigen-specific total IgG and IgG(T) antibodies were evaluated. Intra- and interassay variability was evaluated as well as the diagnostic sensitivity and specificity of the ELISA. Antigen specific IgG(T) antibodies gave a better distinction between positive and negative horses than IgG. Using mean optic density (OD) values, the intraassay coefficient of variation (CV) values were 7 % and the interassay CV based on the mean of the OD values were 27 %. In comparison, when using the normalized percentage of positive (PP) control values, the interassay CV was 10 %. Using 100 serum samples from horses with infection status verified on necropsy, the diagnostic sensitivity and specificity were found to be 0.61 and 0.70, respectively, while using a cut-off value of 26 PP. The ELISA shows promise for diagnosing prepatent *S. vulgaris* infection with migrating larvae. The assay could be useful in clinical settings and as a research tool for epidemiological studies.

A case control study of foal diarrhoea: rotavirus detection by reverse transcription quantitative polymerase chain reaction in foals

K.E. Bailey, S.J. Symes, C.A. Hartley, G.F. Browning, J.M. Devlin, and J.R. Gilkerson
Centre for Equine Infectious Diseases, Faculty of Veterinary Science, The University of Melbourne, Victoria, 3010, Australia

Foal diarrhoea is both labour intensive and costly to manage, and although diarrhoea is a common disease in foals, the aetiological agent is often not determined. The potential infectious causes of foal diarrhoea are numerous, however few contemporary studies have investigated the clinical significance of those pathogens present in foals with and without diarrhoea. The aim of this study was to conduct a prospective age-matched case control investigation of a range of pathogens present in foals, while developing a sensitive molecular diagnostic screening panel. The results presented here describe a reverse transcription quantitative polymerase chain reaction (RT-qPCR) to detect rotavirus in foal faeces. Faecal samples were collected on five Thoroughbred breeding farms in New South Wales, Australia, from foals with diarrhoea and from an age-matched control. A case was defined as any foal with diarrhoea on the day of sampling. A control was defined as a foal without diarrhoea on the day of sampling, born within 7 days of the case foal. Faecal samples were also collected from foals with gastroenteritis at an equine intensive care hospital, however, no control samples were collected for this population. To enable investigation of a range of bacterial and viral pathogens, faecal samples were aliquoted into RNAlater, glycerol and cooked meat broth. A Syto 9 based rotavirus RT-qPCR assay targeting an 87bp region of the rotavirus non-structural protein 3 1 was performed on nucleic acids extracted from faecal samples in RNAlater using the MoBio PowerSoil DNA Isolation Kit. Of the 1021 foals born on the farms during this study, 234 foals were sampled and included in the age-matched case control series. Rotavirus RT-qPCR products with an appropriate dissociation curve were amplified from 25% of cases and 7% of controls. In this study, the odds of detecting rotavirus in a case were 4.49 times that of detecting rotavirus in a control (OR = 4.49, 95%CI: 2.05-9.82). Of the 25 cases of foal diarrhoea from the equine intensive care hospital, 3 (12%) were rotavirus RT-qPCR positive. The results of this study indicate that rotavirus is an important pathogen in this foal population, despite use of an inactivated maternal vaccine on four of the five farms. The detection of rotavirus in control foals needs to be investigated further to establish the role of these foals in disease transmission. Importantly, in 75% of the cases in this study rotavirus was not detected and investigation of other infectious causes of foal diarrhoea in this sample set is ongoing.

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Equine neonatal salmonellosis 114 cases: clinical features, prognostic indicators, and racing performance of surviving Thoroughbreds (2004-2010)

B.S. Barr VMD, DACVIM [1], C.M. Childers DVM [1], and J. Cheetham VetMB, PhD, DACVS [2]
[1] Rood and Riddle Equine Hospital, Lexington, Kentucky,
[2] Cornell University, Ithaca, New York

Salmonellosis is one of the most common causes of enteritis in the foal. The purpose of this study was to describe the clinical and clinicopathologic characteristics, treatments, and short-term outcome associated with equine neonatal salmonellosis, to assess risk factors for survival, and to evaluate the racing performance of Thoroughbred survivors. Medical records of Rood and Riddle Equine Hospital from 2004-2010 were searched to identify cases of equine neonatal salmonellosis, defined as a foal less than 14 days

of age with a positive fecal culture within 72 hours of hospitalization. Information was obtained from the medical record including clinical signs, clinicopathologic results, diagnostics, treatment and outcome. For surviving Thoroughbred foals, information regarding racing performance was obtained from each foal and all maternal siblings from an online database. Differences between the outcome groups (survivors or nonsurvivors) were determined using one-way ANOVA with Tukey's *post hoc* tests where appropriate. For categorical variables Chi-squared tests were used. Statistical analysis was performed using and JMP (SAS Institute, Cary, North Carolina, USA). Significance was set at $p < 0.05$ throughout. There were a total of 114 foals that met the inclusion criteria. The two most common clinical signs at admission were diarrhea 56% (64/114) and fever 25% (29/114). Plasma was administered to 31% (35/114) of foals. The most common complications during hospitalization included the presence of at least one septic joint, 18% (20/114) and pneumonia, 6% (7/114). Fecal cultures were collected throughout hospitalization on 106/114 accompanying dams; 39% (44/106) cultured positive for *Salmonella*. Survivors had a significantly higher serum IgG at admission (mean 1222.6 mg/dl; SD 595.9) compared to nonsurvivors (mean 622.89 mg/dl; SD 537.52) ($p=0.017$). The serum glucose was significantly different between the two groups. Foals that survived had a significantly higher glucose (mean 148.91 mg/dl; SD 38.24) compared to nonsurvivors (mean 110.31 mg/dl; SD 47.62) ($p=0.001$). There were 79 out of 114 foals (69%) that survived to discharge from the hospital. Of the 35 foals that did not survive, 60% (21/35) were euthanized. Risk factors for nonsurvival included abnormal mucous membrane color, fever and a low WBC during hospitalization. The only prognostic indicator for survival identified was the presence of a capillary refill time of less than 2 seconds at admission. Of the 79 foals that were discharged from the hospital, 63 were Thoroughbreds. Of these registered horses, 74% (26/35) of hospitalized cases compared to 77% (135/175) of their maternal siblings started at least one race. There was no significant difference in regards to number of starts per year and total earnings per year between the two groups. In conclusion, foals with salmonellosis that are intensively managed have a fair prognosis for survival (69%). Foals that survive long enough to be registered with the Jockey Club raced at a level equivalent to their maternal siblings.

Biological control using the fungi *Duddingtonia flagrans* against cyathostomins of horses

A. Buzatti [1], C.P. Santos [2], U.Y. Yoshitani [1], L.K. Sprenger [1], F. Kloster [1], J.D. Antunes [3], and M.B. Molento [1,4]
[1] Federal University of Parana, UFPR, Curitiba, PR, Brazil, [2] North Fluminense State University, [3] DMV, Haras Sao Jose da Serra, Brazil, [4] National Institute of Science and Technology, INCT, Belo Horizonte, MG, Brazil

Gastrointestinal parasitism in horses can affect all categories with evident clinical signs. Control is made with the regular use of chemical products, which has cause resistance to all classes of drugs [1, 2]. New alternative control strategies are needed and biological control with the fungi *Duddingtonia flagrans* is an appropriate option. The fungi pass through the intestinal tract of the horse acting on the parasite free-living stages. The chlamydospores need to grow in particular environmental conditions for the hyphae to be able to predate the eggs and larvae [3]. The objective of this study was to evaluate the activity of *D. flagrans* against nematode parasites in naturally infected horses. The study was performed at the Sao Jose da Serra Stud in Pinhais, Brazil. Twenty Thoroughbred animals were divided based on their faecal egg count (EPG) values in 3 treatment groups and one control (n=5). Chlamydospores of a Brazilian isolate of the *D. flagrans* were orally administered together with grinded-corn at G1: 250.000, G2: 500.000, and G3: 1.000.000 per kilogram of live weight. The animals were treated daily during 5 days. Faecal samples were collected during the 5 days of treatment and 5 days after treatment for EPG counts, which was performed with a 1:25 concentration, individually, as well as the coproculture. Recovered larvae from coproculture were counted on triplicates from each sample, calculating the average and standard deviation. Statistical analysis was performed using Tukey test with GraphPad Prims 5 at 5% significance ($P < 0.05$). The average EPG counts from all groups had a large (53%) daily variation during the experiment and did not show any statistical difference between groups (data not shown). *Cyathostomum* sp. was the predominant (above 94%) genus present at the faecal cultures in all groups. The fungi started to have an effect on the L_3 stages 4 days after initiating the treatment reducing more than 97% ($P < 0.01$) compared to the Control group. The predatory effect of the hyphae persisted for 4 days after the treatment was interrupted significantly ($P < 0.01$) reducing larvae number (Figure 1). *Duddingtonia flagrans* showed a significant activity against infecting free-living larvae of *Cyathostomins* of horses.

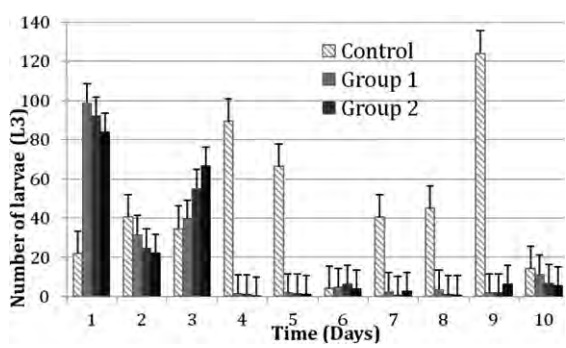

Figure 1. Average and standard error count of infecting larvae per day after *Duddingtonia flagrans* treatment in *Cyathostomins* of horses.

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Evaluation of rectal mast cell responses as a novel method to estimate equine cyathostomin burdens

R.J.M. Clements [1], T.M. McNeilly [1], K.J. Pickles [2], J.A. Keen [2], and J.B. Matthews [1]
[1] Moredun Research Institute, Midlothian, EH260PZ,
[2] R(D)SVS, University of Edinburgh, Midlothian, EH259RG

Cyathostomins are a prevalent and potentially life threatening pathogen to equidae worldwide. Infected animals may be asymptomatic or show clinical signs which include weight loss, diarrhoea and colic. Mast cell serine proteinases, tryptase and chymase, have been shown to aid nematode expulsion in other species by contributing to innate immunity and tissue remodelling, through proteolysis of matrix proteins. Mast cell infiltration in the large intestine of horses with cyathostomin infection has been previously demonstrated. Antibodies directed against two purified equine mast cell proteinases (equine tryptase and equine mast cell proteinase-1 (eqMCP-1)) demonstrated a significant positive linear relationship between caecal mast cell counts and cyathostomin burden such that total cyathostomin burden accounted for 57% of the variation in tryptase labelled mast cell counts. The objective of this study is to further investigate the recruitment of mast cells and expression of their proteinases in cyathostomin infection and to evaluate the potential for rectal mast cell enumeration or mast cell proteinase assays to be used in diagnostic tests to estimate cyathostomin burden. Equine tissue samples from the caecum, right ventral colon and rectum were collected from an equine abattoir (n=16) and the R(D)SVS (n=6). Trans-mural illumination and pepsin digestion of the tissues were performed to enumerate cyathostomin encysted larvae to calculate a total mucosal burden (TMB). Faecal egg counts and luminal counts were also performed. Rectal biopsies were taken from the aboral rectal mucosa using endometrial biopsy forceps (Equivet uterine biopsy forceps, Kruuse). Tissue sections were preserved in Carnoys fixative (24 hrs) and then transferred to 70% ethanol. Mast cells were enumerated following rehydration and overnight staining with 0.5% Toludine Blue in 0.5M HCL, pH 0.5 and counterstaining with 1% eosin in 70% ethanol. Immunofluorescent labelling with polyclonal rabbit antibodies was used to demonstrate expression of equine tryptase and eqMCP-1. Correlations will be performed, comparing TMB with both mast cell populations and proteinase expressing populations. Preliminary results show a significant correlation between mucosal and submucosal mast cells in the caecum and right ventral colon ($p < 0.0001$). There is also a significant correlation between the submucosal mast cell numbers and cyathostomin burden in the right ventral colon ($p < 0.05$). Further defining the recruitment and activation of mast cells and the production of equine tryptase and eqMCP-1 using immunofluorescence will allow investigation into the utility of these cells and enzymes as diagnostic markers of cyathostomin infection.

Molecular typing of equine *Lawsonia intracellularis* isolates

C.J. Gebhart, M.R. Kelley, and Y. Chander
College of Veterinary Medicine, University of Minnesota, St. Paul, MN 55108

Lawsonia intracellularis is an obligate intracellular bacterium and causative agent of proliferative enteropathy (PE) in pigs, horses and a variety of other species [1]. Due to its intracellular nature, few isolates exist in pure culture making traditional molecular typing techniques difficult [2]. Previous analyses of *Lawsonia* isolates using variable number tandem repeat (VNTR) typing methods demonstrated a clear distinction between the isolates obtained from porcine and equine species [3]. The objective of this study was to expand the database of *Lawsonia* genotypes to include individual and multiple equine isolates obtained from various sites in different geographic locations. A further objective was to compare the VNTR typing method to another widely used molecular typing method, multilocus sequence typing (MLST), to determine whether phylogenetic relationships between *Lawsonia* isolates from horses and other species obtained by VNTR typing are further reflected by a typing technique (MLST) with lower resolution. *Lawsonia* isolates of geographic and temporal diversity from 3 pig herds, 14 horse sites (including both sites with individual cases and outbreaks of PE), and various other animal species were used to determine if there were geographic or temporal variability of VNTR typing profiles among equine *Lawsonia* isolates. VNTR typing was performed as previously described [3]. The MLST system was established using six *Lawsonia* pure-culture isolates of porcine, equine, and rodent origin. Primers were designed for the seven housekeeping loci and their specificity was confirmed by PCR using other enteric bacteria and closely related species. The VNTR typing profiles as well as isolate description, source, year of isolation, origin, passage number and disease status of *Lawsonia* isolates used for determining genetic relationships were noted. Slight variations in VNTR typing profiles between equine isolates from different geographic locations were found. Moderate variation in VNTR types were found between isolates from horse sources and those obtained from other animal species, with the most marked differences found compared to pig isolates. The allelic profiles or sequence types generated from the seven housekeeping loci showed that MLST typing confirmed the VNTR results and further demonstrated that, while the porcine isolates had identical sequence types, the equine and rodent isolates were distinctly different from both the porcine isolates and from each other. These molecular typing results will further enhance our understanding of the transmission dynamics and epidemiology of PE within and between equine sites. Furthermore, application of both MLST (discriminates isolates from different species) and VNTR (further discriminates isolates from different sites) typing techniques will allow future phylogenetic analyses and molecular epidemiological studies of *Lawsonia*.

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Egg-productivity of horse strongylids: why fecal egg counts can be misleading

T.A. Kuzmina [1], E.T. Lyons [2], and S.C. Tolliver [2]
[1] Institute of Zoology NAS of Ukraine, vul. B. Khmelnyts'kogo, 15, Kyiv, 01601, Ukraine, [2] Department of Veterinary Science, Gluck Equine Research Center, University of Kentucky, Lexington, KY 40546-0099, USA

Currently, 64 strongylid (Nematoda: Strongylidae) species are described in wild and domestic equids worldwide. The main measure of the horse infection by these parasites is the number of strongylid eggs per one gram of feces (EPG). However, no direct relation between EPG value and number of worms in the horse intestine has been observed. The aim of our study was to determine fecundity of main strongylid species parasitizing domestic horses. All possible relations between numbers of eggs in female uteri, size of eggs and nematodes as well as the influence of egg-productivity on proportion of species in the strongylid community were analyzed. Twenty-five specimens from each of 15 of the most widespread strongylid species (*Strongylus vulgaris, S. edentatus, Triodontophorus serratus, T. brevicauda, T. tenuicollis, Cyathostomum catinatum, Coronocyclus coronatus, Cylicocyclus nassatus, C. insigne, C. leptostomum, Cylicostephanus calicatus, C. goldi, C. longibursatus, C. minutus* and *Poteriostomum imparidentatum*) were examined. Nematodes were collected after necropsy of horses never treated with anthelmintics. The reproductive system was extracted from the female body and macerated to release eggs. All eggs were removed to a drop of creosote solution and counted and measured under the light microscope. Proportion of every species in the strongylid community was calculated using published data from the same herd of horses (Lyons et al., 2001). The Paleontological Statistics Software (PAST) was used for analysis of the results obtained. Significant differences in number of eggs in female uteri of various strongylid species were observed for all species examined (Kruskal – Wallis test; $p < 0.0001$). The lowest number of eggs was in *C. longibursatus* (average – 49) and *C. leptostomum* (average – 63); the highest egg numbers were in *S. edentatus* (average – 5918) and *P. imparidentatum* (average – 3225). Linear correlation between size of nematodes and number of eggs in female uteri was observed ($p < 0.0001$). Negative correlation was observed between number of eggs in female uteri and proportion of species in the strongylid community ($p < 0.0001$). Rare species with the lowest prevalence and proportion in the community (0.04–0.3%) appeared to have the highest egg number. Our data support previous observations on lack of correlation between strongyle EPG and number of adult worms in horse intestine. As a horse is parasitized by 10-15 strongylid species, the same EPG value can be shown by thousands of small strongylids with low egg productivity, or by a dozen of large strongyles producing thousands of eggs. Besides, taking into account differences in pathogenesis of various strongylid species, we can conclude that selective treatment of the horses with the highest EPG values, especially when *Strongylus spp*. are present, should not always replace routine deworming of all horses at farms and be considered as the only effective method of strongylid control.

The spatial distribution of strongyle eggs in horse faeces

H. Lester [1], D. Bartley [1], E. Morgan [2], J. Hodgkinson [3], and J. Matthews [1]
[1] Moredun Research Institute, EP26 0PZ, UK, [2] University of Bristol, BS8 1UG, UK, [3] Institute of Infection and Global Health, University of Liverpool, L69 3BX, UK

Faecal egg counts (FEC) are routinely used for monitoring parasite infection in equids to determine treatment requirements and for assessing anthelmintic efficacy. There are several underlying factors leading to variation in FEC, which present difficulties in interpretation, potentially leading to under-/over-estimating treatment requirements or more importantly, the misclassification of resistance. If FEC is the foundation of evidence-based equine parasite control, there is a need to ensure that FEC methodologies and procedures are investigated and optimised. The spatial distribution of strongyle eggs in faeces was investigated, to determine whether overdispersion (OD) or clumping could lead to variability in FEC. A single entire motion was collected from three horses daily, at the same time, for three consecutive days. Each motion was divided into individual boli (average of 24 boli/horse) and two, 1g subsamples were taken from each and analysed using a centrifugal-flotation method (sensitivity up to 1 epg). The distribution of eggs within each motion, between boli and between subsamples was tested for overdispersion by multiplying the variance to mean ratio by the degrees of freedom, and comparing the result with the chi-square distribution. Where OD was confirmed ($p \leq 0.05$), the negative binomial distribution was fitted to the data using maximum likelihood estimation. Bonferroni correction was used to determine the chi-square critical value to test for overdispersion between samples within each bolus and at cuvette level. Results demonstrate that eggs were overdispersed ($p < 0.01$) within the entire motion. At the bolus level results suggests that if egg density is high, eggs are randomly distributed compared to when egg density is lower (<50 EPG). This finding has implications when assessing post anthelmintic treatment samples, where it may be expected that egg density will be low and eggs therefore potentially clumped. Eggs may either be missed and a treatment may be declared efficacious incorrectly (type ll

error), or eggs numbers will be over-estimated, particularly if a less sensitive FEC method is used. When comparing subsample 1 and 2 there was little evidence of OD, suggesting that the FEC procedure employed, including weighing and mixing, etc had little impact on clumping. Strongyle eggs are overdispersed within horse faeces and egg density appears to have an impact on OD at bolus level. This has implications leading to potential misclassification when assessing anthelmintic efficacy and requires consideration when conducting FEC. Further work will be conducted to compare FEC methodologies to investigate the impact of multiplication factors on the performance and interpretation of FEC, in the context of treatment recommendations and efficacy. The effect of mixing faeces and faecal sample size prior to sub-sampling and the size of subsample will be investigated to determine whether this minimises variation in equine FEC as a recommendation for future equine FEC protocols.

A comparison of three methods for calculating anthelmintic efficacy in equids

Hannah Lester [1], David Bartley [1], Eric Morgan [2], Jane Hodgkinson [3], Claire Stratford [4], and Jacqueline Matthews [1]
[1] Moredun Research Institute, EH26 0PZ, UK, [2] University of Bristol, BS8 1UG, UK, [3] University of Liverpool, L69 7BE, UK, [4] Royal (Dick) School of Veterinary Studies, EH25 9RG, UK

The Faecal Egg Count Reduction test (FECRT) is considered the gold standard for assessing anthelmintic efficacy *in vivo* and is well defined for use in small ruminants although less so for equids [1]. The current WAAVP-recommended method for calculating FECR makes various assumptions regarding distribution of the egg count data. However, low group mean egg counts, high variability and small sample size of egg count data likely affects the underlying statistical validity of this method in horses. Furthermore, published studies use different methods and criteria for classifying resistance which leads to the potential for misclassification and prevents meaningful comparisons between studies. A validated, standardised and easily computable method of FECR data analysis in equids is required in order to ensure widespread future implementation. Faecal egg count (FEC) data were collected from equine yards on Day 0 and Day 14 post anthelmintic administration. Strongyle FECs were determined by a centrifugal-flotation method sensitive up to one egg per gram. Three different methods for estimating percentage efficacy and 95% confidence limits (CL) were applied to FECR data for four anthelmintic compounds (fenbendazole, pyrantel, ivermectin and moxidectin), totalling 32 field data sets.

1. Percentage change in arithmetic group mean FEC before and after treatment (WAAVP method [1]).
2. Percentage change in arcsine-transformed proportional reduction in FECR in each individual horse, 95% CL estimated by using the standard deviation from the mean of individual FECR proportions [2].
3. Percentage change in FECR in each individual, with 95% CL estimated by non-parametric bootstrapping of post-treatment individual proportional reduction.

The three methods generated different assumptions regarding anthelmintic efficacy within the same nematode populations (Table 1). Overall, Method 1 gave higher percentage efficacy and lower CL compared to Methods 2 and 3. Methods 2 and 3 gave similar percentage reduction in FEC, although Method 2 demonstrated larger CL. These differences may be irrelevant for anthelmintics whose efficacy falls well above or below the designated sensitivity threshold, as the conclusions generated will be the same. However, for anthelmintics with field efficacy close to the designated sensitivity threshold i.e. 90%, these differences could potentially lead to a misclassification of resistance, as each method may estimate a different conclusion. The statistical processes and practicality of different methodologies for calculating and interpreting FECR should be discussed and the position clarified so that the most appropriate method is employed as the gold standard in equine studies to reduce potential misclassification of resistance.

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Table 1
Mean percentage faecal egg count reduction calculated by three different methods

		Fenbendazole								Pyrantel					
		Method 1		Method 2		Method 3				Method 1		Method 2		Method 3	
Yard ID	*n*	% FECR	LCL	% FECR	LCL	% FECR	LCL	Yard ID	*n*	% FECR	LCL	% FECR	LCL	% FECR	LCL
CC	6	46.9	40	42.4	39.7	42.1	31.1	CC	8	90.7	*82*	93.2	*62.8*	92.9	*81.8*
LAS	14	-5.2	*	*	*	*	*	LAS	14	92.2	*69*	84.45	64.6	84.9	65.5
MTL	5	15.8	*	*	*	*	*	MTL	4	99.2	99	99.4	97.4	99.3	98.7
OXD	6	83.4	20.4	71.7	63.0	78.1	63.3	OXD	4	99.9	99.9	94.8	94.4	100	100
PEN	11	58.3	31.1	*	*	54.3	32	PEN	7	97.3	*86*	89.4	85.6	93.3	93.6
SWA	12	82	52	77.8	67	59	40	SWA	12	93	*89*	90.8	*88*	94	*89*
SEA	4	26.6	-23	*	*	23.1	-9.3	SEA	12	98	93	95.2	92.1	98	95

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Table 1
(Continued)

		Moxidectin								Ivermectin					
		Method 1		Method 2		Method 3				Method 1		Method 2		Method 3	
Yard ID	*n*	% FECR	LCL	% FECR	LCL	% FECR	LCL	Yard ID	*n*	% FECR	LCL	% FECR	LCL	% FECR	LCL
CC	4	100	100	100	100	100	100	CC	5	100	100	100	100	100	100
LAS	45	100	99.8	99.92	99.5	99.9	99.7	DSS	52	100	100	100	100	99.8	99.2
MTL	8	99.4	98.6	99.5	96	99.5	98.6	LAS	38	99.8	98.2	98.6	96.5	98.7	95.2
OAK	7	100	100	100	100	100	100	OAK	3	99.8	98.4	99.4	95.2	99.4	98.3
OXD	3	100	100	100	100	100	100	WHI	4	100	100	100	100	100	100
PEN	10	100	100	100	100	100	100	PEN	11	100	100	100	100	100	100
SEA	10	100	100	100	100	100	100	OXD	3	100	100	100	100	100	100
SWA	13	99.9	96.0	99.9	99	98.7	96.1	SEA	4	100	100	100	100	100	100
WHW	12	100	99.1	99.9	99.8	99.7	99.1	SWA	11	100	100	100	100	100	100

% Reduction in bold = resistance.
% Reduction in *italic* = LCL < 90%.
* Too many negative reduction in individuals to calculate % reduction and/or LCL.

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Influence of the variability of McMaster results on selective anthelmintic treatment in horses

K. Meier [1], A.M. Becher [2], M. Reist [3], and K. Pfister [2]
[1] Tierärztliche Gemeinschaftspraxis für Pferde, Dres. Meister, Schöberl, Nahr, D-92339 Beilngries, Germany, [2] Comparative Tropical Medicine and Parasitology, Vet. Faculty, Ludwig – Maximilians -University, D-80802 Munich, Germany, [3] Veterinary Public Health Institute, Vetsuisse-Faculty, University of Berne, CH-3097 Liebefeld, Switzerland

Formerly the McMaster method was predominantly used for faecal egg count reduction tests in order to evaluate the efficacy of anthelmintic drugs. Thereby the variability of the McMaster results (faecal egg counts = FEC) might pose a problem for the interpretation of the results. In recent years, selective anthelmintic treatment is facing an increasing interest in equine parasite control. Thereby, treatment decisions are based on FEC of individual horses. The aim of this study was to analyze the influence of the variability of the McMaster results in the context of selective anthelmintic treatment procedures. Over a period of one year 2214 faecal samples were collected in 4-week intervals from 202 horses (Mean age 5 ± 6 years) from10 farms. The samples were analysed twice with a modified McMaster method with a sensitivity of 30 eggs per gram feces (epg). The mean difference of the second FEC compared to the first FEC was 5.5 %, the standard deviation was 46.5 %. In the Table 1 are presented the numbers of samples which had results below a given treatment threshold in the first FEC and accordingly, the number of samples with the 1st FEC < threshold and 2nd FEC ≥ the same threshold. The major objective of the diagnostic part of selective anthelmintic treatment is to identify horses which are shedding high numbers of strongyle eggs. The repeated analysis demonstrates that only a very low percentage (2.8 %-0.4 % depending on the threshold) of samples exceeds the threshold just in the second analysis. This clearly shows that a very low number of horses would not have been treated when doing only one FEC. The herein presented results associated with the in-borne characteristic "strongyle egg shedding consistency" and the use of subsequent analyses in selective anthelmintic treatment allow the following conclusion: The variability of the McMaster results does not affect selective anthelmintic treatment in a way that matters to the equine practitioner.

Table 1
Numbers of samples with results below a given treatment threshold in 1st FEC; number of samples with the 1st FEC < threshold and 2nd FEC ≥ the same threshold

Threshold	Number of samples with 1st FEC < threshold (% of all samples)	Number of samples with 1st FEC < threshold and 2nd FEC ≥ threshold (% of all samples)	Max epg of the same samples in the 2nd FEC
200 epg	1799 (81.3%)	61 (2.8%)	360epg
250 epg	1883 (85.0%)	51 (2.3%)	360epg
300 epg	1911 (86.3%)	39 (1.8%)	420epg
400 epg	2007 (90.7%)	20 (0.9%)	570epg
500 epg	2042 (92.2%)	9 (0.4%)	630epg

Implementation of selective anthelmintic treatment in an equine-practice in Upper Bavaria (Germany): 1st experiences

M. Menzel [1,2], A.M. Becher [2], M. Reist [3], and K. Pfister [2]
[1] Tierarztpraxis Thurmading, D-84568 Pleiskirchen, Germany, [2] Comparative Tropical Medicine and Parasitology, Vet. Faculty, Ludwig – Maximilians - University, D-80802 Munich, Germany, [3] Veterinary Public Health Institute, Vetsuisse-Faculty, University of Berne, CH-3097 Liebefeld, Switzerland

So far selective anthelminic treatment and especially the interval between the faecal samples have not yet been tested scientifically under practical conditions for their ability to reduce pasture contamination with strongyle eggs. In 2011 selective anthelmintic treatment was introduced in an equine practice in Upper Bavaria (Germany) for horses older than 2 years. A combined sedimentation/flotation technique plus a modified McMaster method with a detection limit of 20 eggs per gram feces (epg) was used to analyse the 1st faecal sample (MS 1). The three subsequent faecal samples (MS 2- 4) of each horse recommended to the horse owners

were analysed with a modified McMaster method only. Horses shedding ≥ 200 strongyle epg were treated with an anthelmintic. The owners were instructed to send the sample MS 2 of their horse 42 days after the analysis of the 1st sample to the laboratory and the samples MS 3 and MS 4 after another 90 days, resp. In total, 1232 faecal samples from 518 horses (121 farms) were analysed. From 518 horses of which faecal samples were sent in as MS 1, the mean FEC was 167 epg. Thereafter, samples from 308 horses sent to the laboratory as MS 2 revealed a mean FEC of 136 epg. Thereafter, although recommended by the practice, the number of samples and horses, resp. considerably decreased. 184 samples sent in as MS 3 had a mean FEC of 112 epg, whereas the mean FEC of 72 MS 4 – samples dropped to 100 epg. Faecal samples exceeding the strongyle cut-off value of 200 epg were found on 16/121 farms (13.2%). 93 horses (18.0%) exceeded the strongyle cut-off value one time, 37 horses (0.7%) two times and 5 horses (0.1%) three times during the study period. Most horse owners did not send in the faecal samples at the recommended time. The difference in epg between two subsequent samples of untreated horses was compared to the interval in days between the analyses of the same subsequent samples. There was no statistically significant difference in the percentage of samples pairs with a difference < or ≥ 200 epg between samples sent in before and after the recommended interval of 42 days and 90 days, resp. The applied faecal sampling scheme has led to a reduced pasture contamination with strongyle eggs. There is no obvious risk for an increase in pasture contamination when extending the interval between the samples for untreated horses. Therefore the interval between the samples can be chosen according to the egg reappearance period of the drug class used for the treatment of the high strongyle egg shedders.

Molecular characterization of Equine Group A Rotaviruses currently circulating in young foals with diarrhea in Argentina

S. Miño [1], J. Matthijnssens [2], M.A. Vissani [1], C. Olguin Perglione [1], M.S. Tordoya [1], L. Becerra [1], V.G. Parreño [1], and M.E. Barrandeguy [1]
[1] Instituto de Virología, CICV y A, INTA Castelar, Buenos Aires, Argentina, [2] Laboratory of Clinical and Epidemiological Virology, Rega Institute for Medical Research, University of Leuven, Leuven, Belgium

Equine Group A Rotaviruses (RVA) are a major cause of neonatal diarrhea in foals under 4 months of age. Based on the VP7 and VP4 outer capsid proteins, RVA strains are classified into 27 G- and 35 P-genotypes, respectively. G3P[12] and G14P[12] are epidemiologically the most important genotypes in horses worldwide. An extended RVA classification system defines genotypes for all 11 genome segments, including I-genotypes for VP6. In Argentina, equine RVA strains were associated with 39% of the foal diarrhea outbreaks during a 17-year surveillance period. From the RVA positive cases, G3, G14 and mixed infections were responsible for 51%, 33% and 4% of the cases respectively. An inactivated RVA vaccine (including G3 P[12] RVA strains H2) is available in Argentina since 1996, and most breeding mares are under vaccination program. Since then, the incidence, severity and duration of foal diarrhea outbreaks has decreased. No foal diarrhea due to RVA was registered in 2001 and 2002. However, an increase in the RVA positive fecal samples was detected from 2006 to 2008, in coincidence with a switch in the prevalent genotype, from G3 to G14. The aim of this study was to report the detection, characterization and molecular epidemiology of RVA strains in foals from 2009 to 2011 in Argentina. One hundred and ninety fecal samples corresponding to 74 foal diarrhea outbreaks were tested. All RVA positive fecal samples were analyzed by PCR and sequencing of the VP7, VP8* and VP6 encoding genes. Phylogenetic analyses including previously identified equine RVA strains were conducted. RVA was detected in 48 out of 190 (25%) fecal samples corresponding to 28 out of 74 (38%) outbreaks of foal diarrhea. Overall, G3 and G14 were detected in 57% and 14% of the samples respectively, whereas the remaining samples are to be typed. G14 was detected in 14%, 34% and 7% of the samples analyzed in 2009, 2010 and 2011 respectively, while G3 was found in 86%, 17% and 67% of these samples. The Argentinean equine G3 and G14 RVA strains form closely related monophyletic clusters. All RVA strains possessed the P [12] genotype. Two VP6 genotypes, I2 (20%) and I6 (80%), were distinguished among the RVA studied. G3 was always found in association with I6, whereas G14 was associated with I2. The re-emergence of equine RVA strains as an important cause of foal gastro-enteritis in Argentina between 2006 and 2011 was caused by the same genotypes (G3 P[12] and G14 P[12]) reported previously, despite the continued vaccination of mares. Whether this re-emergence may be due to changes in any of the other 9 RVA gene segments (including VP6), is currently under investigation.

Selective therapy for strongyle parasite control: Reemergence of *Strongylus vulgaris*?

M.K. Nielsen [1], A.N. Vidyashankar [2], S.N. Olsen [3], J. Monrad [4], and S.M. Thamsborg [4]
[1] M.H. Gluck Equine Research Center, Department of Veterinary Science, University of Kentucky, Lexington, Kentucky, USA, [2] Department of Statistics, George Mason University, Fairfax, Virginia, USA, [3] Department of Large Animal Sciences, University of Copenhagen, Denmark, [4] Danish Center for Experimental Parasitology, Department of Veterinary Disease Biology, University of Copenhagen, Denmark

Strongyle nematodes are ubiquitous in grazing horses, and the large strongyle *Strongylus vulgaris* is considered the most pathogenic. This parasite was originally found widely prevalent, but decades of frequent anthelmintic treatment have reduced the prevalence dramatically. Increasing levels of anthelmintic resistance in cyathostomin parasites have led to implementation of selective therapy regimens to reduce further development of resistance, and it has been hypothesized that *S. vulgaris* could reoccur under these treatment circumstances. The aim with the present study was to evaluate the occurrence of *S. vulgaris* and possible association with usage of selective therapy. A total of 43 horse farms in Denmark were evaluated for the presence of *S. vulgaris* using individual larval cultures and real-time

PCR. Farms were either using a selective therapy principle based on regular fecal egg counts from all horses, or they treated strategically without using fecal egg counts. A total of 662 horses were included in the study. The overall prevalence of *S. vulgaris* was 12.2% at the individual level and 64.3% at the farm level. Farms using selective therapy had horse and farm prevalences of 15.4% and 83.3%, respectively, while the corresponding results for farms not using selective therapy were 7.7% and 38.9%. These findings were found statistically significant at both the horse and the farm level. In addition, the occurrence of *S. vulgaris* was significantly higher in horses that were dewormed more than six months prior to the study. The results suggest that basing all anthelmintic treatments on fecal egg counts can be associated with an increased prevalence of *S. vulgaris*. Treatment intensities on the studied farms were considerably lower than what is seen in other countries, and modifications of the parasite control programs could be considered. It remains unknown to which extent the occurrence of *S. vulgaris* can be associated with increased health risks for infected horses.

Lawsonia intracellularis-associated Necrotizing Enteritis in Four Weanling Horses

A.E. Page [1], L.H. Fallon [2], U.K. Bryant [3], D.W. Horohov [1], T.W. Luna [4], P.S. Marsh [4], N.M. Slovis [2], K.A. Sprayberry [2], and A.T. Loynachan [3]
[1] Department of Veterinary Science, University of Kentucky, Lexington, KY, 40546, [2] Hagyard Equine Medical Institute, Lexington, KY, 40511, [3] Veterinary Diagnostic Laboratory, University of Kentucky, Lexington, KY, 40511, [4] Equine Medical Associates, PSC, Lexington, KY, 40583

Lawsonia intracellularis, the causative agent of equine proliferative enteropathy (EPE), has not been reported to cause necrotizing enteritis and acute death in multiple horses. Four weanling horses from central Kentucky were included after presentation to the University of Kentucky Veterinary Diagnostic Laboratory for necropsy following acute clinical deterioration. The top differential diagnosis, for all weanlings, was EPE based on the initial clinical signs and clinicopathologic data. Each weanling was euthanized, or died, within 96 hours after development of clinical signs. Necrotizing enteritis (subacute or chronic) was observed in each of the cases at necropsy. This is believed to be the reason all four weanlings demonstrated antemortem and/or postmortem signs of systemic endotoxemia and/or bacteremia, including fulminant DIC in one weanling. Additionally, each case was positive for *L. intracellularis* within the small intestine and gross thickening of the mucosa was present. Based on the presence of necrotizing enteritis in each of the cases, as well as the acute clinical deterioration noted, we propose that this entity be referred to as a "necrotizing form of EPE" (N-EPE). Despite the correct preliminary diagnosis and appropriate initial treatment, all four of these weanlings succumbed to complications arising from EPE. This suggests that suspect N-EPE cases should be treated aggressively with broad-spectrum antimicrobials, anti-thrombotics, and anti-endotoxic measures. While more work is needed to understand risk factors, practitioners must watch for signs associated with N-EPE in an effort to avoid acute deterioration and death.

The evaluation of African and UK bioactive plant extracts for the control of equid gastrointestinal nematodes

L.E. Peachey [1], G.L. Pinchbeck [1], C.E. Scantlebury [1], G. Tefera [2], M. Getachew [3], D. Etana [4], F.A. Burden [3], A.F. Trawford [3], J.B. Matthews [5], and J.E. Hodgkinson [1]
[1] University of Liverpool, Liverpool, UK, [2] Society for The Protection of Animals Abroad, Debre Zeit, Ethiopia, [3] The Donkey Sanctuary, Sidmouth, UK, [4] The University of Hawassa, Hawassa, Ethiopia, [5] Moredun Research Institute, Edinburgh, UK

In the developed world the control of equid gastrointestinal (GI) nematodes, in particular cyathostomins, is increasingly challenging due the threat of anthelmintic resistance. In developing countries such as Ethiopia despite high parasite burdens, access to genuine anthelmintic treatment is limited. In both situations there is a need for alternative treatment and management regimens for effective parasite control and consequently there has been increasing interest in the use of bioactive plant extracts (BPEs) [1]. This study identified candidate plants in the UK and Ethiopia and evaluated their *in vitro* efficacy against cyathostomin populations derived from donkeys. A participatory rural appraisal (PRA) approach was used to identify five ethnoveterinary medicines for use in donkeys in Ethiopia. In the UK three plants were identified following extensive review of literature citing efficacy against GI nematodes of other host species and two extracts were provided courtesy of Prof Jerzy Behnke, University of Nottingham. Hydro-alcoholic extraction of dried plant material was carried out for the eight extracts prior to reconstitution in both water and DMSO. Efficacy of each extract was evaluated by egg hatch assay (EHA) and larval migration assay (LMA) using eggs and larvae recovered from the faeces of donkeys at the Donkey Sanctuary, UK. Dose response curves were produced and ED-50 values were calculated using probit analysis. Of the five Ethiopian plant extracts tested, four showed efficacy in the EHA and/or LMA. The two most efficacious were *Acacia nilotica* and *Rumex abyssinicus* in the EHA with ED-50 values of 0.72mg/ml and 1.29mg/ml respectively. Of the five UK extracts four showed efficacy in the EHA and/or LMA. The two most efficacious were *Carica papaya* (papaya) in the LMA and *Allium sativum* (garlic) in the EHA with ED-50 values of 18.9μM and 0.65mg/ml respectively. The two BPEs most efficacious *in vivo* for Ethiopia and the UK are to be carried forward to *in vivo* trials. This study has demonstrated *in vitro* efficacy of nine plant extracts against cyathostomins. There is evidence in the literature that these plant extracts show efficacy both *in vitro* and *in vivo* against GI nematodes in other species. Therefore these results have identified potential

alternatives to synthetic anthelmintics for the treatment of cyathostomins that require further investigation.

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High seroprevalence to *Anoplocephala perfoliata* despite annual treatment with pyrantel: evidence of anthelmintic resistance?

A.S. Peregrine, and L. Trotz-Williams
Department of Pathobiology, Ontario Veterinary College, University of Guelph, Ontario N1G 2W1, Canada

Between April 2006 and September 2007, ceco-colic intussusceptions were diagnosed in four standardbred horses aged 1-3 years on a well-managed farm in southern Ontario, Canada. Throughout this period there were approximately 90 standardbred horses maintained on the farm. Surgery on all four horses with ceco-colic intussusceptions revealed the presence of large numbers of *Anoplocephala perfoliata* in all four animals. Blood samples were collected from all horses on the farm in October 2007 and examined for antibody to a 12/13 kDa *A. perfoliata* secretory protein [1,2]; 61 of 67 (91%) horses were seropositive and the mean optical density (OD) of all horses was 1.391 (median = 1.538). Among the seropositive horses, 4 (6% of total) and 57 (85% of total) had OD values indicative of moderate (0.201-0.600) and high (>0.600) infection intensity, respectively. This is in contrast to a 2003-2004 study of 234 horses in southern Ontario of multiple breeds, 117 with colic and 117 controls; 131 (56%) were seropositive to *A. perfoliata* and the mean OD of all horses was 0.509 (median = 0.243). Among the seropositives, 65 (27.9% of total) and 66 (28.1% of total) had OD values indicative of moderate and high infection intensity, respectively. Assuming data independence, horses on the standardbred farm were significantly more likely to be seropositive than the 2003-2004 reference population (odds ratio = 8.0; 95% CI = 3.3-19.6; $p < 0.0001$). The reason for the high prevalence and high intensity of *A. perfoliata* infection among horses on the standardbred farm are unclear. In November each year, for multiple years, all yearlings and adult horses on the farm were treated for tapeworms with double-dose pyrantel. In contrast, only 23% of the reference population had received treatment with a cestocide in the year prior to sampling. Since neither underdosing nor use of outdated product appear to have been a concern, it is possible that the problem was associated with resistance to pyrantel in *A. perfoliata*. However, because of the lack of a sensitive diagnostic method for current infection, this was not possible to prove.

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Characterisation of IgE antibody responses to *Anoplocephala perfoliata*

C.E. Pittaway, G.C. Coles, and A.D. Wilson
School of Veterinary Sciences, University of Bristol, Langford House, Bristol BS40 5DU UK

Anoplocephala perfoliata is the most common intestinal tapeworm of horses, with abattoir surveys showing a 40-50% prevalence. Mature *A.perfoliata* shed gravid proglottids that break up releasing eggs which are not readily detected by faecal counts. This has prompted the development of antibody based methods of detection. Proudman and Trees [1] demonstrated elevated IgG(T) antibody levels to excretory/secretory (E/S) antigens in *A.perfoliata*-positive horses. However due to persisting IgG antibodies the assay has trouble distinguishing current from previous infections [2].

IgE, the antibody class found at the lowest concentrations in serum, is thought to have evolved as a specific defensive mechanism against parasitic infections. The half-life of IgE within serum is only 7-10 days, therefore raised serological IgE has the potential to be a more accurate marker of current *A.perfoliata* infection. Serum samples were collected from horses at an abattoir and a definitive diagnosis of *A.perfoliata* infection made by post mortem examination of the terminal ileum and caecum. Serum IgE, IgA, IgG and IgG(T) responses to E/S antigens were measured by ELISA. High levels of IgG and IgG(T) antibodies were present in serum from infected horses but also gave some false positive results. The OD values for IgE were very low compared to those for IgG, nevertheless elevated IgE antibodies were detected to E/S antigen in the serum of some horses but false negative results were common and the IgE ELISA offered no advantage over IgG(T) ELISA as a diagnostic test. In contrast immunohistology of frozen sections of *A.perfoliata* detected high levels of IgE antibody in the serum of infected horses binding to the surface layers of the proglottids, while IgG gave only a marginal signal suggesting the main antigenic target of the IgE response was surface structures of the worm and not E/S antigens. Parasite surface carbohydrate exhibited strong binding of the lectin *Ulex europaeus* agglutinin (fucose) and to a lesser extent peanut agglutinin (galactose). Dual staining showed that IgE and lectin binding co-localized and in many horses the IgE antibody completely blocked lectin binding implying the IgE antibodies are directed at a carbohydrate epitope. Immunohistology of fixed colon sections revealed large numbers of Toluidine blue staining mast cells and IgE positive cells in the lamina propria of both *A.perfoliata* positive and negative horses. Whereas lymphoid follicles in the mucosa of healthy horses showed no IgE staining; follicles from *A.perfoliata* infected horses contained IgE positive cells which may indicate that a local production of IgE by mucosal lymphoid tissue does play a role in the immune response to *A.perfoliata*.

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Transmission of *Lawsonia intracellularis* to weanling foals using feces from experimentally infected rabbits

N. Pusterla [1], D. Sanchez-Migallon Guzman [1], F. Vannucci [2], S. Mapes [1], A. White [1], M. DiFrancesco [1], and C. Gebhart [2]

[1] School of Veterinary Medicine, University of California, Davis, CA 95616, [2] College of Veterinary Medicine, University of Minnesota, St. Paul, MN 55108

Equine proliferative enteropathy (EPE) is an emerging enteric disease of foals caused by *Lawsonia intracellularis.* Affected foals, generally less than one year of age, display lethargy, anorexia, fever, weight loss, peripheral edema, diarrhea and colic. Transmission of *L. intracellularis* is thought to occur through the ingestion of feed or water contaminated with *L. intracellularis*-infected feces from free-living or domestic animals. *Lawsonia intracellularis* has been detected by PCR in the feces of a variety of domestic and wild animals. Recently, the authors found that 7.5% of fecal samples and 27% of serum samples from cottontail rabbits from a farm with endemic occurrence of EPE tested positive for *L. intracellularis* by PCR and serology, respectively. Of interest was that on this farm, a large population of rabbits lived in the hay barn and directly contaminated hay fed to the horses. The goal of this study was to determine if feces from rabbits experimentally infected with *L. intracellularis* could be the source of infection for naïve weanling foals. Two 9-week-old New Zealand white rabbits were experimentally infected with 2.5 x 10^9 *L. intracellularis* of equine origin via nasogastric intubation, while two rabbits served as uninfected controls. Eight weanling foals randomly assigned to one of two groups (infected and control) received daily feces from the infected or control rabbits mixed with feed or water. All rabbits and foals were monitored daily for the development of clinical abnormalities and were weighed once weekly for the duration of the study. Feces were collected every day to every other day for the quantitative molecular detection of *L. intracellularis* via real-time PCR. Blood was collected weekly for the measurement of concentrations of total solids and serologic analysis. None of the infected rabbits or foals developed any clinical signs or hypoproteinemia compatible with PE. Onset of fecal shedding of *L. intracellularis* was detected by PCR on days 3 and 9 post-challenge in the 2 infected rabbits. The duration of fecal shedding was 6 and 9 days for the 2 infected rabbits. All infected foals began to shed *L. intracellularis* between days 10 and 14 post-infection, and fecal shedding lasted between 4 and 10 days. Feces and rectal swabs indicated that control rabbits and control foals, respectively, remained PCR negative for *L. intracellularis* throughout the entire study period. A humoral immune response was detected in all infected rabbits and foals. This study represents the first report documenting the successful feco-oral transmission of *L. intracellularis* using infectious fecal material from rabbits. Lagomorphs may represent an effective reservoir/amplifier host for *L. intracellularis* due to their large population, their close contact to horses, their short reproductive cycle and their world-wide distribution.

Emerging outbreaks associated with equine coronavirus in adult horses

N. Pusterla [1], S. Mapes [1], C. Wademan [1], A. White [1], R.L. Ball [2], K. Sapp [2], P. Burns [3], C. Ormond [4], K. Butterworth [5], J. Bartol [6], and K.G. Magdesian [1]

[1] School of Veterinary Medicine, University of California, Davis, CA 95616, [2] Bracken Equine Clinic, San Antonio, TX 78266, [3] Elkhorn Veterinary Clinic, Elkhorn, WI 53121, [4] Oak Hill Veterinary Services, Orinda, CA 94563, [5] SRH Veterinary Services, Ipswich, MA 01938, [6] New England Equine Medical & Surgical Center, Dover, NH 03820

Equine coronavirus (ECoV) has been identified by electron microscopy, culture and more recently by PCR in feces of foals with and without enteric disease. A recent study reported on the isolation of ECoV from the feces of 2- to 4-year-old horses with pyrogenic and enteric disease living in stables of a racetrack in Japan. However, little is known about ECoV, especially with regard to molecular diagnostics of field samples and the clinical significance of ECoV PCR positive fecal results. The purpose of this study was to describe clinical, hematological and fecal PCR results from 161 horses involved in outbreaks associated with ECoV. The outbreaks happened at four separate boarding facilities between November 2011 and April 2012 in the States of California, Texas, Wisconsin and Massachusetts. The population of horses per stable ranged from 28 to 65 horses. Following the molecular detection of ECoV in the feces from the initial index cases, the remaining herdmates were closely observed for the development of clinical signs. Fecal samples were collected from sick and healthy horses for the PCR detection of ECoV. Clinical pathology from sick horses was evaluated when available. All four outbreaks involved primarily adult horses ranging in age from 1 to 29 years (median 15 years). Fifty-eight horses developed clinical signs with 12 to 16 sick horses per outbreak. The main clinical signs reported were anorexia (52), lethargy (46) and fever (43). Changes in fecal character, ranging from soft-formed to watery consistency and colic were observed in 12 and 4 horses, respectively. Clinical signs generally resolved within 1-4 days with supportive care. Four horses from 3 different outbreaks were euthanized or died due to rapid progression of clinical signs. The cause of death could not be determined with necropsy evaluation in 2 horses, while septicemia secondary to gastrointestinal translocation was suspected in 2 horses. Blood work was available from 10 horses with clinical disease and common hematological abnormalities were leucopenia due to neutropenia and/or lymphopenia. Feces were available for ECoV testing by real-time PCR from 44 and 99 sick and healthy horses,

respectively. 38/44 (86%) horses with abnormal clinical signs tested PCR positive for ECoV, while 89/99 (90%) healthy horses tested PCR negative for ECoV. The overall agreement between clinical status and PCR detection of ECoV was 89%. The study results suggest that ECoV is associated with self-limiting clinical and hematological abnormalities in adult horses. Real-time PCR is a sensitive and fast diagnostic tool to document the presence of ECoV in feces from horses with unspecific clinical signs.

Parasitologic, Physiologic, and Performance Parameters of Yearling Horses Receiving Daily Pyrantel Tartrate

C.R. Reinemeyer [1], J.C. Prado [1], B. Schricker [2], and T. Kennedy [2]
[1] East Tennessee Clinical Research, Rockwood, TN, USA, [2] Central Life Sciences, Phoenix, AZ, USA

Daily pyrantel tartrate is approved in horses for the prevention of *Strongylus vulgaris* larval infections, and for the control of adults and L4s of other large strongyle species, cyathostomins, pinworms and ascarids. A masked, controlled, clinical trial was conducted to evaluate the effects of daily pyrantel tartrate on additional parasitologic, physiologic, and performance parameters of yearling horses exposed to repeated, mixed strongylid infections. Twenty-eight yearling horses were treated with strongylid-larvicidal regimens and randomly assigned to receive Purina Strategy (placebo) or pyrantel tartrate (Equi-Aid; ~2.64mg/kg) once daily with each morning feeding. Twenty-five *Strongylus vulgaris* L3s and ~5,000 pyrantel-susceptible, infective, cyathostomin larvae were added to the evening ration once or five times weekly, respectively, for 5.5 months. At 2-week intervals, Body Condition Scores (BCS) and body weights were recorded, and feces and blood were collected to measure strongylid egg counts, packed cell volume, and total protein and serum albumin concentrations. Prior to Day 120, "rescue" treatment with pyrantel pamoate paste (13.2 mg/kg) was administered to any individual horse that achieved an egg count ≥1,000 EPG. Horses remained on a common pasture for 154 days, were transferred to individual stalls for 14 days, and then euthanatized and necropsied. Aliquots (1%) of the large intestinal contents were collected for total worm counts, and additional tissues were harvested for parasitologic assessments. Daily pyrantel tartrate significantly reduced strongylid egg counts ($P < 0.05$) by 84.4% to 98.9% from Day 28 until trial termination. Body weights, packed cell volumes, and total protein levels did not differ significantly between groups. Mean BCS of treated horses were significantly greater than controls ($P < 0.05$) after Day 112, confirming that performance benefits of parasite control may not become apparent for months following implementation. Serum albumin concentrations were frequently higher ($P < 0.05$) in treated geldings, suggesting that male horses may have less resilience against the harmful effects of parasitism than females. Daily pyrantel tartrate reduced the numbers of *S. vulgaris* larvae in arterial lesions by 98.7% ($P=0.0002$). Treated horses harbored significantly fewer lumenal cyathostomins than controls ($P < 0.05$; efficacy = 93.98%), and accumulated only 55% as many encysted mucosal larvae. The ceca and ventral colons of treated horses weighed significantly less (as a percentage of antemortem body weight) than those of controls, ostensibly due to less inflammation and associated edema. Group differences in strongylid numbers would likely have been far greater if 11/14 controls had not received rescue treatment, and if the groups had grazed separate pastures. No adverse events were associated with the daily administration of pyrantel tartrate to 14 pastured, juvenile horses over a 5.5-month period. Daily pyrantel tartrate provided excellent prophylaxis against infections with strongylin and cyathostomin nematodes, as confirmed by significantly lower egg counts and larval and adult worm numbers. Ancillary benefits included improved BCS scores, higher serum albumin levels, and less gut inflammation.

Laboratory animal models for *Lawsonia intracellularis*: discovering the truths of cross-infection

F. Sampieri [1], A.L. Allen [1], F.A. Vannucci [2], N. Pusterla [3], A.J. Antonopoulos [1], K.R. Ball [1], P.M. Dowling [1], J. Thompson [4], D.L. Hamilton [1], and C.J. Gebhart [2]
[1] Western College of Veterinary Medicine, University of Saskatchewan, Saskatoon, SK, Canada, [2] College of Veterinary Medicine, University of Minnesota, St. Paul, MN, USA, [3] School of Veterinary Medicine, University of California-Davis, Davis, CA, USA, [4] Canadian Light Source, Saskatoon, SK, Canada

Lawsonia intracellularis causes proliferative enteropathy (PE) in a variety of animal species worldwide, with equine and porcine PE (EPE and PPE, respectively) being of economic relevance. To date PPE infection models in pigs and rodents have been repeatedly published, but EPE is experimentally reproduced only in foals. To develop an EPE model, we attempted to infect hamsters and rabbits, as they are naturally affected by PE. Inocula derived from EPE and PPE diseased animals were used to infect rabbits and hamsters in 2 experimental trials each. Controls, when involved, were sham-treated. Trial-1: 9 New Zealand white does were assigned to 2 groups: 3 uninfected controls and 6 EPE-challenged does. Infected rabbits were challenged with cell-cultured EPE-strain *L. intracellularis*. Trial-2: 6 does were inoculated with PPE-strain *L. intracellularis.* Trial-3: 29 weanling Golden Syrian hamsters were randomly divided in 9 uninfected controls and 20 EPE-challenged subjects. Trial-4: 24 weanling hamsters were randomly divided in 6 uninfected controls, 9 EPE-challenged and 9 PPE-challenged subjects. In both rabbit trials, onset of clinical signs was monitored for 21 days post infection (DPI). Only EPE-challenged rabbits developed mild depression and moderate weight-loss at the disease peak (14DPI). Hamsters were observed for 21DPI (Trial-3) to 24DPI (Trial-4) and never developed clinical signs. Diagnostic techniques used in horses were applied. *Antemortem:* serology was tested weekly (rabbits) and/or at euthanasia (both species); and fecal DNA shedding was analyzed through qPCR. *Post-mortem*: routine H&E staining and immunohistochemistry (IHC) labelling with *L.*

intracellularis-specific mouse monoclonal antibody were used on intestinal tissues. For macroscopic observation and collection of tissue samples, 1 control rabbit, 2 EPE-strain (Trial-1) and 2 PPE-strain (Trial-2) infected rabbits were euthanized once weekly; whereas hamsters of each group were randomly euthanized on 7, 11, 14, 17, 21DPI (Trial-3) and 17, 21, 24DPI (Trial-4). In EPE-challenged rabbits, serologic responses were detected starting at 14DPI and increased by 21DPI. *L. intracellularis*-DNA was detected in EPE-infected rabbits' feces starting at 7DPI; gross lesions were apparent starting at 14DPI; and *L. intracellularis* antigen was confirmed through IHC-labelling within enterocytes on 7, 14, and 21DPI. In PPE-challenged rabbits, no typical intestinal lesions or IHC-labelling were found; fecal shedding was reduced; and the serological response (14DPI) was lower (P=0.0023). Both species' controls were negative. EPE-challenged hamsters did not develop infection when compared with PPE-challenged hamsters (P=0.009 (IHC); =0.0003 (PCR)). These studies describe rabbits as an infection model to simulate EPE- but not PPE-natural infection, as diagnostic criteria for EPE were fulfilled. Conversely, hamsters represent a valuable infection model for PPE, but not for EPE.

Investigations on equine habronematosis in a stable in Dubai, UAE

R.K. Schuster [1], Saritha Sivakumar [1], and Mieke de Rijck [2]
[1] Central Veterinary Research Laboratory, P.O. Box 597, Dubai, UAE, [2] Sharjah Equine Hospital, P.O. Box 29858, Sharjah, UAE

Habronematosis is a vector borne parasitic disease in equines and camels caused by spiruroid nematodes of the family Habronematidae with 3 species occurring in horses. The adult dioecious nematodes (*Habronema muscae, H. microstoma* and *Drascheia megastoma*) live in the stomach of the horse and produce eggs/larvae that are passed with the faeces. Flies of different species act as intermediate hosts. Gastric habronematosis usually does not develop clinical signs while cutaneous forms (summer sores) may require conventional treatment or even surgical intervention. Pulmonal habronematosis is the severest form but has been rarely diagnosed so far. *Habronema muscae* is the only representative of Habronematidae family detected so far in the UAE. In February 2012 we got knowledge of cases of summer sores in a stable in Dubai. Amongst 49 horses (endurance and recreation) of different gender, age and breeds in 3 different barns, 3 animals had developed granulomatous wounds on both frontal fetlocks as well as on the prepuce. Prior to intervention, 430 flies were caught by net and with specially designed weir traps. Individually taken feacal samples from all 49 animals were placed into a cage containing laboratory bred and parasite free house flies. The faecal samples were removed the next day and checked for the presence of fly eggs or 1st stage larvae. After fly larvae had consumed all nutrients from horse faeces they were fed with a wet mixture of wheat bran, alfalfa flour and molasses. Flies emerged from this cultivation were immobilized by placing them for 5 min in a deep freezer and sliced into parts. Caput, thorax and abdomen were separately examined for the presence of *Habronema* larvae. Horse faeces were also examined with routine sedimentation and $ZnCl_2$ flotation methods. Out of 320 *M. domestica* caught in the stable 90 flies harboured *Habronema* larvae. A second muscid species (*Stomoxys calcitrans,* n= 110) present at the farm was negative for nematode larvae. The *Habronema* prevalence in *M. domestica* in the 3 barns was 18.2, 35.2 and 40.0%, respectively with average burdens of 4.8, 4.25 and 6.9, respectively. The maximum burden found in one fly was 35 larvae. Routine parasitological methods failed to detect *Habronema* development stages. Two thousand four hundred and fifty specimens of *M. domestica* experimentally grown on individual faeces of the 49 horses were examined for the presence of *Habronema* larvae. This examination revealed that 18 out of these 49 horses had a patent *Habronema* infestation. Faeces of the remaining 31 horses were negative for *Habronema* larvae. A further examination of faecal samples using the same diagnostic procedure one week after oral administration of an oral moxidectin gel revealed that this treatment stopped the excretion of *Habronema* development stages. Destruction of muscid breeding sites and rising temperatures in the following months after treatment reduced the fly population on the farm.

Equine salmonellosis. The use of an enhanced rapid test system (Reveal®2.0 Salmonella Test System) for early detection of *salmonella* in feces and environmental samples

N.M. Slovis, and J.P. Elam
Hagyard Equine Medical Institute, Lexington, KY

In veterinary medicine, a rapid, inexpensive and accurate diagnosis of horses infected with Salmonella spp. is important, so that necessary control and preventative measures can be implemented to reduce the risk of disease transmission to other horses or personnel. Conventional diagnosis of Salmonella spp. is conducted by testing fecal samples using bacteriologic procedures in the laboratory. However, it can take up to 3 to 5 days to obtain laboratory results. Several studies have developed PCR tests for detection of Salmonella spp. in fecal and environmental samples but there is evidence of an increased frequency of Salmonella-PCR positive results in horses without clinical signs of salmonellosis that test negative to Salmonella spp by culture on multiple fecal samples, perhaps due to the use of primers targeting a non-specific Salmonella spp gene fragment that may cross-react with other enteric or non-enteric organisms. The objective of the study is to assess the diagnostic accuracy of an enhanced rapid test system (Reveal®2.0 *Salmonella* test system) for the detection of Salmonella spp. in fecal and environmental samples, compared to bacteriological culture identification.The samples were initially plated to a hektoen agar and inoculated in a selenite broth. After 24 hours, the hektoen agar was examined for black or green colonies. The selenite broth was subbed to a hektoen agar, incubated for 24 hours and then examined for black or green colonies. The Reveal®

test would be performed on any black or green colonies found on either hektoen agar. Our lab has performed 810 Reveal® tests on suspicious cultures from January 16, 2010 to December 31, 2011. There were 150 Reveal® positive samples and 660 negative samples. There was 100% correlation (100% sensitivity and 100% specificity) of the Reveal® test results to the BBL Crystal identification results. The Reveal® test has been shown in our lab to save 24 hour to 48 hours in notifying the veterinarian of a positive Salmonella culture. Further studies are warranted with the Reveal® test system on Selenite broths. If similar correlations are noted with the broths then *Salmonella* results may be able to be obtained in a 24 hour turnaround time.

Antitoxic activity of serum and colostrum antibodies induced by prepartum immunization of mares with binding domains of Toxin A and B of *Clostridium difficile*

J.F. Timoney [1], S.C. Artiushin [1], L.H. Fallon [2], and R.A. Rathberger [2]
[1] University of Kentucky, Lexington, KY, [2] Hagyard Equine Medical Institute, Lexington, KY

Enterocolitis caused by *C. difficile* is a serious sometimes fatal disease of neonatal foals and older horses [1, 2 and 3]. There are no reports of use of vaccines or immunotherapeutics against the disease. Results achieved for humans and laboratory animals provide a strong rationale for pursuit of an immunoprophylaxis approach in horses. Toxins A and B produced by *C. difficile* (TcdA and B) are well documented as important virulence factors of this bacterium. Our approach involved the use of the receptor binding regions of these toxins to generate antibodies to block toxin binding to cell receptors and thus prevent toxin entry and resultant diarrhea. The objective of this work was therefore to determine whether these antibodies generated in the pregnant mare will neutralize the toxicity of TcdA and B. Sequences encoding binding domains of toxins were isolated by PCR amplification from *C. difficile* JF09, a foal isolate, and cloned into pET15b. Thirteen pregnant mares were immunized twice two weeks apart with 0.2 mg of each recombinant protein and 2 mg Quil A two months prior to foaling. Antibodies were assayed in sera and colostrums by ELISA and for ability to block cytopathic activity of each toxin for equine endothelial cells. Significant ($P < 0.01$) elevations of IgG antibodies specific for each recombinant binding domains were detected in serum and colostrum of mares as well as foal sera 2 days after suckling. ELISA values for pre- and postimmune mare sera for both TcdA and TcdB were not correlated. Positive correlations were determined for IgG reactivity to TcdA and TcdB in postimmune sera/colostrum and in colostrum/foal serum. Cytotoxicities of TcdA and B were not inhibited by most pre-immune mare sera, control mare sera, control foal sera and control colostrums. Post immune mare sera, colostrums and foal sera completely inhibited or substantially reduced cytotoxicity of TcdA and B. Therefore, our studies showed that immunization of pregnant mares with recombinant binding domains of TcdA and B of *C. difficile* resulted in production of specific antibodies in serum and colostrum that blocked their cytopathic activity. These results support the feasibility of a prepartum vaccine against *C. difficile* enterocolitis in foals.

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Equine strongylid egg re-appearance period after ivermectin or moxidectin treatment in Italy

D. Traversa [1], A. Frangipane di Regalbono [2], D. Otranto [3], R.P. Lia [3], P. Beraldo [4], A. Di Cesare [1], B. Besognet [5], and T. Geurden [5]
[1] Department of Comparative Biomedical Sciences, University of Teramo, Italy, [2] Department of Veterinary Medicine, Production and Health, University of Padua, Italy, [3] Department of Animal Health and Zootechny, University of Bari, Italy, [4] Department of Food Science, Division of Veterinary Pathology, University of Udine, Italy, [5] Pfizer Animal Health

Drug resistance in cyathostomins is a potential threat for horse health, welfare and production. Recent studies have shown a reduction in egg reappearance period (ERP) after treatment with ivermectin (IVM) [1,2]. Although moxidectin (MOX) is still highly effective [3,4], recent studies suggest a decline in ERP for MOX as well [1,5]. Therefore, a survey was carried out in 2012 in Italy to investigate the ERP after treatment with both IVM and MOX in naturally strongylid-infected horses. Ten sites were selected on the basis either of a <100% efficacy of MLs in a previous survey or of a history of frequent use of macrolactones. In each site, ten horses were selected on the basis of a minimum pretreatment faecal strongylid egg count (FEC) of 50 (range 50 to 2425) per gram of faeces (EPG). Animals were randomly allocated to one of two treatment groups based on pretreatment FEC and treated either with IVM or MOX on Day 0. Individual faecal samples were taken from all animals included every two weeks until Day 56, and additionally on Day 70 and on Day 84 from the MOX-treated horses. All samples were examined using a modified McMaster technique with a sensitivity of 25 EPG. The results will be presented and discussed at the Conference.

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Drug Resistant Cyathostomins in Donkey Herds; Lessons in Management for All Equids

A.F. Trawford, and F.A. Burden
The Donkey Sanctuary, Sidmouth, Devon, UK

The Donkey Sanctuary has over 2500 donkeys on its farms in the UK. Endoparasite control in these herds has been a major focus for the last 10 years. Monitoring of the herds has identified significant levels of anthelmintic resistance in the cyathostomins parasitising the donkey herds. Lack of efficacy of ivermectin, moxidectin, pyrantel and fenbendazole has been identified on multiple sites and in a number of cases treatment of donkeys harbouring such parasites is becoming increasingly challenging. Changes in management practices and approach to parasite treatment is discussed. Research to establish the extent of drug resistance and clinical significance of parasitism in donkeys is of primary concern; projects have focussed on identification of drug resistance, impacts of pasture management, validation of thresholds of faecal egg counts for treatment and alternatives to anthelmintics. Significant changes in management practices have been based upon results of these studies and new ideas and techniques are being developed. Treatment of all donkeys is carried out on the basis of a strongyle faecal egg count which is carried out on a 4-weekly basis throughout the year. Preliminary data and clinical experience has shown that increasing the FEC treatment threshold for healthy, mature donkeys to $\geq$ 1000 strongyle epg is sufficient to control clinical disease whilst reducing pasture contamination. Application of new treatment thresholds have reduced the reliance on chemical treatment. The mean number of anthelmintic treatments per year per animal was one in 2011; this is a significant reduction from previous dosing regimes where animals would be treated a minimum of four times per year. Animals are monitored frequently with problematic herds being 100% sampled every four weeks; such monitoring allows individualised treatment programmes and highlights reduced egg reappearance periods early. Reduced ERPs are investigated immediately with suspect animals undergoing a faecal egg count reduction test (FECRT) at the next treatment. Failed FECRTs trigger an alternative approach to treatment and further investigation. It is evident that some individual cases are becoming increasingly difficult to treat effectively; future trials of combinations of anthelmintics are being considered. Effective parasite control has also led to an increased emphasis on pasture management; dung is closely managed and carefully composted. Pasture is also rested in 6 month blocks with mixed species grazing instigated where possible. Good pasture management practices produce significant drops in pasture larval counts and is the primary mechanism used to reduce parasitism. Research and practical experience has emphasised the importance of good pasture management, treatment based upon faecal egg counts and routine assessment of efficacy of treatment. Researching and trialling higher dosing threshold has also led to significant decreases in the reliance of the organisation on anthelmintics with no recorded clinical effects. The lessons learnt from management of these large herds of donkeys may provide salient and important foundations for future equid herds that undoubtedly will experience similar problems in the future.

Differentiation of cyathostomin species from horses with shortened Egg Reappearance Period after treatment with ivermectin

D.C.K. van Doorn, F.N.J. Kooyman, H.W. Ploeger, M. Eysker, and J.A. Wagenaar
Department of Infectious Diseases and Immunology, Faculty of Veterinary Medicine, Utrecht University, the Netherlands

At the introduction of ivermectin the Egg Reappearance Period (ERP) was 8 weeks. Currently a shortened ERP was reported several times. It is unknown to which cyathostomin species these eggs belong. In the current study these eggs were cultured to L3 and subsequently differentiated. 5 farms with 10 egg shedding horses each were included. The horses were aged between 1-3 years and were last treated with an anthelmintic at a minimum of 8 weeks before start of the study. Weight of the animals was estimated with a girth tape. Horses were treated with ivermectin (Eraquell®) dosed according to the product label. Fecal egg counts and larval cultures were performed after each visit (day-3, d0, d14, d21, d28, d35, d42, d70, d84,) Egg counts were performed with a McMaster method with a sensitivity of 50 eggs per gram. Larvae were cultured for 10 days at room temperature and larvae per gram were determined for all treated horses. On 2 farms 2 horses with shortened ERP and a horse with ERP of $\geq$ 8 weeks on each farm were selected. On these 2 farms the larvae, from in total 6 horses, were investigated at 3 different time points. Once before treatment, the second time the day that the egg counts were $\geq$ 10% of the egg counts at day 0. The third time when the 4 horses with shortened ERP on d42 and the other two after d56. Forty of these cyathostomin larvae per animal per time point were randomly selected from the cultures and differentiated using a Reverse Line Blot technique. This method is based on hybridization of PCR products from individual L3s with species specific probes. The ERP on all 5 farms was shortened to 6 weeks on average, with large individual differences. Preliminary results from the differentiation of the L3s showed a relative increase of *Cylicocyclus leptostomum* and *Cylicocyclus ashworthi* during the shortened ERP. The shortening of the ERP can be caused by

more rapid development of the larvae, reduced residual effect of treatment or a combination of both. These data show an alteration from the situation as was found when ivermectin was introduced and can be considered as an early warning for the development of ivermectin resistance.

Evidence of host adaptation in *Lawsonia intracellularis* infections

F.A. Vannucci [1], N. Pusterla [2], S.M. Mapes [2], M. Kelley [1], and C.J. Gebhart [1]
[1] Department of Veterinary and Biomedical Sciences, College of Veterinary Medicine, University of Minnesota, St. Paul, MN 55108, [2] Department of Veterinary Medicine and Epidemiology, School of Veterinary Medicine, University of California, Davis, CA 95616

Lawsonia intracellularis is the causative agent of proliferative enteropathy (PE), an emerging concern in horses and an endemic disease in pigs. Enterocyte hyperplasia is a common lesion in PE but there are differences regarding clinical and pathological presentations among affected species. We hypothesize that host susceptibility to *Lawsonia* infection depends on the species of origin of the bacterial isolate. The objective of this study was to evaluate the susceptibilities of horses and pigs to *Lawsonia* infection using equine and porcine isolates. Twelve 4-month-old foals were divided into three groups (n=4/group) and infected with an equine [1] or a porcine [2] isolate and a saline solution (negative-control group). An identical experimental design was applied to 18 3-week-old pigs divided into three groups (n=6/group). Two pigs from each group were euthanized 21 days post-inoculation (PI) for evaluation of gross lesions and the level of infection by immunohistochemistry (IHC). The animals were monitored for clinical signs, average daily weight gain, fecal shedding of *Lawsonia* [2], and humoral serological response [1] during 56 days PI. Fecal shedding (Figure 1A; $p < 0.05$) and serologic response were higher and longer in foals infected with the equine isolate compared with foals infected with the porcine isolate or with the negative-control group. One equine-isolate infected foal developed severe clinical signs, did not respond to the supportive care and was euthanized 24 days PI. Typical lesions and marked presence of *Lawsonia* antigen was identified by IHC. Similarly, reduced average daily gain and diarrhea were observed in pigs infected with the porcine isolate. Only porcine isolate-infected pigs demonstrated proliferative lesions associated with the presence of specific *Lawsonia* antigen by IHC. Additionally, these animals showed higher and longer shedding of bacteria in the feces (Figure 1B; $p < 0.05$) and serologic response compared with equine isolate-infected pigs. Clinical signs, longer periods of shedding and stronger serologic immune responses were observed in animals infected with species-specific isolates. The results support our hypothesis that host susceptibilities can be driven by the origin of the bacterial isolate. Currently, comparative genomic analysis is being conducted in order to associate these phenotypic characteristics with potential genomic variations between porcine and equine isolates.

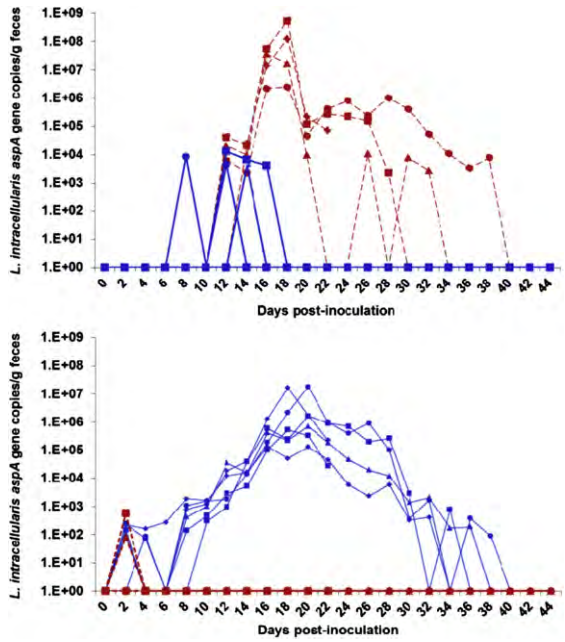

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Figure 1. Fecal shedding of *Lawsonia* (blue-solid line = porcine isolate-infected animals; red-dashed line = equine isolate-infected animals).

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Equine coronavirus, a possible cause for adult horse enteric disease outbreaks

R. Vin [1,4], N. Slovis [2], P.J. Henney [3], U.B.R. Balasuriya [3], and C.M. Leutenegger [4]
[1] Myhre Equine Clinic, Rochester NH, [2] Hagyard Equine Medical Institute, Lexington, KY, [3] Maxwell H. Gluck Equine Research Center, Lexington, KY, [4] IDEXX Laboratories, Inc., Sacramento, CA

Little information about the clinical and pathological consequence of equine coronavirus (ECoV) infections in adult horses is currently available in the literature. In this retrospective study, we determined the prevalence of ECoV in fecal samples from horses that had diarrhea using a fecal real-time PCR panel, and used viral isolation, history, clinical signs, and laboratory results to assess the pathophysiological significance of ECoV as an etiological agent in equine entero pyrogenic disease in the adult horse. A total of 560 fecal samples from 560 horses of all ages were tested using a panel of ten real-time PCR assays specific for equine bacterial, viral and protozoal agents. Of the 560 fecal samples, thirty five (6.25%) tested positive for ECoV nucleic

acid by real-time RT-PCR. All ECoV positive horses in this study were older than one year. In addition to diarrhea, clinical presentation of ECoV PCR positive horses included colic and pyrexia. The most common laboratory abnormality was neutropenic leukopenia. The 525 fecal samples negative for ECoV serves as strong control showing that ECoV is not a common incidental finding in horses fecal samples. 16/35 horses were from outbreaks (clusters of more than one horse with a positive clinical presentation and positive ECoV PCR result within a single farm). 3/16 horses associated with outbreaks tested positive for co-infections with Cryptosporidium species, but all 3 were associated with horses only positive for ECoV within the same farm. Of the 35 total ECoV positive horses, 8 were co-infected: 4 with cryptosporidium species, 1 with equine rotavirus, 1 with C. difficile toxin A & B, 1 with C. perfringens enterotoxin A, and 1 with salmonella species. ECoV outbreaks were identified in 4 states: CA, WA, ID, and NJ. ECoV was successfully isolated from multiple fecal samples submitted from outbreaks in ID and WA. Sporadic ECoV PCR positive cases with clinical signs were found in 11 states: CA, TX, MI, PA, ID, MT, NM, NJ, VA, NH, and WA. The presence of clinical signs, laboratory abnormalities and the molecular and virological findings suggest that ECoV is associated with diarrhea outbreaks in adult horses. Results of this retrospective study indicate that ECoV should be considered as an important enteric, potentially contagious, viral pathogen of adult horses. More experimental study is needed to better define the virulence and pathophysiology of US ECoV strains in horses to further the understanding of coronavirus infections in the horse.

Parasitological, clinical and serological examinations on the progress of *Parascaris equorum* infections in foals

M. Völlger [1], J. Demeler [1], M. Lämmer [2], and G. von Samson- Himmelstjerna [1]
[1] Institute for Parasitology and Tropical Veterinary Medicine, Freie Universität Berlin, Germany, [2] Lewitz Stud, Neustadt-Glewe, Germany

Parascaris equorum causes infections in young horses worldwide. Despite the ubiquitous prevalence of this infection there is still a considerable lack of information e.g. on the course of infections in stud farms, its clinical relevance also in the context of other infectious diseases and the serological conduct of the infection. To monitor the course of *P. equorum* infection we performed coproscopical examinations and took blood samples on a weekly basis on 190 foals from a big German stud over a period of 25 weeks. The faecal samples were examined using the FLOTAC technique and the blood samples were frozen until the serological examinations. During the course of the study, the copromicroscopic analysis showed a *P. equorum* infection rate of approx. 60 per cent in this herd. The mean weekly faecal egg per gram (epg) counts started to become positive in foals 9 weeks of age. This is somewhat earlier than expected according to the prepatency period of at least 10 weeks and indicates that foals are being exposed to ascarid eggs directly after birth. The mean epgs steadily increased reaching with a peak epg of 171(SD ±345, range 0-2292) at the end of the trial when foals were 25 weeks old. Ascarid eggs isolated from the faeces were cultivated to generate larvae which were used for producing a larval excretory-secretory antigen (ES). This antigen was examined SDS-PAGEchromatography. By Western Blot analysis using sera from five coproscopically positive and negative foals, each, marked similarities within each of the two groups and explicit difference between groups were encountered. Thus, the larval ES antigen preparation appeared to be suitable for use in ELISA tests. In first tests with positive and negative sera and a secondary antibody directed against IgG antibodies a differentiation was found to be possible. Ongoing analyses aim at the evaluation and optimization of the ELISA test and the characterization of the serological profile during the course of infection.

Occurrence of *Anoplocephala perfoliata* in Swedish horses and possible associations with colic

H. Back, and E. Osterman Lind
National Veterinary Institute (SVA), Department of Virology, Immunobiology and Parasitology, SE-751 89 Uppsala

The tapeworm *Anoplocephala perfoliata* is common worldwide in horses; the prevalence varies from 50 to 65%. Nevertheless, the significance of tapeworm infections is uncertain and still under debate. The parasite was previously thought to be of little or no clinical significance. However, studies in the UK suggest that tape worm infections are associated with ileo-caecal colic, especially when the infection intensity is high. Currently, little is known about the association between *A. perfoliata* infections and colic in horses under Scandinavian conditions. The aim of this study is to evaluate a possible association between infection with *A. perfoliata* and colic signs in Swedish horses. The study, which started in 2011 and will include 150 horses, (75 cases and 75 control horses) visiting either of two equine veterinary clinics in the south of Sweden. The cases are horses treated for colic symptoms. For each colic horse, a non-colic horse of the same age and with no history of colic is chosen as control. Faecal and blood samples are taken from all horses and submitted to the National Veterinary Institute (SVA). Moreover, a questionnaire on deworming routines, grazing history colic symptoms, colic treatment etc is filled in by the horse owner and the clinician. The fecal samples are analyzed for tapeworm eggs with a modified flotation technique based on 30 g of faeces. Sera will be tested for the presence of *A. perfoliata* antibodies by the ELISA method of Proudman and Trees. So far, a total of 120 samples and questionnaires have been submitted to SVA. The prevalence for *A. perfoliata* in the case group is 18%, while it is 6% in the control group. Fisher's 2-sided test presented a P-value of 0,095. An association between colic and the presence of *A. perfoliata* could not be ruled out based on the current data generated. More data points are needed to complete this analysis. Results from the assays and the questionnaires will be analyzed and presented.

Polymorphism at the 167 and 200 Allele of the β-Tubulin Gene in Adults and Larvae of *Cyathostomin* sp

R. Canever [1], U. Ioshitani [1], and M.B. Molento [1,2]
[1] Federal University of Parana, UFPR, Curitiba, PR, Brazil,
[2] National Institute of Science and Technology, INCT, Brazil

Cyathostomins are the most prevalent parasites in horses causing colic, diarrhea and death. The control is difficult due to the large number of different species and routine treatment has favor the selection of resistant population, especially in Brazil [1]. One of the problems is the late diagnostic to monitor resistance and molecular tools must be developed to detect specific alleles (SNPs). Point mutations on the β-tubulin isotype 1 gene may induce mutations on the 167, 198, and 200 codons, which are associated with benzimidazole (BZM) resistance. The TTC/TAC polymorphism at the 167 position has being incriminated as the mechanism of resistance to the BZMs [2]. The objective of this work was to determine the frequency of the polymorphism at the codons 167 and 200 of the β-tubulin isotype 1 gene in BZM-resistant adults and larvae of *Cyathostomin* sp. Twenty adult worms were collected from one horse from the Sao Jose da Serra Stud, a BZM-resistant (0% to fenbendazole) farm. The larvae were collected from 10 pool samples from BZM-resistant horse farms after faecal egg count reduction test (FECRT). Specific primers [3] were used to amplify the codons 167 and 200 for β-tubulin isotype 1 gene using, 167: forward primers 5'-GCTAACT CACTCACTTGGAGGA-3'; reverse: 5'-CTTTGGTGAGGGAACA ACG-3' and 200: forward primers 5'- TACAATGCTACCCTAT CCGTTCAT-3'; 5'-GAAGTGAAGACGAGGGAATGGA-3'. Samples were sequenced with ABI3130 and analyzed with Mega 5. It was possible to identify the DNA amplified bands (167 and 200 = TTC) to all samples (Figure 1) with double peaks 167 (6/20) and 200 (2/20) which corresponded to heterozygous worms (TTC/TAC) on chromatograms. All larvae had double peaks for the 167 and 7 for the 200. We found only the homozygous (TTC/TTC) susceptible genotype in adults and also the heterozygous (TTC/TAC) to benzimidazole in larvae. As suggested by other authors we consider that the mutation at the codon 167 may be involved with the mechanism of resistance in the *Cyathostomin* sp. and that molecular markers using SNPs could be used to detect BZM-resistant worms, most certainly for the codon 167 in adults and larvae.

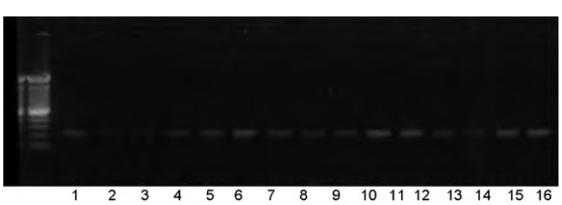

Figure 1. DNA bands of *Cyathostomin* sp β-tubulin isotype 1. Column 1: molecular marker (100 bp), columns 2 to 8: adults, 9 to 16: larvae.

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Counting of *Anoplocephala eggs* in equine faecal samples

J. Cima, and G.C. Coles
School of Veterinary Sciences, University of Bristol,
Langford House, Bristol BS40 5DU, UK

A simple reliable method to count cestode eggs in equine faecal samples would be of value to possibly indicate a) numbers of mature worms present in the horse, b) whether horses required treatment and c) whether products were fully effective. Previous attempts at counting eggs of *Anoplocephala perfoliata* have not been very successful [1,2]. With the introduction of a novel egg counting technique sensitive to 1 e.p.g., FLOTAC [3], the possibility of using this technique to count cestode eggs in horse faecal samples was examined. Adult *A.perfoliata* and faeces from horses with negative worm burdens or known numbers of worms were collected from a licensed horse abattoir. Mature segments of worms were homogenised in tap water and washed through a 150μm sieve onto a 50 μm sieve. A known number of eggs were re-suspended and added to 10 g of faeces from uninfected horses. Counts were performed using the FLOTAC technique and different flotation solutions. When eggs were added directly to the FLOTAC cell the recovery was 100%. Percentage recovery of eggs added when undertaking the full FLOTAC process using three flotation solutions without faeces were: saturated sucrose 46%, saturated sodium chloride 21% and saturated zinc sulphate 13%. To eliminate the possibility of the eggs sticking during the process, Tween 20 was added to the flotation solutions at different concentrations. Recovery rates for the addition of 5% Tween 20 with no faeces added were: sucrose 81%, sodium chloride 79% and zinc sulphate 74%. When testing different solutions and Tween 20 concentrations, it was found that 10% Tween 20 and 50% sucrose produced the best results. With the FLOTAC technique using this combination and 10g faeces, a recovery rate of 72% was produced. When eggs were added to egg free faeces, eggs were always found in the sample, but the results were still variable. The repeatability of naturally infected faecal samples from heavily worm burdened horses was low, varying from horse to horse, suggesting eggs were not evenly distributed in the faeces. Further experiments with FLOTAC indicated that egg loss occurred at an early stage when the faecal suspension was first filtered suggesting that the coarse nature of the faecal fibres trapped the eggs. This was confirmed by the high recovery of eggs (88%) when added to cattle faeces. Novel methods of estimating worm burdens in horses are therefore required.

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The prevalence of helminths with tissue associated stages in horses in the south-west of England

G.C. Coles, D. Fews, H.V.F. Jones, B. Hayes, and K. Shaddick
School of Veterinary Sciences, University of Bristol, Langford House, Bristol BS40 5DU, UK

The transmission of a horse strain of *Echinococcus granulosus* occurs in the UK between hounds fed raw horse meat and horses with infection levels at 60% [1]. To re-estimate the prevalence, livers of horses slaughtered at a licenced abattoir were inspected. The opportunity was also taken to examine for the presence of *Strongylus vulgaris*, *Strongylus edentatus* and *Onchocerca cervicalis*. Horses at the abattoir were primarily private horses brought in by owners with some animals from common grazing on moorland. A total 921 horses were visually inspected for the presence of *E granulosus*, *S.edentatus*, and *Setaria equina*. Of these 264 were also examined for *S.vulgaris* and *O.cervicalis.* Aneurisms caused by *S.vulgaris* were dissected out and numbers of larvae counted. To detect *O.cervicalis* fresh 8mm skin biopsies were collected and thin slices incubated over night at 37C. A questionnaire was sent out to 17 hunts on the feeding of raw meat and treatment of dogs with praziquantel. Hydatid cysts were present in 17.4% of livers. Most livers had only 1-2 cysts between 1 and 10 cms in diameter. As most horses were old when slaughtered this represents the probable rate of infection up to 20 years previously. One decade later *E.granulosus* cysts are still being found but at a reduced rate of 5.2% (in 1205 horses). 18% of kennels stated that they regularly fed raw horse meat and 59% said they did this occasionally. Only 53% stated that they were using a praziquantel based product so the infection rate is not really surprising. 5.7% of the 264 horses had *S.edentatus* in the peritoneal cavity and firm white foci in the liver. Of the 18 horses with aneurisms caused by *S.vulgaris* 3 had no worms and the remainder a mean of 16.7 worms (range 1-52). Although large strongyles have been greatly reduced in prevalence due to the use of macrocylic lactones they are clearly still present in the horse population possibly representing failure to use anthelmintics correctly. Small numbers of larvae of *O.cervicalis* were only found in October and not during the rest of the winter, *S.equina* was not found in any horses. The low numbers of *O.cervicalis* and lack of *S.equina* probably represents the widespread use of macrocylic lactones and the disruption of their transmission.

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Diarrhea in Thoroughbred foals is still a disease of concern in Argentina

M. Ferrari [1], S. Miño [2], J. Raffo [1], J. Mondelli [1], M. Etchepareborda [1], A. Vissani [2], V. Parreño [2], and M. Barrandeguy [2]
[1] La Biznaga Stud Farm, Estación Islas, Buenos Aires, Argentina, [2] Instituto de Virología, CICVyA, INTA Castelar, Buenos Aires, Argentina

Diarrhea in foals is considered a major sanitary problem in thoroughbred breeding farms worldwide. The ethiology of diarrhea can be bacteria, viruses, parasites, or a range of non-infectious agents or conditions such as toxins, lactose intolerance, or “foal heat” diarrhea. During the 1990s, the morbidity rates of diarrhea in foals varied, reaching 100% in some Argentinean farms. Group A Rotavirus (RVA), G3 P[12] and G14 P [12], were associated to 39% of the outbreaks of foal diarrhea studied during a 17-year surveillance period. The introduction of a systematic Rotavirus vaccination program in pregnant mares significantly reduced the morbidity rates, reaching levels of only 15% in some thoroughbred farms. The aim of this work is to describe the occurrence of diarrhea in thoroughbred foals in a farm with high standard management in Argentina. The farm has a history of foal diarrhea outbreaks of variable intensity, which have occurred almost every year, and, as in other farms, the incidence and severity of diarrhea seem to have increased in the last years. The pregnant mares are vaccinated with a commercial inactivated rotavirus vaccine. The foals are born in a clean and disinfected stall, and under the care of a veterinarian, and get good-quality colostrums, in sufficient amounts, within the first 18 hours of life. Those mares with foal on foot are kept in a small individual paddock and under veterinarian supervision for 7 additional days. After this period the mares are grouped on pastured paddocks under daily veterinarian supervision. During the 2011 breeding season 40% (62/156) of the foals developed diarrhea. The first case took place in July 31st, and the last one in December 28th, while the maximum incidence occurred in October, when 24% (32/134) of the foals were affected. The age of affected foals ranged from 8 to 132 days, but in the majority of cases the age was between 10 and 20 days at the moment of the initiation of the disease. The duration of the disease was also variable, but the disease lasted, in average, 7 days. No deaths were registered. All of the affected foals received an immediate supportive symptomatic treatment. Twenty-one samples (diarrheic feces), obtained as soon as diarrhea was taken notice of (first day of disease), were analysed for RVA infection by commercial rapid test (*FASTest* ROTA Strip Megacor). RVA was detected in 38% (8/21) of the samples, and was characterized as G3 P[12]. In the present study, the number of diarrheic foals that did not shed the virus in feces on the first day of disease is remarkable, and emphasises the need for further research. Other infectious agents could be associated to diarrhea in young foals. Vaccine breakthrough should not be disregarded.

Lawsonia intracellularis: Clinical presentation and long term effects of disease

M.L. Frazer
Hagyard Equine Medical Institute, Lexington, KY 40511

Equine proliferative enteropathy caused by infection with *Lawsonia intracellularis* (LI) is a commonly diagnosed infectious disease in the equine population. A retrospective review of medical records at Hagyard Equine Medical Institute in Lexington, Kentucky was performed to characterize typical clinical presentation. The study included 57 horses diagnosed with LI infection based on the inclusion criteria of hypoalbuminemia of less than 2.5 g/dL (reference range 3.5-4.5 g/dL) and a positive fecal polymerase chain reaction (PCR) for LI, a positive (>60) serum immunoperoxidase monolayer assay (IPMA), or both. The most common clinical signs of LI infection in this group of horses were ventral edema, poor body condition, and lethargy, as well as diarrhea, fever, and colic. Horses were usually diagnosed with disease in the fall or early winter. Affected horses were most often foals and weanlings between two and eight months of age. The majority of horses treated for the disease survived. A second retrospective review of medical records was performed to determine if horses diagnosed with LI infection had long term effects from disease. Horses included in this study had the same inclusion criteria as the first study, although only Thoroughbreds were included. 116 horses met the initial criteria. Sale price of horses sold at public auction as yearlings were compared to average sale price of all yearlings by the same stallion as the affected horse. The sale price of the 36 horses sold at public auction was significantly less than the sale price of the control horses. Monetary earnings from racing were compared to average monetary earnings of progeny by the same stallion as the affected horse. Monetary earnings were not significantly different between the LI infected horses and the control horses. 12 horses both sold at public auction and raced. As well as being included in the total number of horses that sold and raced, their sale records and monetary earnings were compared to the averages from their respective stallion as a separate group. This group of horses also sold for significantly less than the control group, but did not have significant differences in monetary earnings. These retrospective studies may aid practitioners in diagnosing LI infection by characterizing common clinical signs, signalment, and season of presentation. In addition, these studies may validate the decision to treat LI infected horses by demonstrating that LI infection does not necessarily affect future athletic potential of the horse.

The effects of windrow composting on the viability of *Parascaris equorum* eggs

J.C. Gould, M.G. Rossano, L.M. Lawrence, and E.T. Lyons
University of Kentucky, Lexington, KY, USA

Parascaris equorum is a parasite common in horses less than 18 months of age and its pathological effects can be severe. Infection occurs when embryonated eggs present in the horse's environment are ingested. Foals exposed to pastures where horse manure was used to fertilize grazing pastures have a significantly higher risk of testing positive for *P. equorum* in a fecal egg count (FEC) evaluation1. Composting manure prior to application on pasture could alleviate this risk. The objective of this study was to evaluate the effects of a windrow composting system on the viability of *P. equorum* eggs at a commercial horse breeding farm. Equine manure, soiled bedding used to build the experimental windrow was provided from stalls housing only mature horses confirmed negative for *P. equorum* eggs. Ingredients in the compost pile were balanced to create a 30:1 carbon nitrogen ratio. Equine feces containing *P. equorum* eggs were sealed in filter bag sentinel chambers. Chambers were exposed to 1 of 3 treatments: constant, or intermittent exposure to the interior of the windrow, or control (stored at 4°C). At day 0, all chambers were placed in the center of 10 locations of the windrow. On days when the windrow was turned, chambers in the constant exposure treatment were returned to the interior of the windrow and chambers in the intermittent exposure treatment were alternated between resting on top of, or inside, the windrow. Chambers from each treatment and controls were removed at days 2, 4, 6, 8, 10, 12, 14, and 18; and incubated for 21 days at room temperature (24°C). After incubation, eggs were recovered from the chambers, evaluated microscopically and classified as viable or nonviable based on whether embryonation had occurred. Results were reported as the mean percent viable eggs for each treatment and time point. A mixed linear model with repeated measures was used to evaluate the influence of experimental day and treatment on the percent viability of *P. equorum* eggs. Chambers treated with constant exposure contained 10.73% (SD = 0.29) viable eggs on day 2 and declined to an average percent of 0.00 by day 8. Chambers exposed to the intermittent treatment contained 16.08% (SD = 0.26) viable eggs on day 2 and decreased to 0.00 by day 6. Control sentinel chambers for days 2, 4, 6, 8, 10, 12, 14, and 18 all had viabilities above 79.00%. A significant fixed effect of experimental day ($p < 0.0001$) and compost treatment ($p < 0.0001$) was observed. The results of this study demonstrate that the windrow composting system was effective at rendering *P. equorum* eggs nonviable when tested under the conditions at a working horse farm.

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Evaluation of anthelmintic treatment options according to horse risk profile

K. Hautala [1], L. Salonen [1], and A. Oksanen [2]
[1] Faculty of Veterinary Medicine, University of Helsinki, Finland, [2] Research and Laboratory Department, Finnish Food Safety Authority (FINPAR), Finland

Increasing anthelmintic resistance is an emerging problem within the equine industry. Overuse of anthelmintics is suspected to be a major reason for this phenomenon. A treatment regimen where all horses on a farm are treated

simultaneously at frequent intervals is still common in Finland, even though horse practitioners are now promoting the use of targeted selective treatment. In order to evaluate selective therapy as an option to reduce anthelmintic use we conducted a study on ten stables. Objectives: 1. To estimate parasite loads of individual horses 2. To analyse risk factors for high egg counts 3. To observe the effects of different anthelmintics to the individual parasite loads. One to eight fecal samples per horse were collected from 215 (86 geldings, 100 mares, 24 stallions) horses (mean age 9.1 yrs, min 0 and max 29) from ten stables in Finland during a one-year study period. The samples were analysed by modified McMaster-technique by the Finnish Food Safety Authority (Evira). Antiparasitic treatment history of each horse was collected by a questionnaire. Majority of the adult horses maintained low epg-values (<200) throughout the study period. Epg-values of young horses (<4 year-olds) were significantly higher than those of adult horses. Post antiparasitic treatment epg-values increased more rapidly in young horses than in adults. Young age and being a mare or stallion are risk factors for high egg counts. The egg count has a negative linear relationship with age. No higher egg count was observed in the oldest age group (>20 years). Majority of the adult horses are able to maintain low epg-values and therefore frequent treatments with anthelmintics to all horses should be discouraged. Horses should be sampled and treated based on individual risk factors and epg-values. Further analysis of the data will bring more information on how the risk of high epg-count is affected by grazing, season and treatment intervals.

Factors influencing the magnitude of strongyle egg-shedding in horses

A. Honeder [1,2], A.M. Becher [2], M. Reist [3], and K. Pfister [2]
[1] Pferdepraxis Dr. Müller, D-83395 Freilassing, Germany, [2] Comparative Tropical Medicine and Parasitology, Vet. Faculty, Ludwig – Maximilians -University, D-80802 Munich, Germany, [3] Veterinary Public Health Institute, Vetsuisse-Faculty, University of Berne, CH-3097 Liebefeld, Switzerland

A relatively new approach in equine worm control is targeted selective treatment. Using this method only those horses are treated with anthelmintic drugs, which are shedding a high number of strongyle eggs. The aim of this study was to evaluate the influence of different factors on the magnitude of strongyle egg-shedding in horses. Faecal samples were collected in 2009 from 314 horses from 35 farms located in the Austrian-Bavarian border area of Salzburg. Altogether, 2703 samples were analysed using a modified McMaster Method (sensitivity: 20 eggs per gram feces) Additionally, the following data was collected for every horse/sample/farm: age, sex, breed, faecal egg count (FEC) data from the previous year, management and hygiene procedures, previous deworming methods and data about climatic conditions before and during the sampling period. With all variables, a univariate Poisson Regression was performed. Variables with a statistically significant influence on the magnitude of strongyle egg shedding were included into a multivariate Poisson Regression model and a backward-elimination was performed. The following variables remained in the multivariate model and were positively associated with the level of the strongyle FEC: mean strongyle FEC in 2008, mean strongyle FEC of the horse population on the same farm, mean strongyle FEC of the pasture-mates, size of large paddocks and number of months since the last anthelmintic treatment). Negatively associated was the age. The effect of hygiene of pasture and of large paddocks on FEC was not consistent. The parameters hygiene of stables and paddocks/bedding of stables/drug class of the last anthelmintic treatment and breed were not significantly associated with the FEC, but remained in the multivariate model as confounders. These results confirm that a reduction of the mean strongyle FEC of pasture-mates can reduce the magnitude of the strongyle FEC of individual horses. This is a very important argument for further developing the method of selective anthelmintic treatment in horses.

The influence of anthelmintic treatment on strongylid (Nematoda: Strongylidae) community structure in domestic and wild equids

T.A. Kuzmina [1], E.T. Lyons [2], S.C. Tolliver [2], and V.A. Kharchenko [1]
[1] Institute of Zoology NAS of Ukraine, vul. B. Khmelnyts'kogo, 15, Kyiv, 01601, Ukraine, [2] Department of Veterinary Science, Gluck Equine Research Center, University of Kentucky, Lexington, KY 40546-0099, USA

Strongylids (Nematoda: Strongylidae) are the group of parasites of domestic and wild equids worldwide. Three groups of anthelmintics (benzimidazoles–BZs, tetrahydropyrimidines–PYR, and macrocyclic lactones) are widely used to control horse strongylids nowadays. The aim of our work was to analyze the influence of different parasite control schemes on the biodiversity and structure of strongylid communities in domestic and wild equids. Our studies were performed during 2004–2011 on 69 domestic horses from various types of horse farms in Ukraine, 31 donkeys from a natural reserve and private farm, and 29 wild Przewalski's horses kept at the "Askania-Nova" reserve and in zoos. Animals had undergone

different schemes of parasite control from "zero-treatment" to regular deworming 4-5 times per year. Strongylid nematodes were collected from all equids *in vivo* after their deworming with the macrocyclic lactone drug "Univerm" (0.2% aversectin C, Russia). Totally, 62,722 specimens were collected and identified. Thirty-six strongylid species were found – 9 species of large strongyles (subfamily Strongylinae) and 27 species of small strongyles (Cyathostominae). In domestic horses, 27 strongylid species from 12 genera were found. The highest biodiversity of the strongylid community (27 species) was observed in horses from farms with rare anthelmintic treatments (1 time per year or less); from 8 to 20 species (average 12.1±3.4) parasitized per host. The lowest biodiversity (19 species) was observed in horses from farms with deworming 4-5 times per year; from 3 to 16 (6.7±2.6) per horse. Eleven cyathostome species (*Cylicocyclus nassatus, C. ashworthi, C. leptostomus, Cyathostomum catinatum, C. pateratum, Cylicostephanus calicatus, C. longibursatus, C. goldi, C. minutus, Coronocyclus coronatus*, and *C. labiatus*) were found to be resistant to benzimidazole anthelmintics. In donkeys, 26 strongylid species were found. In donkeys from the "Askania-Nova" reserve with rare anthelmintic treatment, 25 strongylid species were found; from 11 to 16 species (13.2±1.7) per host. In donkeys regularly treated with BZ and PYR, 14 species were found; from 3 to 10 (5.6±1.9) per host. In Przewalski's horses from the "Askania-Nova" reserve, 31 strongylid species were found; there were 11 to 18 species (15.2±1.9) per host. Przewalski horses from zoos harbored 21 strongylid species; from 7 to 13 (10.4±2.1) per host. The structure of the strongylid community was bimodal (with dominant and rare species) in all regularly dewormed equids. Multimodal structure (with dominant, subdominant, background and rare species) was observed in never treated animals or with rare dewormings. Future studies are necessary to clear up whether such a decrease in biodiversity and change in strongylid community structure caused by deworming programs can lead to increasing the pathogenic influence of these parasites in horses or provoke development and spreading of anthelmintic resistance in strongylids.

Equine rotavirus detection: sensibility and specificity of two commercially available diagnostic kits

S. Miño [1], M.A. Vissani [1], A. Kern [2], V.G. Parreño [1], and M.E. Barrandeguy [1]
[1] Instituto de Virología, CICVyA, INTA Castelar, Buenos Aires, Argentina, [2] MEGACOR DIAGNOSTIK GmbH Lochauer Str. 2 A - 6912 Hörbranz – Austria

Group A rotaviruses (RVA) are important pathogens associated with diarrhea in human infants and the young of several animal species including foals. Currently a variety of methods are commercially available for rapid laboratory diagnosis, mainly designed for human stool samples, i.e., enzyme immunoassays (ELISA), latex agglutination and strip tests. The sensibility and specificity of such tests have been ascertained in human stools, but the applicability in veterinary diagnosis has been poorly studied. The aim of this study was to compare the sensibility and specificity of two commercially available diagnostic kits for detection of RVA in stool samples from diarrheic foals. A total of one hundred and ninety stool samples collected from young foals with diarrhea during 2009, 2010 and 2011 breeding seasons, in stud farms located in the Buenos Aires province, were analyzed. ELISA Pathfinder (BioRad, Marnes-la-Coquette, France) and immunochromatographic *FAST*est ROTA Strip** (MEGACOR DIAGNOSTIK GmbH, A-6912 Hörbranz, Austria) assays were used, following the manufacturer's instructions. All the samples were also tested by an in-house ELISA, and samples showing discordant results were analyzed by RT-PCR targeting the VP6 gene. A sample was considered "true positive" when two or more assays detected rotavirus on it and, contrarily, "true negative" when three or more assays were unable to detect rotavirus. Of the 190 samples tested, 48 and 142 met the criteria to be considered rotavirus positive and negative, respectively. ELISA Pathfinder detected 16 (33%) true positive and 137 (97%) true negative samples, while *FAST*est ROTA Strip detected 44 (92%) true positive and 135 (95%) true negative samples. Five samples were detected as positive only by ELISA Pathfinder and 7 only by *FAST*est ROTA Strip. Thirty two true positive samples were not detected by ELISA Pathfinder and four by *FAST*est ROTA Strip. According to ROC analysis, the sensibility and specificity for diagnosis of equine RV (ERVA) were 95% and 92% for *FAST*est ROTA Strip, and 33% and 96% for ELISA Pathfinder, respectively. The low sensibility of ELISA Pathfinder to detect ERVA could be due to a variation in the VP6 protein (I2 and I6 genotypes) of the Argentinean ERVA field strains. Antigenic variations in the viral VP6 epitope could have the effect of diminishing or completely eliminating the capacity of the monoclonal to detect the virus. Phylogenetics and further epitope mapping studies are needed to confirm this hypothesis. On the light of the obtained results, the use of *FAST*est ROTA Strip is by far more suitable and efficient to the screening of Rotavirus infection in young foals with diarrhea than ELISA Pathfinder. Moreover, the strip format is easier and faster to carry out, and does not require any additional expensive equipment, skilled personnel and/or additional reagents.

Real-time PCR determination of *Strongylus vulgaris* in horses on farms with different anthelmintic regimens in Denmark and Central Kentucky

M.K. Nielsen [1], S.N. Olsen [2], E.T. Lyons [1], J. Monrad [3], and S.M. Thamsborg [3]
[1] M.H. Gluck Equine Research Center, Department of Veterinary Science, University of Kentucky, Lexington, Kentucky, USA, [2] Department of Large Animal Sciences, Faculty of Health and Medical Sciences, University of Copenhagen, Denmark, [3] Danish Center for Experimental Parasitology, Department of Veterinary Disease Biology, Faculty of Health and Medical Sciences, University of Copenhagen, Denmark

Strongyle parasites are ubiquitous in grazing horses, and the large strongyle *Strongylus vulgaris* is considered the most pathogenic helminth parasite of horses. Investigations have suggested an association between occurrence of this

parasite and usage of targeted selective therapy regimens basing treatment decisions on individual fecal egg counts from all horses on the farm. The established diagnostic method for *S. vulgaris* involves larval culture and subsequent morphological identification of third stage larvae under the microscope. Recently, a real-time PCR assay was developed and validated for the detection and semi-quantification of *S. vulgaris* eggs in equine fecal samples. The purposes of the present study were a) to determine the presence of *S. vulgaris* by real-time PCR in Danish and American horses on farms using vastly different anthelmintic treatment regimens and b) to evaluate the association between larval culture results and the PCR. A total of 991 horses representing 53 different horse farms in Denmark and Central Kentucky were studied. Fresh fecal samples were collected from all horses, and strongyle eggs retrieved for DNA extraction and subsequent real-time PCR analysis. Individual larval cultures were performed on the Danish part of the data set (662 horses on 42 farms). On the Danish farms, the *S. vulgaris* PCR prevalence was found to be 9.2% on farms not basing parasite control on fecal egg counts, and 14.1% on farms using selective therapy. None of the 328 horses evaluated on 11 farms in Central Kentucky were PCR positive. Kappa-values indicated a moderate agreement between PCR and larval culture results, while a McNemar's test revealed no statistical difference between the paired proportions. Statistically significant associations were found between PCR cycle of threshold (Ct) value groups and larval culture counts. Results indicate that both diagnostic methods can be useful for determining the occurrence of *S. vulgaris* in horses on farms, but that they both are affected by potential sources of error. Agreement between tests was moderate and no direct linear association could be found. The PCR results confirmed previous findings suggesting that *S. vulgaris* is highly dependent on the anthelmintic treatment intensity, and that selective therapy regimens can be associated with higher occurrence of this parasite.

Gasterophilus larvae in association with primary parasitic periodontitis

E. Osterman Lind [1], J. Chirico [1], and T. Lundström [2]
[1] National Veterinary Institute (SVA), Department of Virology, Immunobiology and Parasitology, SE-751 89 Uppsala, [2] Animal Dental Clinic (Djurtandvårdskliniken), V. Husby, SE-605 96 Norrköping

Findings of *Gasterophilus* (Insecta: Diptera: *Oestridae*) larvae in periodontal pockets of horses are well described in the literature but for many years, bot flies have been rare in Sweden owing to a long tradition of using effective anthelmintic drugs in late autumn. However, in recent years, increasing numbers of larvae have been observed at oral examinations. The female fly deposits eggs on hairs, where larvae hatch and then either migrate into the oral cavity or are ingested by the horse. The larvae remain in the mucosa of the mouth for several weeks before they move into the stomach or duodenum where they stay until the following summer. The study included horses that for various reasons visited a Swedish dental clinic for examination of the oral cavity. The horse owners reported any symptoms that had been observed. Larvae recovered from periodontal pockets were placed in 70% ethanol and sent to the National Veterinary Institute (SVA) for species identification. From the second week of August until the third week of December, bot fly larvae were detected in periodontal pockets in totally 93 out of 475 examined horses. Second stage larvae of *Gasterophilus intestinalis*, *G. nasalis* and *G. haemorrhoidalis* were identified; sometimes co-infections occurred. Oral findings associated with *Gasterophilus* larvae included necrosis of gingival papillae, deepened periodontal pockets and lingual lesions. A majority of the sampled horses recovered within three weeks but deepened pockets remained in a few individuals for as long as nine months. Signs of discomfort at performing, excessive salivation, lingual irritation and chewing problems were registered in all the sampled horses. Multiple signs were expressed by 48% of the horses. Here, we present three cases of myiasis caused by *Gasterophilus* spp. Owing to altered deworming routines which mainly focus on helminths and are based on faecal egg output, bot flies are becoming more prevalent in Sweden. It is important to assess whether, and to what extent the local reactions in the gingiva cause discomfort for the horse.

Antimicrobial resistance of equine *Salmonella* isolates in the Northeastern United States (2001-2010)

G.A. Perkins [1], K. Cummings [1,2], S.M. Khatibzadah [1], J. Stiler [1], L.D. Warnick [1], and C. Altier [1]
[1] Cornell University, Ithaca, NY, [2] Texas A&M University, College Station, TX

The objectives of this study were to describe the antimicrobial resistance status of equine *Salmonella* isolates recently cultured in the northeastern U.S. and to identify trends in resistance over time. Data were collected retrospectively for all equine *Salmonella* isolates with antimicrobial minimum inhibitory concentrations (MICs) that were isolated at the Cornell University Animal Health Diagnostic Center from January 1, 2001 to December 31, 2010. MICs of isolates were determined using the microbroth dilution method (TREK Sensititre). A total of 459 equine *Salmonella* isolates with antimicrobial susceptibility testing were retrieved (median of 45 isolates per year; range 20 - 83). The majority of isolates were from clinical submissions (feces) either from regional veterinarians or the Cornell University Equine Hospital. Most isolates (98.4-100%) were susceptible to amikacin, enrofloxacin and imipenem. Individual Cochran-Armitage tests showed a significantly decreasing trend over time ($p \leq 0.005$) in prevalence of resistance to amoxicillin/clavulanic acid (AUG), ampicillin (AMP), cefazolin, cefoxitin (FOX), ceftiofur (TIO), chloramphenicol (CHL) and tetracycline (TET). Increasing resistance was not found for any of the 16 antimicrobials tested. There were 380 isolates that tested nine available National Antimicrobial Resistance Monitoring System (NARMS) panel drugs. The most

common resistance patterns were pan-susceptible (38.2%), AUG-AMP-FOX-TIO-CHL-TET (16.6%), AUG-AMP-FOX-TIO-CHL-TET-Timethoprim/sulfamethoxazole (SXT) (12.4%), AUG-AMP-FOX-TIO-CHL-TET-SXT-Gentamicin (GEN) (11.3%), and AMP-TIO-CHL-TET-SXT-GEN (7.6%). Although antimicrobial resistance among equine clinical *Salmonella* isolates did not increase for any drug and declined for several antimicrobial agents, multi-drug resistance was common in the region with 60.5% of isolates showing resistance to two or more drugs on the NARMS panel.

Anthelmintic resistance in horse cyathostomins in France

D. Traversa [1], G. Castagna [1], G. von Samson-Himmelstjerna [2], S. Meloni [1], R. Bartolini [1], T. Geurden [3], M.C. Pearce [3], E. Woringer [4], B. Besognet [4], P. Milillo [1], and M. D'Espois [4]
[1] Department of Comparative Biomedical Sciences, University of Teramo, Italy, [2] Institute for Parasitology and Tropical Veterinary Medicine, Freie Universität Berlin, Germany, [3] Pfizer Animal Health, Veterinary Medicine Research and Development, Belgium, [4] Pfizer Animal Health, Paris, France

Anthelmintic resistance in horse Cyathostominae (small strongyles) is globally distributed. Results from a study conducted using the faecal egg count reduction test (FECRT) showed that single or multiple resistance in cyathostomins occurs in the UK, Germany and Italy: resistance to fenbendazole (FBZ) and pyrantel (PYR) is widespread but reduced efficacy of ivermectin (IVM) and moxidectin (MOX) is very low [1]. Resistance to benzimidazoles (BZs) in France has been reported since the early 1990s [2] but recent data on the efficacy of commonly used anthelmintics are lacking. A FECRT was performed to evaluate the efficacy of oral FBZ, PYR embonate, IVM and MOX in 445 horses from 30 farms in France. At each site, 12 to 20 horses were enrolled: 4 or 5 horses were included in each of 3 or 4 treatment groups on each farm. When no more than 12 horses showed sufficient FECs for inclusion, the FBZ group was excluded from the study. Calculation of the mean FECR and 95% confidence intervals (95% CI) around the mean was performed using bootstrap analysis, based on arithmetic means. Resistance to FBZ was found on 17 of the 18 (94.4%) farms at which FBZ was evaluated, with a mean reduction of 57.0% (95% CI: 38.5–71.2%). For PYR suspected resistance was found at 6 of 30 farms and confirmed at another 3, with a mean reduction for PYR of 94.7% (95% CI: 88.9–98.5%). Reduced efficacy simultaneously to FBZ and PYR was found at 7 (23.3%) sites. Reduced efficacy of IVM and MOX treatment was detected in one horse on one farm each and occurred simultaneously with resistance against FBZ and/or PYR. This study showed that single and multiple drug resistance in equine cyathostomins is present in France. FBZ was shown to be ineffective, consistent with similar findings in other European countries and the Americas. Resistance against PYR is present in France, but less commonly than elsewhere. Macrocylic lactones proved to be effective molecules, with indications of reduced efficacy for IVM and MOX in two horses only. These results illustrate the necessity to use anthelmintics in appropriate worm control programmes.

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Transcriptional profiling of pathogenic and non-pathogenic homologous *Lawsonia intracellularis* isolates

F.A. Vannucci [1], D.N. Foster [2], and C.J. Gebhart [1]
[1] Department of Veterinary and Biomedical Science, College of Veterinary Medicine, University of Minnesota, St. Paul, MN 5510, [2] Department of Animal Science, College of Food, Agricultural and Natural Resource Science, University of Minnesota, St. Paul, MN 5510

Non-pathogenic isolates of *Lawsonia intracellularis* obtained through multiple passages in cell culture do not induce proliferative enteropathy (PE). Conversely, isolates at low cell passage induce typical clinical and pathological changes. Standard DNA-based techniques have shown identical genotypes in both pathogenic and non-pathogenic strains. Therefore, any phenotypic differences may occur at the transcriptional level. We hypothesize that genes differentially expressed between pathogenic and non-pathogenic homologous *L. intracellularis* isolates encode potential bacterial virulence factors. Intestinal piglet epithelial cells were infected with pathogenic (passage 10) and non-pathogenic (passage 60) homologous *L. intracellularis* (PHE/MN-01) isolates. On day five post-infection (approaching peak of infection) total RNA was extracted from the infected cell monolayers. Subtractive hybridizations were performed to enrich bacterial mRNA. High-throughput sequencing technology (RNA-seq) was used to generate single-end 76 bp reads. These transcripts were mapped onto the *L. intracellularis* reference genome and compared according to the number of readings per kilobase per million readings (RPKM). Overall, 731 protein-encoding genes were mapped against the 1340 genes present in the reference genome. A total of 401 genes were exclusively expressed by the pathogenic strain, including ABC transporters and two-component system and non-mevalonate pathway genes. This wider transcriptional landscape of the pathogenic strain was distributed in the chromosome and three plasmids (Figure 1). Only transcripts from the pathogenic strain were mapped onto plasmid A, suggesting a major role of this plasmid in the pathogenesis of PE. There was no significant difference ($Log_{2\ (RPKM)} + 2$; p-value < 0.05) in the comparison of all 319 genes

commonly expressed in both strains. Surprisingly, those genes demonstrated a positive correlation (r^2=0.81; $p < 0.05$), indicating the involvement of gene silencing (switching off) mechanisms to attenuate virulence of the pathogenic strain through cell passages. The results were validated by RT-qPCR using a set of 10 selected genes. This study is the first report characterizing the transcriptional profile of *L. intracellularis*. Specific properties of genes expressed at high levels in both or exclusively in the pathogenic strain may now be studied in order to: (i) understand the pathogenesis of PE; (ii) develop new diagnostic tools and (iii) develop recombinant vaccines. We believe the results support our hypothesis and open a new research field for studying target genes involved in the pathogenesis of PE.

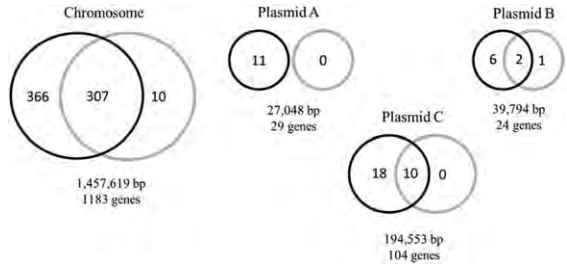

Figure 1. Schematic representation of the *L. intracellularis* genomes showing the distribution of genes expressed by the pathogenic (black circles) and the non-pathogenic (gray circles) variants. Overlapping zones represent genes expressed in both variants.

Diagnostic

Real-time PCR for detection of *Leptospira interrogans* serovar Pomona type kennewicki in equine clinical specimens

S.C. Artiushin [1], J.F. Timoney [1], U.B. Balasuriya [1], E. Erol [2], and S.F. Sells [2]
[1] The Gluck Equine Research Center, University of Kentucky, Lexington, USA, [2] The Veterinary Diagnostic Laboratory, University of Kentucky, Lexington, USA

Leptospirosis has serious negative impacts on the horse industry including losses due to abortion and stillbirth. Other clinical manifestations of infection include renal and liver dysfunction and recurrent uveitis, a major cause of equine blindness worldwide. The control of the infection relies both on prophylactic antibiotic treatment and management practices based on identification of seropositive animals by MAT as well as detection of leptospira in urine and other body fluids. A serological survey of farms with a history of recent leptospiral abortion demonstrated seropositivity of 76% to Bratislava, 49% to Icterohaemorrhagiae, 20% to Grippotyphosa, 16% to Pomona type kennewicki, 16% to Hardjo and 1% to Canicola [2]. However, over 80% of abortions in USA are caused by *Leptospira interrogans* serovar Pomona type kennewicki (Lk) and 10% by serovar Grippotyphosa [1]. The goal of these studies was to develop a real-time PCR assay to detect and differentiate Lk from other pathogenic leptospira in specimens from horses. Type strains of different serovars of leptospira and isolates of Lk were grown at 30°C in EMJH medium (Difco Laboratories). DNA's were isolated from 3-5 ml of stationary-phase cultures using ZR Genomic DNA II kit (Zymo Research Corp.). Concentration and purity of DNA's was determined using a NanoDrop spectrophotometer (Thermo Scientific). The primers and probe sets (IDT, Coralville, IA) specific to pathogenic leptospira and Lk were designed to the conserved regions of *lipL32* and sequence upstream of *lk73.5* using PrimerQuest program. The PCR assays were performed using TaqMan Universal Master Mix II (Applied Biosystems) according to manufacturer's instruction in a 7500 Fast Real-Time PCR System. Pathogenic but not saprophytic leptospira or other spirochetes gave positive results in real-time PCR assay specific for *lipL32*. Positive results in real-time PCR specific for *lk73.5* were shown only for horse isolates of Lk and type strain of *L. interrogans* serovar Pomona. Analytical sensitivity of *lipL32* and *lk73.5* specific real-time PCR assays were 0.23 and 0.14 pg of DNA or 46 and 28 genome copies respectively. The specificity and sensitivity of these assays were confirmed by using clinical samples from horses with an established diagnosis of leptospira infection. The real-time PCR assays developed in these studies allowed detection of pathogenic leptospira and differentiation of Lk from other pathogenic leptospira, including the horse adapted, avirulent *L. interrogans* serovar Bratislava.The assays will provide for rapid and specific diagnosis of leptospirosis in horses caused by Lk or other leptospira and identification of urinary shedding.

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Validation of an equine arteritis virus antibody cELISA according to OIE protocol

C. Chung [1], C. Wilson [1], E. Adams [1], D.S. Adams [1], J. Evermann [2], P. Timoney [3], A. Clavijo [4], S. Rogers [4], S.S. Lee [5], and T.C. McGuire [1]
[1] VMRD Inc., Pullman, Washington 99163, [2] WADDL, Pullman, Washington 99163, [3] University of Kentucky, Lexington, Kentucky 40546, [4] TVMDL, College Station, Texas 77841, [5] Department of Statistics, University of Idaho, Moscow, Idaho 83844

Equine arteritis virus (EAV) is the cause of Equine Viral Arteritis characterized by conjunctivitis, nasal discharge, dependent edema, abortion, and infrequently, death in young foals. OIE defines a horse as seropositive if the EAV serum neutralization (SN) antibody titer is $\geq$1:4. The SN test

takes 72h to complete and requires certain laboratory facilities, equipment, and technical expertise to perform. Furthermore, non-specific cellular cytotoxicity of particular samples and inter-laboratory variation in SN results have been reported. For these reasons an alternative serologic test is desirable. None of the previously reported tests have shown equivalent sensitivity and specificity compared to the SN. In attempting to produce a better alternative assay, a cELISA was developed using EAV gp5-specific neutralizing monoclonal antibody (MAb) 4B2 which performed moderately well. This cELISA has been significantly improved by substituting 4B2 antibody with the non-neutralizing monoclonal antibody 17B7. In the present study, the improved cELISA was validated according to the OIE-recommended validation protocol. As part of an in-house validation procedure of the EAV antibody cELISA, the following five analyses were performed: 1. the primary assay was calibrated with the OIE approved reference serum panel for EVA, 2. repeatability of the assay was evaluated within and between runs, 3. analytical specificity was evaluated using sera specific to related viruses, 4. analytical sensitivity was evaluated with sera collected from horses vaccinated with the modified live virus vaccine against EVA (Arvac®, Pfizer Animal Health), and 5. the duration of the positive cELISA antibody detection was evaluated following EVA vaccination. The outside validation of the cELISA utilized three laboratories including one OIE reference laboratory and two AAVLD-accredited state laboratories. Each laboratory assayed their panel of field sera (150-200 sera) to evaluate diagnostic specificity and sensitivity of the cELISA, and VMRD prepared an inter-dependency panel (25 sera in duplicate) to evaluate the robustness of the cELISA in all three laboratories. The cut-off of the cELISA was evaluated by ROC plot analysis. As a result, the original cELISA using MAb 4B2 was significantly improved by switching to 17B7 and incorporating other test procedures. The analytical sensitivity of the new cELISA was comparable to the SN assay in that it detected EAV-specific antibody as early as 6 days post-vaccination. The duration of EAV-specific antibody detected by cELISA was over six years post-vaccination. In the field trial, the relative specificity of the new cELISA was 99.5% and the relative sensitivity was 98.2%. This field trial data also showed a significant correlation between SN and cELISA results (r^2=0.79, P < 0.0001). These results indicate that new EAV antibody cELISA is a reliable, simple alternative to the SN assay for detecting EAV-specific antibodies in horses.

Evaluation of immunofluorescent antibody and immunoblot tests for the detection of antibodies against *Besnoitia bennetti* tachyzoites and bradyzoites in donkeys

S.L. Ness [1], G. Schares [5], J. Peters [2], J.P. Dubey [6], L.D. Mittel [3], H.O. Mohammed [3], D.D. Bowman [4], and T.J. Divers [1]
[1] Departments of Clinical Sciences, [2] Biomedical Sciences, [3] Population Medicine and Diagnostic Sciences, [4] Microbiology, Cornell University College of Veterinary Medicine, Ithaca, NY 14853, [5] Friedrich-Loeffler-Institut, Federal Research Institute for Animal Health, Wusterhausen, Germany, [6] Animal Parasitic Diseases Laboratory, United States Department of Agriculture, Beltsville, MD 20705

Besnoitiosis is caused by infection with protozoan *Besnoitia* spp, which are cyst-forming coccidian parasites that affect multiple host species worldwide. *Besnoitia bennetti* is the species known to infect equids and has been reported in both horses and donkeys in Africa and Asia, and more recently, donkeys in the United States. Clinical disease is characterized by a miliary dermatitis with parasitic cysts in the skin, mucous membranes, and sclera (Figure 1). Several serologic assays have been validated for the diagnosis of bovine besnoitiosis, caused by infection with *Besnoitia besnoiti*. We hypothesize that assays validated for the diagnosis of bovine besnoitiosis will also be effective for identifying equine infection. The goal of the present study was to evaluate the utility of three serologic assays for the detection of *B. bennetti* antibodies in donkeys. Sera from donkeys confirmed to be infected with *B. bennetti* via histopathology (cases; n = 13) and donkeys with no clinical signs of besnoitiosis (controls; n = 126) were tested for antibodies against *B. bennetti* using an immunofluorescent antibody test and 2 immunoblot assays specific for bradyzoite and tachyzoite antigens, respectively. For all tested hypotheses, values of $P < 0.05$ were considered significant. Donkeys with besnoitiosis had significantly higher geometric mean immunofluorescent antibody titers than control donkeys (case mean geometric titer 2.87 [reciprocal titer 741] vs control mean geometric titer 2.0 [reciprocal titer 100]). There was a significant difference in the median number of bradyzoite immunoblot bands between cases and controls (7 vs. 1), as well as in the median number of tachyzoite immunoblot bands between cases and controls (6 vs. 0). There were positive correlations among geometric titer and number of bradyzoite and tachyzoite immunoblots bands. Immunofluorescent antibody and immunoblot tests are effective at identifying donkeys with besnoitiosis and provide a more efficient and less invasive diagnostic alternative to histopathology.

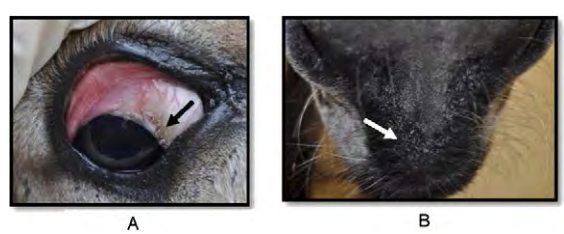

Figure 1. *Besnoitia* bennetti lesions on the sclera (A) and muzzle (B) of an infected donkey.

Development of a rapid isothermal assay to detect the causative agent of strangles

S.E. North, P. Das, P.R. Wakeley, and J. Sawyer
Technology Transfer Unit, Specialist Scientific Support Department, Animal Health Veterinary Laboratories Agency – Weybridge, United Kingdom

We have developed a Loop mediated AMPlification (LAMP) assay to detect *Streptococcus equi* subspecies *equi* (*S. equi*), the causative agent of equine strangles. The

LAMP assay proceeds at a constant temperature which negates the need for expensive, sophisticated equipment. The reaction is fast (under 30 minutes) and the products of amplification can be detected in real time or on a lateral flow device (LFD). Nasal swabs, abscess swabs and guttural pouch washes from suspected strangles cases were used to inoculate solid culture media before a simple boiling method was used to extract DNA from the swabs, enabling a comparison with conventional culture methods. The real time LAMP assay was tested against a panel of 38 bacterial species to determine the analytical specificity of the assay. The analytical sensitivity of the real time LAMP assay was calculated using a dilution series of target DNA. The LAMP assay (using real time and LFD detection) was tested against a panel of 30 positive and 30 negative DNA extracts from clinical isolates (as determined by culture and real time PCR). The LAMP assay successfully amplified a 215bp fragment of the *eqbE* gene of *S. equi*. The LAMP primers did not cross-hybridise with any of the 38 different bacteria tested to determine specificity, including closely related species *e.g. S. equi zooepidemicus* and known equine commensal bacteria. DNA was successfully extracted from nasal and abscess swabs and guttural pouch washes by a simple boiling method. The LAMP assay was tested against a panel of 60 clinical strangles swabs (30 positive and 30 negative DNA extracts as determined by culture and real time PCR). All of the culture negative DNA extracts were correctly identified by the LAMP assay, irrespective of whether detection was in real time or LFD. When the culture positive DNA extracts were tested, and the results detected in real time, 26 of the 30 were correctly identified. When the products of amplification were detected using LFDs, 24 of the 30 were correctly identified. Those DNA extracts that were culture positive but RPA negative had high real time PCR C_T values, indicating they had low levels of target DNA present. The real time LAMP assay detected as few as 48 gene copies in less than 25 minutes. We have successfully developed a LAMP assay to identify *S. equi*, the causative agent of strangles. This test provides a fast (less than 30 minutes) and accurate means of identification that, following further validation may enable rapid diagnosis, treatment and help prevent the spread of strangles.

New real-time PCR assay using allelic discrimination for detection and differentiation of equine herpesvirus-1 strains with A_{2254} and G_{2254} polymorphisms

Kathryn L. Smith [1], Yanqiu Li [1], Patrick Breheny [2], R. Frank Cook [1], Pamela J. Henney [1], Stephen Sells [3], Stéphane Pronost [4], Zhengchun Lu [1], Beate M. Crossley [5], Peter J. Timoney [1], and Udeni R. Balasuriya [1,3]

[1] Maxwell H. Gluck Equine Research Center, Department of Veterinary Science, [2] Department of Biostatistics, University of Kentucky, Lexington, KY 40546, USA, [3] University of Kentucky Veterinary Diagnostic Laboratory, Lexington, KY 40512, USA, [4] Frank Duncombe Laboratory, Animal Health Department, IFR146 ICORE, 14053 Caen, France, [5] California Animal Health and Food Safety Laboratory, School of Veterinary Medicine, University of California, Davis, CA 95616, USA

A single nucleotide polymorphism in open reading frame 30 (ORF30) of equine herpesvirus-1 (EHV-1) has been linked to the neuropathogenic phenotype of this virus. Identification of this polymorphism led to the development of allelic discrimination, real-time PCR (rPCR) assays to distinguish between potential neuropathogenic (G_{2254}) and non-neuropathogenic (A_{2254}) EHV-1 strains. While rPCR assays are potentially an expedient, accurate means for detection and genotyping of EHV-1 in clinical specimens, the need to improve such assays for routine diagnosis still exists. A new EHV-1 rPCR assay (E_1) was developed and compared, in terms of sensitivity and specificity, with a previously published PCR assay (E_2). Viral DNA purified from serial 10-fold dilutions (10^{-1}-10^{-8}) of tissue culture fluid, containing either EHV-1 A_{2254} or G_{2254} strain, were used to ascertain the analytical sensitivity of each rPCR assay. E_1 and E_2 were also evaluated using 76 archived EHV isolates and 433 clinical specimens from cases of suspected EHV-1 infection. Nucleotide sequence analysis of ORF30 was used to confirm the presence of EHV-1 and characterize the genotype in all of the archived isolates, as well those from 168 clinical samples. E_1 was ten times more sensitive than E_2, with a lower detection limit of 10 infectious virus particles. Furthermore, the E_1 assay accurately identified all

Table 1
E_1 and E_2 real-time PCR results

	E_1					E_2				
	EHV-1 E_1 rPCR		EHV-1 E_1 Genotype			EHV-1 E_2 rPCR		EHV-1 E_2 Genotype		
Specimen Type	Negative	Positive	A_{2254}	G_{2254}	A_{2254}+G_{2254}	Negative	Positive	A_{2254}	G_{2254}	A_{2254}+G_{2254}
Archived EHV TCF (n=76):										
USA & UK (n=54)	16*	38	17	21	0	18*	36	17	19	0
France (n=22)	0	22	15	7	0	1	21	14	7	0
Clinical Samples (2001-2011) (n=433):	229†	204	107	94	3	289†	144	70	53	21
Nasal swabs (n=260)	151	109	60	46	3	178	82	42	19	21
Buffy coat cells (n=173)	78	95	47	48	0	111	62	28	34	0

* Sixteen samples that were positive for EHV-4 by standard PCR were included as negative controls.
† Forty-three samples that were positive for EHV-4 by DNA sequencing were included as negative controls.

A_{2254} and G_{2254} genotypes, together with identifying three cases of dual infection (A_{2254}+G_{2254}). In comparison, the E_2 produced 20 false dual positive results and one confirmed A_{2254}+G_{2254} genotype (Table 1). This increased sensitivity of E_1 probably results from this assay having a shorter amplicon length compared to E_2, resulting in elevated efficiency and/or less susceptibility to secondary DNA structural effects. The findings of this study demonstrate that E_1 is significantly more sensitive for the diagnosis of EHV-1 and less prone to produce false dual positive results than E_2. E_1 is clearly a reliable and sensitive means for the detection and A_{2254}/G_{2254} genotyping of EHV-1, making this improved rPCR assay a very valuable diagnostic tool when investigating outbreaks of EHV-1 infection.

Development and initials characterization of serotype specific RT-qPCR assays for African horse sickness virus

C.T. Weyer, A.J. Guthrie, and C. Joone
Equine Research Centre, Faculty of Veterinary Science, University of Pretoria, Pretoria, South Africa

Historically, diagnosis of African horse sickness (AHS) has been done using viral isolation and serotyping is then done with Viral Neutralisation tests (Guthrie and Quan, 2009). This process is time-consuming. Recently a number of PCR assays have been developed for the diagnosis of AHS. Advantages to this approach are that they have the potential to be rapid, sensitive and versatile, and can supplement the older conventional methods. These assays can also be applied to samples that do not necessarily contain live virus, or have very low viral levels, making these tests ideal for screening tests. Whilst serotype specific conventional and real-time PCR's have been described for AHSV (Sailleau et al 2000, Koekemoer 2008) and these assays have limitations and have not been applied routinely to determine the serotype of AHSV involved in a specific outbreak. The aim of this study was to develop 9 serotype specific TaqMan assays for AHSV and to characterize these assays using archived field samples from AHS cases from which viruses had been previously isolated and serotyped. The assays were designed to target the neutralizing epitope region of the VP2 protein of each serotype of AHSV. Sequences of the gene coding for VP2 of each of the serotypes of AHS were obtained from our own database and those available on the National Centre for Biotechnology Information's Genbank website (www.ncbi.nlm.nih.gov). Primers and probes were designed for each AHSV serotype using Primer Express 3. The assay was characterized using three different sets of reference viruses and at least 8 representative archived viruses of each serotype available within our laboratory. In brief, nucleic acid was extracted from 100μl of blood or homogenised organ sample and then subjected to a RT-qPCR using three separate triplex assays for the 9 serotypes of AHSV. RNA extraction was performed using MagMAX™ Pathogen RNA/DNA kits (Life Technologies) and a Kingfisher 96 Magnetic Particle Processor (Thermo Scientific) according to the manufacturer's protocols. The PCR will be performed on a StepOnePlus™ Real-Time PCR System (Life Technologies) using the VetMax One-Step RT-PCR Kit (Life Technologies) using the protocol recommended by the manufacturer. The serotype specific AHSV RT-PCR assays developed in this study specifically identified the serotype of 99 reference and archived viruses examined in this study. Furthermore, the assays have been applied to incurred field samples received from South Africa, Namibia and Kenya and have identified the serotype these incurred viruses. These assays can be applied to extracts from the original blood and organ samples from suspect AHS cases and can be completed within 4 hours of receipt of samples. The application of these assays to incurred samples from outbreaks of AHS have greatly assisted with the selection of vaccines containing appropriate serotypes of AHS thereby assisting in the control and management of AHS outbreaks in the field.

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Establishment of real-time PCR assays for rapid detection of foreign, emerging and zoonotic pathogens of equines

U.B.R. Balasuriya, S. Artiushin, and Z. Lu
Maxwell H. Gluck Equine Research Center, Department of Veterinary Science, University of Kentucky, Lexington, KY

The availability of sensitive and highly specific molecular diagnostic assays that could detect foreign, emerging and zoonotic equine viral and bacterial agents is critical for the rapid diagnosis and control of infectious disease outbreaks. Therefore, the objective of this study was to establish or develop real-time PCR (rPCR) assays for the detection of three foreign viral pathogens (African horse sickness virus [AHSV], equine encephalosis virus [EEV] and Rift Valley fever virus [RVFV]) and three reemerging bacterial pathogens of horses (*Streptococcus equi* [Strangles], *Leptospira interrogans* and *Taylorella equigenitalis* [contagious equine metritis or CEM]). Several PubMed literature searches were conducted to determine the availability of published rPCR assays for the detection of the above viral and bacterial pathogens. If multiple assays were already published for an agent, the most suitable assay(s) for the detection of that agent was selected based on sensitivity, specificity and assay validation. Furthermore, comparative sequence analysis was performed using the sequence data available in GenBank to see whether the primer and probe binding regions were conserved among known field strains of that particular agent. All three viral agents are foreign animal pathogens and thus, the genes of interest were cloned into a plasmid vectors using recombinant DNA technology. The

linearized plasmids were used to generate *in vitro* transcribed RNA (IVT RNA) for the assay development. The genomic DNA from prototype strains of the three bacterial pathogens was used for the development of the assays. We have established six previously published rRT-PCR assays in our laboratory targeting the conserved regions of the VP7, NS1 and NS2 genes of all nine AHSV serotypes, as well as an assay targeting the L gene of RVFV. Furthermore, we have developed three new rRT-PCR assays targeting the NS3 gene of the EEV. These three assays were designed and optimized to detect all seven serotypes of EEV including the recently identified clade B strains circulating in Israel. We have also developed new rPCR assays for the detection and differentiation of *S.equi* from *S. zooepidemicus* (three assays targeting the *seM* gene, 3′ terminus of *Seq4* prophage and *eqbE* gene), *L. interrogans serovar kennewicki* from other pathogenic and nonpathogenic leptospira (two assays targeting the LipL32 and Lk73.5 genes), and *T.equigenitalis from T. asinigenitalis* (16S gene). In addition, we have modified the probes of a previously described rPCR assay targeting the published 16S RNA gene to increase its sensitivity. Studies are underway to further validate these rPCR assays in collaboration with various national and international laboratories. These reagents and protocols developed in this study have broadened the existing panel of rPCR assays available at the Maxwell H. Gluck Equine Research Center for the diagnosis of equine infectious diseases. These reagents can also be used for absolute quantification of these viral pathogens in research laboratories. The long term goal is to make these protocols and reagents readily available to the veterinary diagnostic laboratories within the United States (NAHLN).

Development of a novel virus-neutralizing test of equine herpesvirus type-1 by a plaque-reduction method using 96-well plates

H. Bannai, M. Nemoto, K. Tsujimura, T. Yamanaka, T. Kondo, and T. Matsumura
Epizootic Research Center, Equine Research Institute, Japan Racing Association, Shimotsuke, Tochigi, Japan

A virus neutralizing (VN) test is an essential method to evaluate a virus-specific immunity in equine herpesvirus type-1 (EHV-1) infection. There are two methods of the VN test for EHV-1. A $TCID_{50}$ method using 96-well plates is applicable for a lot of samples and widely used. However, it takes 4 to 5 days to induce clear cytopathic effects to cells. Another method is a 50%-plaque reduction method using 24-well plates, in which the sample number is limited, and it requires 3 to 5 days to induce visible plaques. In this study, we established a novel VN test by a 50%-plaque reduction method using 96-well plates by which we can test a large number of samples in a shorter period. Paired sera from horses which seroconverted in an EHV-1-specific gG-ELISA (n = 20) were used to optimize experimental conditions. Sera from foals before suckling colostrum (n = 30) were used as negative controls. Madin-Darby Bovine Kidney cell and EHV-1 89C25p strain were used. Dilution of the virus was determined so that 25 to 40 plaques were yielded in each control well. Serial two-fold diluted sera were incubated with EHV-1 at 37°C for 1 h. The incubated samples were inoculated onto duplicate wells of one-day monolayer cells on 96-well plates and were incubated at 37°C for 2 h. After removal of the supernatant, an overlay medium containing Avicel, a microcrystalline cellulose, was added. Because Avicel has lower viscosity than methylcellulose, it can be added to the 96-well plates easily. After designated incubation time, the cells were fixed and immuno-stained with anti-EHV-1 gp14 monoclonal antibody (clone 121-3) and a secondary antibody conjugated with horseradish peroxidase. Plaques were visualized with TrueBlue peroxidase substrate (KPL Inc., MD, USA). The antibody titer was expressed as reciprocal of the highest dilution that had reduced number of plaques more than 50% from that of control virus. An optimal incubation time after viral inoculation was determined to be 36 h, so that an appropriate plaque size was yielded. We set 1:10 as a cut-off titer because all tested negative control sera had titers less than 1:10. A significant increase in the VN titers (more than 4-fold between pre- and post-sera) was observed in the horses infected with EHV-1 (Fig. 1). The titers by the new method were relatively higher (2.55-fold, mean) than those by the conventional method using 24-well plates with a few exceptions. In conclusion, the VN test for EHV-1 by the 50%-plaque reduction method using 96-well plates was established. This method is applicable for a large number of samples, and can perform the test in a shorter period than the conventional methods.

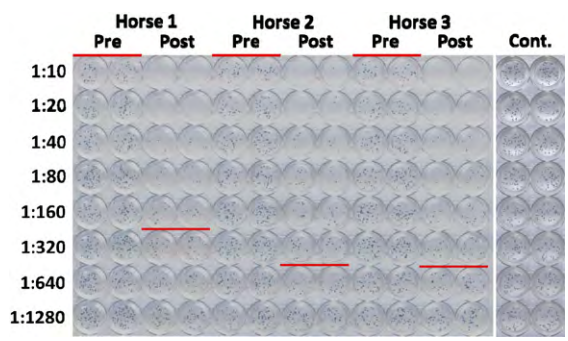

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Figure. 1. The new method detecting the increased VN titers in horses infected with EHV-1. Bars, end-point.

Evaluation of a Nucleic Acid Hybridization test (GeneQuence®) for identification of *Salmonella spp* in equine feces and environmental samples

B.S. Barr VMD, DACVIM, and M. Swintowsky MT
Rood and Riddle Equine Hospital, Lexington, Kentucky

Infection control is an essential component of an equine hospital. Nosocomial infections with *Salmonella spp* have resulted in the temporary closure of equine hospitals thus surveillance for this organism is critical. The gold standard for detection of *Salmonella spp* in fecal or environmental samples is bacterial culture. The disadvantage is that results are not available for 3-5 days. With PCR testing the results are available within 24-36 hours but there is a higher risk of

false positive results and the equipment requires a very controlled setting. In the food industry nucleic acid hybridization is used to rapidly and accurately identify contamination with *Salmonella spp*. This test targets the pathogen's ribosomal RNA. The purpose of this study was to use this technology to detect *Salmonella spp* in fecal samples and environmental samples from an equine hospital and compare the results to bacterial culture. Routine fecal samples and environmental samples collected from April 2010- April 2011 at Rood and Riddle Equine Hospital were used for the study. The fecal samples were enriched in selenite broth and incubated for 24 hours, then plated on Hekton agar and incubated for 24 hours. The environmental samples were incubated in peptone broth for 24 hours, then tetrathionate broth for 24 hours. Afterwards the samples were plated and incubated on Hekton agar for 24 hours. After appropriate incubation any black colonies on the Hekton agar plates were gathered and transferred to the GeneQuence® D2 automated system for nucleic acid hybridization and to BBL crystal identification system for the final phase of microbiological culture. The GeneQuence® results were available within 2 hours whereas the bacterial culture results were available within 24 hours. A total of 1494 samples were analyzed. The results were as follows: 1274 samples were GeneQuence® negative and culture negative, 210 samples GeneQuence® positive and culture positive, four samples GeneQuence® negative and culture positive, and six samples Gene Quence® positive and culture negative. The overall sensitivity was 98.1% and the specificity was 99.5%. The positive predictive value was 97.2%, whereas the negative predictive value was 99.6%. GeneQuence® results were available 1 day sooner and were almost as accurate as the bacterial culture. An advantage of GeneQuence® over PCR testing is that there are fewer false positive results. A disadvantage is that bacterial culture is still required to identify an antimicrobial sensitivity. A rapid and reliable test for identification of *Salmonella spp* would be beneficial for equine veterinarians. Future projects include decreasing enrichment time thus making the results available sooner.

European Union Reference Laboratory for equine diseases: towards improved and harmonized diagnosis of West Nile disease

C. Beck, S. Lowenski, S. Zientara, and S. Lecollinet
ANSES, EU-RL for equine diseases, UMR1161 Virologie
INRA, ANSES, ENVA, 23 avenue du Général de Gaulle,
94703 Maisons-Alfort, FRANCE

In July 2008, the European Commission officially designated the French Agency for food, environmental and occupational health safety (ANSES) as the European Union Reference Laboratory (EU-RL) for equine diseases (apart from African horse sickness). West Nile disease, exotic Equine Encephalitis, Vesicular stomatitis, glanders and melioidosis are studied at ANSES Laboratory for animal health located in Maisons-Alfort (near Paris), while the Laboratory for equine diseases located in Normandy is dedicated to Contagious Equine Metritis, Dourine, Equine Viral Arteritis, Equine Herpes viruses and Equine Infectious Anaemia. The EU-RL on equine diseases aims at providing technical and scientific support to the European National Reference Laboratories and the European Commission, as well as improving, harmonizing and developing new diagnostic tools for the diagnosis of equine diseases (through workshops, trainings, proficiency tests). A proficiency test (PT) on West Nile (WN) serological and molecular diagnosis was organized in 2010, in order to assess the performances of different WN diagnosis tools and to improve the results homogeneity among European countries. 22 and 25 laboratories participated to the serology and RT-PCR proficiency test, respectively. This PT indicated that IgG or competition ELISA tests were well established, on the contrary to IgM ELISA and virus neutralization tests. Moreover, there is a need to develop more specific IgG ELISA. WN real-time RT-PCR diagnosis is functional and efficient in most EC countries, however some participants (8) failed to detect lineage 1b and 2 strains. The detection capacity of lineage 1b and 2 strains needs to be improved, in particular in Europe where a pathogenic lineage 2 virus has been identified since 2004. In conclusion, this European PT highlighted the need for improved, adapted and harmonized diagnosis on WN in Europe.

Recent progress of the diagnosis for equine piroplasmosis

I. Igarashi, N. Yokoyama, and X. Xuenan
National Research Center for Protozoan Diseases, OIE Reference Laboratories on equine piroplasmosis, Obihiro University of Agriculture and Veterinary Medicine,
Obihiro, Hokkaido 080-8555, Japan

Equine piroplasmosis, caused by intra-erythrocytic protozoa *Theileria equi* and *Babesia caballi*, is a tick-borne disease of equids. The disease is characterized by fever, anemia, icterus, and hemoglobinuria. Horses that recover from the initial infection often be carriers of parasites. In such case, it is very difficult to detect the parasites in microscopic examination, and the horses become potential disseminators of the parasites. Since the disease is distributed worldwide including Asia, Europe, Africa, South America, the disease has posed a threat to the horses in the piroplasmosis free area and severe restriction has imposed on international movement of equids to avoid the spread of the infection from an endemic area. Current diagnosis of equine piroplasmosis relies on microscopic examination, serological assays and other molecular tools. OIE is recommending the several diagnostic tests including microscopic detection, complement fixation test (CFT), the indirect fluorescent antibody test (IFAT) and competitive enzyme-linked immunosorbent assay (cELISA). However, there is no gold standard reference test for equine piroplasmosis. Each country utilizes one or more tests from OIE recommending tests and each test has disadvantages. Microscopic detection from blood smears has been used for the most standard diagnosis of equine piroplasmosis; however, the technique is relatively laborious and it is difficult to detect the parasites in the case of low parasitemia. Several serological tests have

been used to detect specific antibodies against the infections, but these assays are also restricted due to their antibody-detected limitation and/or potential cross-reactivity to other pathogens. For example, reading of IFAT results is very difficult to read results and not objective at borderline between positive and negative titers. Results of IFAT may also change not only with different origin of antigens, but also with different lots of FITC-conjugated anti-horse IgG antibody. Recently, new diagnostics such as immunochromatographic test (ICT) and loop-mediated isothermal amplification (LAMP) assay have been developed for the detection of *B. caballi* and/or *T. equi* infections. ICT has similar sensitivity to ELISA and is a rapid and simple method to detect antibodies [1]. LAMP assay can rapidly amplify DNA samples within one hour under an isothermal condition (at 60 to 65°C). LAMP has higher specificity and sensitivity than those of PCR [2]. Therefore, both ICT and LAMP are practical pen-side diagnostic methods for equine piroplasmosis. The objectives of this presentation are to review diagnostic methods and problems of the current diagnostic methods, to introduce newly developed diagnostics, and to discuss how these methods can contribute the strengthening of quarantine for equine piroplasmosis.

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Development of one-step TaqMan® real-time reverse transcription-PCR and conventional reverse transcription PCR assays for the detection of equine rhinitis A and B viruses

Zhengchun Lu, Peter J. Timoney, Jena White, and Udeni B.R. Balasuriya
Maxwell H. Gluck Equine Research Center, Department of Veterinary Science, University of Kentucky, Lexington, KY 40546

Equine rhinitis virus A and B (ERAV and ERBV) are common equine respiratory viruses belonging to the family *Picornaviridae*. There is evidence that these two viral infections are prevalent in countries in which sero-surveillance studies have been undertaken. Currently there is a lack of rapid and reliable diagnostic methods for virus detection and antibody determination. The sensitivity of virus isolation varies between laboratories and is confounded by difficulties in isolating virus from the urine of carrier horses. The objective of this study was to develop molecular diagnostic assays (real-time RT-PCR [rRT-PCR] and conventional RT-PCR [cRT-PCR] assays) capable of detecting and distinguishing ERAV from ERBV without the inherent problems associated with the current laboratory diagnosis of these infections. Three rRT-PCR assays targeting the 5'-UTR of ERAV and ERBV were developed. One assay was specific for ERAV, whereas the remaining two assays were specific for ERBV. In addition, six cRT-PCR assays targeting the 5'-UTR and 3D polymerase regions of ERAV and ERBV were also developed. RNA extracted from prototype strains of ERAV and ERBV as well as 21 archived tissue culture fluid samples which were previously determined to be positive by virus isolation for ERAV or ERBV with mono-specific rabbit antisera were used to evaluate the sensitivity and specificity of these assays. Using serial decimal dilutions (10^{-1} to 10^{-10}) of ERAV and ERBV prototype strains, the detection limits of the rRT-PCR and cRT-PCR assays were evaluated and compared. The rRT-PCR and cRT-PCR assays targeting ERAV detected all ERAV isolates (n=11) and did not cross-react with any ERBV isolates (n=10). Similarly, the rRT-PCR and cRT-PCR assays targeting ERBV only detected ERBV isolates and did not cross-react with ERAV isolates. None of the rRT-PCR or cRT-PCR assays cross-reacted with any of the other common equine respiratory viruses. With the exception of one cRT-PCR assay, the detection limit among all of these assays was 1 plaque-forming unit per ml (pfu/ml). The newly developed rRT-PCR and cRT-PCR assays appear to provide improved diagnostic tools for the detection and differentiation of ERAV and ERBV. While the results of this study are very promising, they will need to be verified by testing a greater number of clinical specimens before each assay is fully validated for the detection of ERAV and/or ERBV.

Development of a rapid isothermal assay to detect *Taylorella equigenitalis*

S.E. North, P. Das, P.R. Wakeley, and J. Sawyer
Technology Transfer Unit, Specialist Scientific Support Department, Animal Health Veterinary Laboratories Agency – Weybridge, United Kingdom

Contagious Equine Metritis (CEM) is an infection of the equine genital tract, caused by the bacterium *Taylorella equigenitalis*. Identifying *T. equigenitalis* from equine genital swabs, and differentiating it from *T. asinigenitalis*, using culture is slow and requires the presence of live bacteria. We have developed a rapid isothermal assay for the detection of *T. equigenitalis* using Recombinase Polymerase Amplification (RPA). RPA reactions occur optimally at 37°C and do not require thermocycling equipment. The reactions can be monitored in real time, based on fluorescence readings, or the amplification products can be detected on a lateral flow device (LFD). Equine genital swabs were used to inoculate solid culture media before a simple boiling method was used to extract DNA from the swabs. This enabled comparisons between culture and the RPA assay. The assay was tested, in real time, against 60 equine genital swabs (30 positive and 30 negative, as determined by culture and real time PCR). The analytical specificity of the assay was determined by testing against a panel of 38 bacterial species, including equine commensals. The analytical sensitivity of the assay was calculated using a dilution series of target DNA. A second probe was introduced into the assay, specific for *T. asinigenitalis*, which enabled detection and differentiation of *T. equigenitalis* and *T. asinigenitalis*. The *T. equigenitalis* assay was adapted, by

use of a labeled probe, to enable detection of amplified products using a LFD. The assay to identify *T. equigenitalis* successfully amplified a 121bp fragment of the 23*S rDNA* genes. The assay detected as few as 46 gene copies in 12 minutes. The assay did not cross-hybridise with any of the 38 different bacterial organisms tested. The RPA assay was tested against a panel of 60 clinical equine genital swabs. All of the culture negative DNA extracts were correctly identified. Of the 30 culture positive DNA extracts, 24 were correctly identified. Culture positive, RPA negative DNA extracts had high real-time PCR C_T values, indicating low levels of target DNA present. The introduction of a second probe into the assay enabled the simultaneous detection and differentiation of *T. equigenitalis* and *T. asinigenitalis*. The *T. equigenitalis* assay was successfully adapted to enable detection of amplification products LFDs. We have successfully developed a rapid and sensitive isothermal assay to identify *T. equigenitalis*. Additionally, the inclusion of a second probe enables the detection of *T. asinigenitalis*. The amplification products can be detected in real time or via LFD. This test provides a fast (under 15 minutes) and accurate means of identification. The low energy requirements, lack of expensive equipment and LFD readout makes this test a suitable candidate for rapid diagnosis in low technology laboratories or near to the point of sampling.

Selection and validation of a real-time rt-PCR assay for the detection of equine arteritis virus and preliminary investigations into the prevalence of carrier stallions in the Netherlands

C. van Maanen [1], R. Buter [1], Th Dijkstra [1], M. van der Lei [1], D. Gaudaire [2], and A. Hans [2]
[1] GD Animal Health Service, Deventer, the Netherlands,
[2] ANSES, Laboratory for equine diseases, Goustranville, France

Equine viral arteritis is a contagious viral disease of equids caused by equine arteritis virus (EAV). The 2 principal modes of transmission of EAV are horizontal and venereal through natural breeding or artificial insemination. In the Netherlands only stallions on EU-AI stations are tested for EAV. The purpose of the present study was to select and validate a sensitive real-time PCR assay for detection of EAV in semen and to perform a preliminary investigation into the prevalence of EAV carriers in the general Dutch stallion population. Three real-time PCR protocols and 3 RNA extraction methods were evaluated. Two real-time PCR methods have been published before[1, 2], whereas one protocol was obtained from SVA, Uppsala, Sweden. Methods were first compared for detection limits, linear range and efficiency for EAV dilution series spiked in undiluted fresh semen and extended semen. Two PCR protocols [1, SVA] were further evaluated combined with a manual and an automated RNA extraction method by testing a 2010 proficiency test panel. Panels of 91 undiluted and 92 extended semen samples, respectively, were subsequently investigated with the preferred method. All PCR protocols demonstrated good efficiency with some variation in detection limits (0.1 – 2.1 $TCID_{50}$/mL). The linear range varied between 4 and 6 ^{10}log dilutions. The real-time PCR method described by Balasurya et al. [1] and the SVA method combined either with manual or automated RNA extraction scored all proficiency test samples correct. Two out of 91 undiluted and 5/92 extended semen samples tested positive. All 7 samples were confirmed and genotyped by the EU-RL for equine diseases (Fig. 1). We have been able to select and implement a sensitive real-time PCR method for EAV detection in either undiluted or extended semen that can be combined with either a manual or an automated RNA extraction method. The overall prevalence of EAV carriers in the panels tested was about 4% and all EAV strains belonged to European subgroup 1.

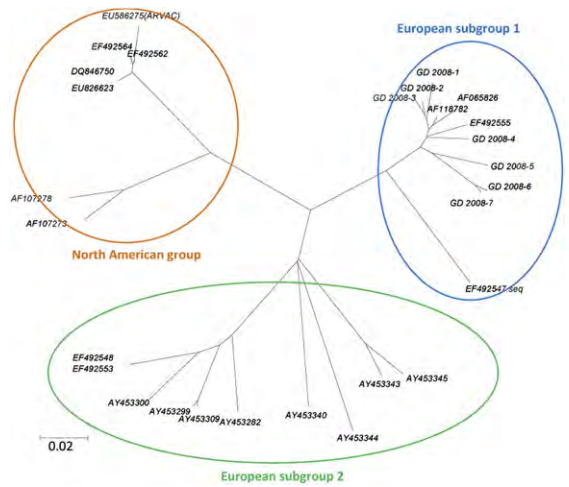

Figure 1. Phylogenetic analysis of the ORF5 of 28 EAV strains. Seven strains were from the Netherlands and 23 have been retrieved from Genbank. The partial ORF5 nucleotide sequences were used for phylogenetic tree construction. Phylogenetic and molecular evolutionary analyses were conducted using *MEGA* version 4.

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Evaluation of three alternative methods for diagnosis of equine herpesvirus abortion

C. van Maanen [1], B. Samplonius [2], R. Buter [1], W. Swart [1], K. Peperkamp [1], and B. van Schaik [1]
[1] GD Animal Health Service, Deventer, the Netherlands,
[2] Pfizer Animal Health, Capelle a/d IJssel, the Netherlands

The gold standard for diagnosing an equine herpesvirus (EHV-1/EHV-4) abortion is a combination of necropsy, histopathology, virus isolation, PCR and/or immunostaining

of the aborted foetus and placenta. The corresponding costs of these diagnostics, including transport logistics of foetuses to a qualified laboratory, are a serious hurdle for many owners in the Netherlands. Therefore three alternative diagnostic methods were hypothesised and investigated: EHV-1/EHV-4 real-time PCR on 1) an aspiration biopsy of the fetal lung; 2) a vaginal swab of the mare collected within 2 days after abortion; 3) detection of EHV-1 specific antibodies in a blood sample of the mare collected within 2 days after abortion. Real-time PCR on aspiration biopsies (n=41) was first optimised by testing 5 different DNA extraction methods in comparison with the gold standard. For EHV-1 real-time PCR primer and probe sequences were generously provided by Dr. Mats Isaakson, SVA, Uppsala, Sweden. EHV-4 real-time PCR was performed as described [1]. EHV-1 and EHV-4 specific antibodies were determined with a commercially available gG-ELISA (Svanova). The three methods were evaluated in populations of mares that delivered normally (group A; n=86), and that aborted their foals (group B; n=83). Aspiration biopsies of foetal lungs (n=41) showed 100% agreement with the gold standard. All EHV-1 positive aspiration biopsies (n=12) were strongly positive with a mean Ct-value of 15.6. In the classical statistical analysis of the field study, using the PCR result of the aspiration biopsy as a gold standard, the relative sensitivity and specificity of EHV-1 PCR on vaginal swabs was 100% (95% CI 66-100%) and 96.8% (95% CI 90-99%), respectively. The relative sensitivity and specificity of the EHV-1 specific gG ELISA was 37.5% (95% CI 14.6-70.1%) and 88.3% (95% CI 77.8-94.2%), respectively. Results of Bayesian analysis are shown in Table 1. Real-time PCR on an aspiration biopsy of the foetal lung, preferably combined with a vaginal swab of the mare collected within two days after abortion, provides a good alternative for diagnosing equine herpesvirus abortion. This diagnostic approach cannot replace pathology in general terms, but provides a cost-effective and practical tool for monitoring and surveillance of viral causes of abortion like EHV-1 and EHV-4 infections.

Table 1
Results Bayesian analysis EHV-1 PCR on vaginal swabs and EHV-1 specific ELISA on serum samples of mares

Parameter	Prior	Posterior		
		Mean	95% Credible Interval	
Se ELISA	49.9-89.6%	67.6%	47.5%	86.0%
Sp ELISA	90.0-97.5%	95.0%	91.8%	97.4%
Se Swab	80.0-95.2%	87.2%	77.8%	94.5%
Sp Swab	90.0-97.5%	93.1%	89.5%	96.2%
P group A	2.5-10.0%	4.6%	2.0%	8.2%
P group B	15.8-68.5%	18.2%	9.6%	28.6%
P diff.		-13.6%	-24.3%	-4.5%

P=prevalence

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A rapid triplex qPCR assay for the detection of *Streptococcus equi*

K. Webb, C. Barker, T. Harrison, Z. Heather, K.F. Steward, C. Robinson, J.R. Newton, and A.S. Waller
Animal Health Trust, Newmarket, UK

Strangles, caused by *Streptococcus equi*, is the most frequently diagnosed infectious disease of horses worldwide, leading to significant welfare and economic cost. It is therefore important that there are highly accurate, rapid and sensitive diagnostic assays for the detection of this pathogen. Here we report the exploitation of the emerging *S. equi* and *Streptococcus zooepidemicus* genome sequencing data to develop a novel diagnostic triplex qPCR assay that targets two genes specific to *S. equi*, *eqbE* and SEQ2190, and a unique 100 bp control DNA sequence, SZIC, which was inserted into the SZO07770 pseudogene of *S. zooepidemicus* strain H70 to create strain *Sz*07770c. Residual material from 213 clinical samples analysed using the AHT's *eqbE* singlex qPCR assay were spiked with 25 μL of killed diluted *Sz*07770c, containing bacteria equivalent to 2000 cfu in the original live culture. DNA was extracted using the GenElute kit (Sigma) according to the manufacturer's instructions and the amount of *S. equi* and SZIC DNA quantified by triplex qPCR. 194 of the clinical samples submitted to the AHT diagnostic laboratories were also tested for the presence of *S. equi* using the traditional culture test. Receiver operator characteristic curves, sensitivity and specificity of the qPCR and culture assays were calculated using STATA (StataCorp LP). The new triplex strangles qPCR assay can be completed in less than 2 hours from sample receipt, has an overall sensitivity of 93.9% and specificity of 96.6% relative to the *eqbE* singlex assay and detects *S. equi* at levels below the threshold of the culture assay regardless of the presence of contaminating bacteria. The culture test identified no positive clinical samples that were not identified by qPCR (singlex or triplex) and had an overall sensitivity of 60.3% and specificity of 100% relative to the combined qPCR results. 15 of the 27 culture negative, qPCR positive samples, contained other β-haemolytic streptococci (10 contained *S. zooepidemicus* and 5 *S. equisimilis*). The mean qPCR copy number of culture positive, qPCR positive samples was 1,388,580 (n=41). Culture negative, qPCR positive samples that did not contain other β-haemolytic streptococci had a mean copy number of 415 (n=12) and culture negative, qPCR samples that contained other β-haemolytic streptococci had a mean *S. equi* DNA copy number of 170,312 (n=15). The use of sensitive qPCR assays will improve the identification and treatment of horses infected with *S. equi*, particularly carriers where intermittent low level shedding of this bacterium is common and can easily be missed by traditional culture and PCR assays. The strangles triplex assay provides a rapid, sensitive and robust method for the detection of *S. equi* infection.

Neurological Diseases

IS-98-ST1 West Nile virus derived from an infectious cDNA clone retains neuroinvasiveness and neurovirulence properties of the original virus

C. Bahuon [1], P. Desprès [2], N. Pardigon [2], J.J. Panthier [2], N. Cordonnier [1], S. Lowenski [1], S. Zientara [1], and S. Lecollinet [1]
[1] ANSES, UMR1161 Virologie INRA, ANSES, ENVA, 23 avenue du Général de Gaulle, 94703 Maisons-Alfort, FRANCE, [2] Institut Pasteur, 25-28 rue du Docteur Roux, 75724 Paris cedex 15, FRANCE

West Nile virus (WNV) is a neurotropic flavivirus mainly transmitted through mosquito bites and whose reservoir hosts are wild birds. Equids and humans are incidental dead end hosts and can develop severe neurological symptoms in 1-10% cases. One specific feature of WNV infections in Europe is the great diversity of WNV strains, belonging to at least 4 lineages causing numerous but limited outbreaks. In 1998, an important outbreak caused by a highly neuroinvasive strain (IS-98-ST1) took place in Israël [1]. To date, much remains to be explored about the neuroinvasiveness properties and virulence determinants of WNV strains circulating in Europe. We aimed at producing a new infectious clone (IC), based on WNV lineage 1a IS-98-ST1, for the characterization of the molecular determinants of European WNV strains virulence. A plasmid encoding the genomic RNA from WNV strain IS-98-ST1 was constructed. *In vitro* transcribed full-length viral RNA molecules were transfected into Vero cells and infectious virus particles were recovered after 3 days. The genomic RNAs from recovered WNV grown on Vero cells and parental virus strain exhibited 100% sequence identity. The growth kinetics of recombinant and parental WNV were similar in Vero cells. Moreover, the phenotypes of recombinant and parental WNV were indistinguishable in terms of viremia, viral load in the brain and mortality in susceptible and resistant mice. Finally, the pathobiology of the infectious clone was examined in embryonated chicken eggs. The capacity of different WNV strains to replicate in embryonated chicken eggs closely paralleled their replication ability in mice, suggesting that inoculation of embryonated chicken eggs could provide a practical *in vivo* model for the study of WNV pathogenesis. A new molecular tool that is useful for the study of molecular determinants of WNV virulence has been generated. We take advantage of the high genetic stability of our one-piece infectious WNV cDNA clone to produce recombinant viruses through the insertion of mutations into the backbone of IS-98-ST1 IC, such as the $NS3_{249P}$ identified as being crucial for the neuroinvasiveness of WNV NY99 strain in American crows [2]. The pathogenicity of recombinant WNV will be assessed *in vitro* and *in vivo*. This project is part of Euro-WestNile project.

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DNA polymerase sequence variation among equid herpesviruses; associations with abortion or with myeloencephalitis?

J.L. Cuxson, J.R. Gilkerson, J.M. Devlin, S.J. Symes, and C.A. Hartley
Centre for Equine Infectious Disease (CIED), Faculty of Veterinary Science, The University of Melbourne, VIC, 3010, Australia

The single nucleotide polymorphism (SNP) in the DNA polymerase gene of equine herpesvirus-1 (EHV-1) that results in an aspartate (D) at position 752 is significantly associated with the neuropathogenic potential of the virus (Nugent *et al.*, 2006). More commonly isolated EHV-1 strains, usually associated with abortion or respiratory disease, encode an asparagine (N) at this position. This N_{752} is unique to EHV-1, since a D or E at position 752 is very highly conserved in most other members of the herpesvirus family. This study aims to (i) determine if N_{752} is unique to EHV-1 by examining the closely related viruses EHV-4 and asinine herpesvirus-3 (AHV-3), and (ii) examine the relationship between N_{752} and abortigenic EHVs by determining the DNA pol sequence of two EHV-4 isolates from abortions. Fourteen EHV-4 isolates comprising 2 isolates from aborted foetal tissue and 12 from cases of respiratory disease were examined. The AHV-3 isolate was cultured from a nasal swab following administration of corticosteroids to a clinically healthy donkey. The full-length sequence of the ORF30 (DNA pol) gene was determined for each virus. The sequence of the ORF68 gene of the EHV-4 isolates was also determined to examine the utility of this gene as a marker of strain variation, as has been suggested for EHV-1 (Nugent *et al.*, 2006). The 14 EHV-4 isolates had a 3663 bp ORF30 sequence containing 11 SNPs compared to the EHV-4 reference strain NS80567, including 6 non-synonymous changes. All EHV-4 strains contained a D at the 752 amino acid position equivalent, and the pattern of SNPs across ORF30 showed no association with pathotype. A single SNP was detected in the ORF68 gene of one EHV-4 isolate, suggesting that unlike EHV-1 ORF68, this gene is not a good marker of strain variation for EHV-4. The 3684 bp ORF30 sequence of AHV-3 was more closely related to

EHV-1 than EHV-4 with 96% and 88% deduced amino acid identities respectively. Like EHV-4, AHV-3 contained a D at the 752 amino acid position equivalent. Even among the most closely related equid alphaherpesviruses, the maintenance of EHV-1 with a DNA polymerase containing N_{752} is unique and suggests a relatively recent evolution. Despite the increasing number of outbreaks of neuropathogenic EHV-1 in the US and Europe in the last decade, this disease manifestation and associated SNP remain less common than EHV-1 abortions and detection of EHV-1 containing a DNA pol with N_{752}. There may be many reasons for apparent fitness advantage of these viruses, including their relative transmissibility that may be greater from high titred aborted foetal tissue compared to levels of virus shed in the respiratory tract.

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Anti-inflammatory drugs will decrease infection of endothelial cell with EHV-1

L.S. Goehring [1], K. Brandes [1], L. Wittenburg [1], F. Olea-Popelka [1], P. Lunn [2], and G Soboll Hussey [3]
[1] Colorado State University, Fort Collins, Colorado, North Carolina State University, [2] Raleigh North Carolina, [3] Michigan State University, East Lansing Michigan

Central nervous system (CNS) endothelial cells (EC) presumably become infected with EHV-1 after contact with infected peripheral blood mononuclear cells (PBMC) during cell-associated viremia. This is likely one of the key steps leading to EHV-1 Myeloencephalopathy (EHM) in horses following primary, respiratory tract infection with EHV-1. Contact between EC and PBMC is thought to be facilitated by inducible adhesion molecules. As some anti-inflammatory drugs have shown to down-regulate adhesion molecule expression, we hypothesize that anti-inflammatory drugs will decrease the interaction between PBMC and EC, and will decrease EC infection. A modified infectious center assay was used in combination with freshly isolated, in vitro EHV-1 infected PBMC or a viral suspension to infect carotid artery-derived, immortalized EC monolayers. In the first series of experiments EC and in vitro infected PBMC, or EC plus viral suspension (EHV-1 Ab4) were incubated for 24 hours with either media alone (NTx), or with published therapeutic equine plasma concentrations (Tx) of one of the anti-inflammatory drugs lidocaine, firocoxib or dexamethasone, before exposure with either PBMC or viral suspensions for 4 hours. Then, monolayers were washed and media containing NTx or Tx were re-applied for additional 48 hrs. In the second set of experiments we focused on cell-to-cell viral spread exclusively by incubating infected PBMC; EC or both with drugs prior to contact, and incubated further with or without added drugs to media for 48 hrs. For statistical evaluation generalized linear and latent mixed models as well as ANOVA were used. Statistical significance was assumed with a p-value of ≤ 0.05. All three drugs significantly decreased the plaque count ($p < 0.001$) in the cell-contact model but not in the viral suspension model (experiment 1). In experiment 2, a plaque count reduction was only noted when both compartments, EC monolayers and PBMC, were pretreated with drugs prior to contact ($p < 0.001$). Physiologically-paired molecular structures on PBMC and EC may play a role in EHV-1 infection of the EC. These results provide a rationale for the use of anti-inflammatory drugs during early phases of EHV-1 infection. Moreover, the use of anti-inflammatory drugs during viremia may aid in preventing EC infection in vivo.

Comparative sequence and phylogenetic analysis of open reading frame 30 (ORF30) of neuropathogenic and non-neuropathogenic EHV-1 strains from the united states and france

Yanqiu Li [1], Frank Cook [1], Stéphane Pronost [2], Beate Crossley [3], Stephen Sells [4], Pamela Henney [1], Kathryn Smith [1], William Laegreid [5], Peter Timoney [1], and Udeni Balasuriya [1]
[1] Maxwell H. Gluck Equine Research Center, [2] Frank Duncombe Laboratory, Animal Health Department, Caen, France, [3] California Animal Health and Food Safety Laboratory, University of California, Davis, CA, [4] Veterinary Diagnostic Laboratory, Department of Veterinary Science, University of Kentucky, Lexington, KY, [5] Department of Veterinary Sciences, University of Wyoming, Laramie, WY

Equine herpesvirus-1 (EHV-1) causes acute upper respiratory tract disease, abortion, neonatal foal death, and neurological disease of varying severity in horses. Although it appears that all EHV-1 strains can induce respiratory disease and abortion in pregnant mares, only certain strains (neuropathogenic) have the potential to cause wide scale outbreaks of equine herpesvirus myeloencephalopathy (EHM) in horses. A previous study suggested a non-synonymous single nucleotide polymorphism ($A_{2254}>G_{2254}$ [$N_{753}>D_{753}$]) within the palm domain of open reading frame 30 (ORF30) that encodes the viral DNA polymerase is responsible for the neuropathogenic phenotype of the virus. The objective of this study was to identify the genetic and evolutionary relationships between EHV-1 strains circulating in United States and in other countries by nucleotide and phylogenetic analyses, as well as to identify additional mutations in ORF30 that may influence the neuropathogneic phenotype of the virus.A panel of archived EHV-1 strains from the USA (1941-2008; [n=41]) and France (2005-2006; [n=21]), fluid (TCF) stocks, along with the modified live virus (MLV) vaccine strain (Rhinomune; Pfizer Animal Health Inc.,) were included in this study. ORF30 and the flanking sequences were amplified with EHV-1 specific primers using Phusion Hot Start DNA polymerase enzyme. Both sense and anti-sense strands were sequenced and sequence data were analyzed with Codoncode Aligner version 2.0.6 and Vector NTI® Suite V.11. Multiple sequence alignments were performed using

CLUSTAL X v1.83. Unrooted neighbor-joining trees were constructed using MEGA5.1. The complete ORF30 was sequenced for 41 US strains and 23 European strains, as well as the modified live virus (MLV) vaccine strain (Rhinomune, Pfizer Animal Health Inc.). Sequence data analysis demonstrated that all ORF30 sequences are very closely related, sharing 96.4-99.9% and 96.6-99.9% nucleotide (nt) and amino acid (aa) sequence identity to the published sequence of V592 strain of EHV-1 (GenBank number AY464052), respectively. However, numerous non-synonymous amino acid substitutions were observed in all six domains of the viral DNA polymerase enzyme. Based on phylogenetic analysis of ORF30, EHV-1 isolates could be segregated into 4 distinct groups, regardless of their year of isolation or geographic location. Interestingly, one strain isolated from an equine placenta had significantly different sequences and this virus grouped very closely with an EHV-9 isolate from the lung of an aborted zebra fetus. Results from this study indicate that additional mutations in ORF30 or other viral genes, as well as host or environmental factors have the potential to contribute to the development of neurologic disease in horses infected with EHV-1.

Investigation of the role of mules as silent shedders of equine herpesvirus-1 during an outbreak of equine herpesvirus-1 myeloencephalopathy in California

N. Pusterla, S. Mapes, C. Wademan, A. White, K. Estell, and E. Swain
School of Veterinary Medicine, University of California, Davis, CA 95616

Equine herpesvirus-1 myeloencephalopathy (EHM) is a relatively uncommon manifestation of EHV-1 infection but can cause devastating losses during outbreaks on individual farms or boarding stables. Although all breeds of horses are susceptible to the neurologic form of EHV-1 infection, the authors are unaware of reports of neurologic EHV-1 clinically affecting donkeys and mules. However, donkeys and mules have shown seroconversion indicating infection with EHV-1 while in contact with affected horses during outbreaks. The aim of this study was to investigate the role of mules as possible silent shedders during an outbreak of EHM occurring at a packing station in northern California. In early September of 2011, an EHV-1 outbreak with several neurologic horses occurred at a packing station located in the eastern Sierra of California. The packing station was visited with the goal to assess the clinical status and exposure rate of the resident horses and mules. Using appropriate biosecurity measures, a physical and neurological evaluation was performed followed by the collection of whole blood and nasal secretions for the quantitative PCR and serological detection of EHV-1. A total of 82 horses and 59 mules ranging in age from 3 to 30 years (median 12 years) were evaluated during the visit. On the day of the visit a total of 5 additional horses showed neurological deficits. Fifty-six horses or mules (39.7%) tested PCR positive for the EHV-1 gB and the ORF 30 (D_{752}) gene. In an attempt to determine statistical differences in viral loads, the PCR positive horses and mules were grouped into asymptomatic horses (27 animals), asymptomatic mules (24) and horses with neurological signs (6). The viral loads in blood were significantly different between the three groups (Wilcoxon-Mann-Whitney tests; $P < 0.05$) with asymptomatic horses having the lowest viral loads and neurologic horses showing the highest viral loads. For nasal secretions, asymptomatic mules had significantly higher viral loads in nasal secretions ($P < 0.05$) compared to asymptomatic horses. A total of 33/141 (23.4%) horses and mules tested seropositive for EHV-1. Based on combined molecular and serological testing 48/82 (58.5%) horses and 32/59 (54.2%) mules had evidence of EHV-1 exposure. The results of this study showed that equal percentages of horses and mules became exposed to EHV-1 during an EHM outbreak; however, neurological disease was only reported in horses. The molecular and serological results support the role of mules as silent shedders, highlighting the need to institute appropriate biosecurity protocols when horses and mules are comingled.

Use of CSF biomarkers as a model to study EHM

G. Soboll Hussey [1], D.P. Lunn [2], S.B. Hussey [3], C. Broccardo [3], C. Broeckling [3], J. Prenni [3], L.V. Ashton [3], and L.S. Goehring [3]
[1] Michigan State University, [2] North Carolina State University, [3] Colorado State University

Equine herpesvirus-1 (EHV-1) infection results in sporadic but devastating outbreaks of neurological disease (equine herpesvirus myeloencephalopathy – EHM) in horses that are caused by a myeloencephalopathy with a poorly understood pathogenesis. Viremia occurs commonly in EHV-1 infection, but only relatively rarely (<10% cases) progresses to EHM. In order to understand how and why some cases of EHV-1 infection lead to EHM, our goal was to develop a model that would allow us to determine whether sub-clinical infection and pathology occurs in EHV-1 viremic horses that do not develop clinical EHM. A study was designed that included 12 yearling horses that were experimentally infected with a neuropathogenic strain of EHV-1. Physical exams, and testing for viremia and viral nasal shedding were performed prior infection and daily for 14 days following infection. In addition, CSF was collected prior to infection and on days 8 and 10 post infection in all horses. Additionally, daily CSF samples were collected in 3 horses via an indwelling subarachnoidal catheter system, Proteomic analysis was conducted on both day 0 and day 10 samples by fractionating digested CSF using 2-dimensional liquid chromatography (2D-LC) consisting of strong cation exchange followed by reverse phase separation on-line with an ion trap mass spectrometer. Metabolomic analysis was performed on deproteinized CSF collected from day -5 to day 12 post-infection, via reverse phase LC-MS. All horses showed the classical clinical signs of EHV-1, exhibited nasal viral shedding through day 5 post-infection and were viremic between days 5-10 post infection. Proteomic analysis revealed 439 proteins across all replicates. Of those

proteins, 17 proteins were significantly different between day 0 and day 10 post-infection. Further evaluations are currently being performed to assess the potential of these proteins as biomarkers for EHM. Metabolomic analysis revealed 313 molecular features that were significantly changed following infection. Metabolite identification is currently underway. Validation and biological evaluation of these candidate protein and metabolite biomarkers has the potential to significantly further our understanding of the pathogenesis of EHM in addition to providing a valuable model for development of vaccines and therapeutics. Further, the techniques developed in this study can be readily applied to the analysis of equine CSF for other disease models, and may provide information relevant for evaluation of human CSF for patients suffering from viral-induced neurologic diseases or stroke.

Analysis of Italian equid herpesvirus type 1 strains

E. Canelli [1], G. Manna [2], G.L. Autorino [2], and P. Cordioli [1]
[1] Istituto Zooprofilattico Lombardia e Emilia Romagna, Brescia, Italy, [2] Istituto Zooprofilattico Lazio e Toscana, Roma, Italy

Among the clinical forms for which equid herpesvirus 1 (EHV-1) is responsible, an increase in the frequency of Myeloencephalopathy (EHM) cases has been observed worldwide in the last decade [4]. These forms are mainly associated to EHV-1 strains commonly classified as mutant because they present a nucleotide substitution of A with G at the 2254 position, resulting in an aminoacid change of N with D at position 752 within the DNA Pol-gene [3]. The objective of this study is to investigate the diffusion of this mutant strain in Italy over a 20 year period, together with its geographical distribution. Eighty-seven EHV-1 strains isolated in Northern (n=69) and Central-Southern Italy (n=17), between 1990 and 2010, were characterised using the Taq-Man® allelic discrimination PCR assay [1]. Eighty of them were from episodes of perinatal deaths and abortion while, 6 were from neurological cases occurring in the last two years of the study period. Results were confirmed as previously described by genetic analysis of ten representative isolates and by comparing them to reference strains Mutant_AB4 and Classic_V592 [3]. The allelic discrimination PCR assay revealed that 61% of the strains presented the mutation D/752 which was detected in all the 6 neurological outbreaks and in 59% of the abortion and perinatal ones. While the two EHV-1 variants were both circulating in Italy since the '90s (63% and 45% isolated respectively from northern and central/southern regions) interesting, is the increase in prevalence of classical strains in the last five year period. Moreover, the genetic analysis detected a new silent substitution at nucleotide position 2874 of G with A in two of the D/752 strains. The association of only type D/752 in the neurological forms supports the hypothesis advanced by other Authors, that the mutation is sufficient in determining this syndrome [2; 3]. The high viraemic titres of mutant D/752 might the reason of the high rate of its presence in foetuses. It is however impossible to retrospectively investigate if the distinct geographical viral strain distribution could be attributable to the presence of different local risk factors. In contrast with other studies [2;4], our results show that the increased viral isolations in neurological forms during the last two years of the study period, might have been associated to the enhanced risk perception consequent to the West Nile epidemics occurring at the same time in Italy, rather than to a real higher incidence of the mutant strain. This, also agrees with the evolutionary hypothesis identifying the variant D/752 as a progenitor of N/752 [3].

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Lineage 1 WNV strains induce distinct brain lesions

E. Donadieu [1], S. Lowenski [1], J.L. Servely [2], E. Laloy [2], S. Zientara [1], S. Lecollinet [1], and M. Coulpier [1]
[1] UMR 1161 Virologie ANSES, INRA, ENVA, Ecole Nationale Vétérinaire d'Alfort, 7 avenue du Général De Gaulle, 94700 Maisons-Alfort, FRANCE, [2] Unité d'Histologie et d'Anatomie Pathologique, Ecole Nationale Vétérinaire d'Alfort, 7 avenue du Général de Gaulle, 94704 Maisons-Alfort Cedex, FRANCE

West Nile virus (WNV) is a neurotropic arthropod-borne flavivirus that is maintained in an enzootic cycle between mosquitoes and birds, but can also infect and cause febrile syndrome and encephalitis in humans and horses. Diverse pathogenic WNV strains circulate in Africa, the Middle East, Asia, the United States and Europe. Their genetic alignments allowed their classification into two major lineages, lineages 1 and 2. So far, little is known about their virulence and neuropathogenicity. To get insight into the neuropathogenicity of WNV, we compared the neuropathogenic properties of two lineage 1 WNV strains known to have caused many (417) or a few (less than 10) human cases, Israel 1998 (IS-98-ST1) and Kunjin 35911 respectively. Four-week old C57Bl6 mice were inoculated intraperitoneally with both strains and strain virulence was assessed, as well as brain viral load and lesions. Histological examination of brain tissues revealed that the Kunjin 35911 strain is able to induce alterations such as spongiosis, gliosis, meningitis, perivascular infiltrates and neuronal death in nearly all the brain structures studied. On the contrary, spongiosis was not observed in the brains of mice inoculated with the same LD_{50} of the IS-98-ST1 strain and meningitis, perivascular infiltrates and neuronal death were strongly reduced compared to the Kunjin strain. In addition, our results suggest that the molecular pathways involved in cell damage vary qualitatively and quantitatively between both strains according to the brain structure considered. In conclusion, although the Kunjin 35911 strain is less virulent than the IS-98-ST1 strain, it was found to induce more severe lesions in the brain, demonstrating that

WNV genotype influences neuropathogenicity as well as virulence. Based on these results, the comparison of WNV strains might help to elucidate the mechanisms of WNV neurovirulence and neuropathogenicity and to promote the development of novel therapeutic targets.

Evidence that inflammation associated with Sarcocystis neurona infection plays a role in clinical signs associated with equine protozoal myeloencephalitis

S.P. Ellison DVM PhD
Pathogenes, Inc., Fairfield, Florida

Sarcocystis neurona is a protozoal pathogen that causes equine protozoal myeloencephalitis (EPM). The commonly recognized neurologic signs that EPM causes are due to the pathological effects of the disease and can be due to infections of somatic and neural tissues by the asexual stages of *S. neurona* that cause cell dysfunction and death. A second component of EPM is the inflammatory host response to the parasitic insult. Inflammation may be as deleterious to the host as the initial insult. Anticoccidial drugs are necessary to treat the infectious aspect of EPM. An objective evaluation of treatment effect on protozoa is measured by a reduction in SnSAG 1, 5, and 6 antibodies. Recurrences of neurologic signs in horses that have diminishing antibody titers was controlled by levamisole HCl and indicate inflammation is a significant component of disease. There are no specific markers of inflammation due to *S. neurona*, therefore clinical exam was used to determine effectiveness of treatment. In a clinical field trial horses with a presumptive diagnosis of EPM based on veterinary clinical exam and the presence of serum antibodies against recombinant SAG's 1, 5, and 6 by ELISA (> 1:16) were treated with 0.5 mg/kg decoquinate and 1 mg/kg levamisole HCl PO for 10 days. A post-treatment clinical examination and antibody titer was determined. Horses that improved and then experienced recurrence of neurologic signs were treated with levamisole HCl PO until the horse resolved the clinical signs. Ninety-four percent of the EPM suspect horses resolved neurological signs within the 10 day treatment period based on clinical exam. Eighty-nine percent showed a reduction in antibody titers at 4-5 weeks and 97% showed a reduction in antibody titers by 12 weeks post treatment. Eleven percent of horses responded incompletely to decoquinate/levamisole or responded completely but experienced recurrence from 7 to 14 days following treatment. Horses that experienced recurrence of signs responded to levamisole HCl and maintained the improvement after discontinuing the 5 week treatment. Horses with a history of chronic relapsing EPM responded to treatment but required a longer period of levamisole HCl. Investigations using c-reactive protein (CRP) as a predictor of relapse post treatment is ongoing.

Table 1
Seroprevalence of antibody against S. neurona in clinically normal horses and those with presumptive EPM.

	Sero (-)	Sero (+)	Mixed Inf.	SAG 1	SAG 5	SAG 6
Normal (n=100)	41%	59%	18%	4%	11%	26%
Suspect EPM (n=100)	16%	84%	48%	22%	6%	8%

Table 2
Results from 141 horses showing decreased clinical signs and decrease in titer following treatment with decoquinate/levamisole. Horses with an incomplete resolution of signs (or recurrence of signs) were treated with levamisole HCl.

Decrease clinical signs	Decrease titer	% recurrence	Response to levamisole	extended use levamisole
132/141	126/141	15	15/15	15-Apr
93.60%	89.30%	11%	100%	26%

Lessons learned from field investigations of outbreaks of equine herpes myeloencephalopathy

K.A. Flynn, W.K. Fowler, and E.M. Wilson
California Department of Food and Agriculture, Animal Health Branch, Sacramento, CA 95814

In January 2011, the California Department of Agriculture (CDFA), Animal Health Branch (AHB) listed Equine Herpes Myeloencephalopathy (EHM) on the California *List of Reportable Conditions for Animals and Animal Products* as a *Regulatory Condition* reportable within forty-eight hours of diagnosis. The case definition for a confirmed EHM case is a horse with a positive laboratory diagnostic test result for Equine Herpesvirus-1 (EHV-1) that is displaying neurologic signs of disease. Upon receipt of laboratory report positive for EHV-1, the AHB veterinarian contacts the submitting veterinarian to obtain an initial case history. The AHB veterinarian performs a field investigation and, as appropriate on a case-by-case basis, imposes quarantine. During the investigation, the veterinarian obtains a complete clinical history of the index case(s), conducts a facility biosecurity risk assessment and provides facility managers biosecurity recommendations addressing any identified risk factors. Based on the outcome of the epidemiologic investigation, additional regulatory actions may be taken to prevent the risk for spread of EHV-1. In January 2012, EHV-1 investigations were conducted at two large equine facilities in California; a large multidiscipline boarding facility with over 365 horses and a large polo facility with approximately 1,000 horses. The large boarding facility had one confirmed case of EHM and fifteen febrile horses exhibiting respiratory signs test positive for EHV-1. Transmission risk factors at this facility included pipe corral stabling with nose-to-nose contact of horses in separate pens, communal water, sharing of equipment, limited availability of an isolation area and inconsistent biosecurity practices. AHB veterinarians met with the boarding facility management, owners, trainers and veterinarians on two occasions to communicate biosecurity and disease control plans. Despite these outreach meetings, implementation of biosecurity measures was challenging due to facility constraints and the number of owners,

trainers and veterinarians involved. At the polo facility, the attending veterinarian promptly isolated the index case, implemented biosecurity measures and provided oversight of thorough cleaning and disinfection of the stabling area and shared equipment. Following rapid progression of neurological impairment, the index case was euthanized; no other horses on the premises were confirmed positive for EHV-1. Lack of EHV-1 transmission at the polo facility is believed to have been due to the prompt response actions by the attending veterinarian and compliance with implemented biosecurity measures by other horse owners on the premises. During both incidents, the CDFA communication plan to ensure availability of accurate up-to-date information for the California equine industry included email notifications to equine practitioners and equine industry representatives and development and maintenance of an EHV-1 Alert webpage. In conclusion, implementation of prompt isolation and enhanced biosecurity practices at equine premises with confirmed EHM cases is critical for controlling transmission of EHV-1. Clear, concise, frequent and consistent messaging to all segments of the equine industry is essential to convey accurate information and recommendations during an infectious disease outbreak.

Outbreak of equine herpesvirus myeloencephalopathy in france. molecular tools, a help to the management

S. Pronost [1,2], L. Legrand [1,2], P. Morreau [3], C. Fortier [1,2], M. Foursin [3], and G. Fortier [1,2]
[1] Frank Duncombe Laboratory, Animal Health Department, 14053 Caen, France, [2] University of Caen Lower Normandy EA 4655 U2RM 14000 CAEN France, [3] Clinique Equine de la Boisrie 61500 Chailloué France

Equid herpesvirus 1 (EHV-1) –associated myeloencephalopathy (EHM) is a disease affecting the central nervous system of horses. The increased interest of researchers in the manifestation of this disease is not only due to the lack of current scientific understanding but also to the associated economic impact. Laboratory diagnosis of EHM is currently based on at least one of the following criteria: detection of EHV-1 in nasal swab or blood by PCR, virus isolation, serological testing (virus neutralization) and post-mortem examination. The ease, the speed and the efficiency of the PCR tests make of this technology a precious tool in the management of the crises. 169 horses (60 mares, 44 foals and 65 yearlings) were stabled at the thoroughbred studfarm. Nasal swabs and blood (EDTA tube) were collected from all horses and analyzed. In addition various tissue samples from horse and foetuses were taken at necropsy, genital swabs taken on aborted mares and all were analyzed by molecular biology techniques. A real-time PCR (qPCR) was used for EHV-1 detection. A duplex real-time Taq-Man PCR assay (SNP-PCR) was used to discriminate between non-neuropathogenic (A2254) and neuropathogenic (G2254) EHV-1 strains. The outbreak commenced with two cases of myeloencephalitis which presents within a 24 hour period. A neuropathogenic strain of the virus was identified Six groups were constituted depending of the origin and status of the horses. One week later several foals presented clinical signs and fever and nasal discharge. The screening revealed positives cases in 3/6 groups. A non neuropathogenic strain of the virus was identified. The status of each foal was investigated each week during 42 days. The viral load varies from 200 to 10^7 viral particules/ml in nasal swabs. The kinetic of decrease of the positive sample allows managing the animals in each group and between the different groups. A total of four cases of EHV-1 related abortion occurred over the 4-6 months following the initial cases. One neuropathogenic strain and 3 non neuropathogenic were identified. This outbreak associated with EHV-1 on a breeding farm in Normandy described the three forms of the disease over a short period. The two different type of EHV-1 were also characterized. Molecular tools help for the management of the outbreak by giving the status of horse in real time.

Comparison of prevalence factors in neurologic horses with and without seropositivity to *Neospora hughesi* and/or *Sarcocystis neurona*

N. Pusterla, E. Tamez-Trevino, A. White, J. Van Geem, A. Packham, P. Conrad, and P. Kass
School of Veterinary Medicine, University of California, Davis, California, USA

Equine protozoal myeloencephalitis (EPM) is a commonly diagnosed neurological disease of horses in North America. The most common cause is *Sarcocystis neurona*, although other protozoal pathogens, such as *Neospora hughesi*, have also been identified in the CNS of horses with EPM. A presumptive diagnosis of EPM is based on the presence of compatible neurologic signs, the exclusion of other potential diseases, the presence of specific antibodies to *S. neurona* and/or *N. hughesi* in serum and/or CSF and the response to antiprotozoal therapy. The objective of this study was to determine the frequency in the sample submission to the School of Veterinary Medicine at Davis of suspected EPM horses testing seropositive to *N. hughesi* and to compare prevalence factors amongst suspected EPM horses testing seropositive or seronegative to *N. hughesi* and/or *S. neurona*. Epidemiological information from neurologic horses tested for specific antibodies to *N. hughesi* and *S. neurona* by means of an indirect immunofluorescent antibody test were reviewed using submission forms submitted to the School of Veterinary Medicine at Davis from 12/1/2010 to 11/30/2011. The submission forms for each horse contained information pertaining to the date of sample collection, the State of residence, signalment and clinical signs. Horses were allocated to one of 4 groups depending on the serological results; *N. hughesi* seropositive only group, *S. neurona* seropositive only group, *N. hughesi* and *S. neurona* seropositive group and *N. hughesi* and *S. neurona* seronegative group. A total of 3,123 of the 4,250 (73.5%) serum samples submitted over the 12 months study period were retained for the study evaluation. The submissions originated from 49 United States. Thirty-eight horses (1.2%) from 21 States were in the *Neospora* only group, 840 horses (26.9%) from 40 States were in the *Sarcocystis* only group, 25 horses (0.8%) from

14 States were in the *Neospora* and *Sarcocystis* group, and 2,220 horses (71.1%) from 49 States were in the seronegative group. There was a significant association between States and the serological groups ($P < 0.0001$). The States contributing to the significance were Oklahoma and Texas. There were more horses than expected in the *Neospora* only group and *Sarcocystis* only group in these two States. Significant associations were made between months of submission, breeds, clinical signs and serological groups. There was no association between age or gender and serological groups. In conclusion, the results of this retrospective study show that *N. hughesi* is, alone or in combination with *S. neurona*, associated with EPM cases. The wide geographic origin of *N. hughesi* seropositive horses, including Eastern, Southern, Midwestern and Western States highlights the need to test for both protozoal pathogens in neurologically affected horses with suspected EPM.

Equine herpesvirus 1 specific antibody seronegativity is a significant risk factor for developing myeloencephalalitis

M.J. Studdert [1], K. Dynon [1], C.M. El-Hage [2], G.A. Anderson [2], and C.A. Hartley [1]
[1] Centre for Equine Virology, [2] Veterinary Clinical Centre, School of Veterinary Science, The University of Melbourne, Victoria, Australia

Most laboratories investigating outbreaks of EHV1 and EHV4 disease including myeloencephalitis (also called myeloencephalopathy) rely on serum neutralization and complement fixation tests. SN and CF tests do not discriminate between EHV1 and EHV4 antibodies. EHV1 and EHV4 are major causes of acute upper respiratory tract infections. EHV1 also causes abortion, perinatal foal deaths and myeloencephalitis. In recent years there has been an increase in the incidence of EHV1 myeloencephalitis particularly in the United States and there is some evidence that a single nucleotide change in the polymerase gene (ORF30) is a major risk factor. In this presentation we update our interpretation of data from an outbreak of EHV1 myeloencephalitis (Studdert et al Vet. Rec. 153, 417, 2003) in which five of 10 pregnant mares developed myeloencephalitis. Three of the five mares became recumbent, developed complications and were euthanized. The diagnosis of EHV1 myeloencephalitis was supported by necropsy findings, polymerase chain reaction identification of the virus and by serology in which an EHV1 specific antibody detection ELISA (Svanovir® EHV1/4 Ab discriminating ELISA; United States Patent 5,922,327) was used (Crabb et al. J. Virol 67, 6332, 1993). The status of EHV1 infection in the five in contact mares was similarly monitored. 3 of 3 (100%) affected mares for which appropriate sera were available had low or borderline EHV1 antibody titers when first tested on either day 6 or 7 and 0/5 (0%) unaffected mares had low or borderline titers when first tested on either day 7 (4 mares) or day 13 (one mare). The P-value (Fisher's Exact test) for this comparison is P=0.018. All 10 mares were EHV4 antibody positive when first tested and these titers remained more of less stable during the episode. An independently developed EHV1/EHV4 antibody detection ELISA based on the same epitope region of glycoprotein G as used in the Svanovir test was developed and applied to the study of outbreaks of EHV1 and EHV4 disease including EHV1 myeloencephalitis in Japan (Yasunaga et al J Vet Med Sci 60, 1133, 1998). In the Yasunga et al. report two of 25 horses (horses 14 and 16 as listed in their Table 1) developed myeloencephalitis. Both horses showed a rising ELISA antibody titer to EHV1 between acute and convalescent phase sera and stable EHV4 ELISA antibody titers. Data from the study in Japan support the central findings of our study. Though the quantum of available data is limited we propose that EHV1 seronegativity is a significant risk factor for the development of EHV1 myeloencephalitis that deserves further study.

A summary of cases investigated from the 2011 multi-state EHV-1 outbreak

J.L. Traub-Dargatz [1], A.M. Pelzel [2], L.H. Creekmore [2], S. Geiser-Novotny [2], B.A. Bischoff DVM [3], T. Kasari [3], A.M. Wiedenheft [1], and E.J. Bush [3]
[1] Colorado State University College of Veterinary Medicine and Biomedical Sciences, Animal Population Health Institute (APHI), Fort Collins, CO 80523, [2] USDA–APHIS–VS Western Regional Office, 2150 Centre Avenue, Building B, Fort Collins, CO 80525, [3] USDA–APHIS–VS Centers for Epidemiology and Animal Health, 2150 Centre Avenue, Building B, Fort Collins, CO 80526

One of the largest EHV-1 outbreaks ever reported was associated with horses that attended the NCHA, Western National Championship that was held April 29–May 8, 2011, in Ogden, UT. Premises and horse-level case surveys were completed by owners/managers of affected horses for both primary (1°= attended the NCHA event in Ogden, UT) and secondary exposed horses (2°= horses exposed subsequently to NCHA event in Ogden). The study was conducted through a collaborative effort between State Animal Health Officials and various units within USDA–APHIS–VS. Participation in the study was voluntary and data are confidential. Among the 35 1° cases, 24 (70.6%) had fever (range 101.5 to 105.8°F) and 20 (57.1%) had neurologic signs with wobbly gait being most common. Thirteen cases had both fever and neurologic signs. Four 1° cases had neither fever nor neurologic signs but tested PCR positive for EHV-1. The earliest reported onset date among 1° cases for fever was May 9 and for neurologic signs was May 10 of 2011. All barns at the event had one or more 1° cases. Among the 15 2° cases 14 had fever (range 101 to 104.7°F) with earliest reported onset of May 15 and 4 had neurologic signs with earliest onset of May 19. Four 2° cases had fever and neurologic signs. Fever and/or neurologic signs were the most common clinical signs in both 1° and 2° cases. All housing locations at the event were represented among the 1° cases. We wish to acknowledge the State Animal Health Officials and VS area offices for their assistance in survey data collection.

The National Cutting Horse Association (NCHA) for their assistance communicating about the epidemiologic study with their members. Judith Rodriguez and Anne Berry of USDA–APHIS–VS–CEAH for data entry and Drs. Brian McCluskey and Bruce Wagner for their input on study design, implementation, and reporting.

Recent Equine Herpes Myeloencephalopathy (EHM) outbreaks in the Netherlands and the impact of (social) media on the public perception

C. van Maanen [1], B. van Schaik [1], and M.M. Sloet van Oldruitenborgh-Oosterbaan [2]
[1] GD Animal Health Service, Deventer, the Netherlands, [2] Department of Equine Sciences, Faculty of Veterinary Medicine, Utrecht University, the Netherlands

Equine herpes virus 1 (EHV-1) strains are associated with respiratory disease, abortion and myeloencephalopathy. Worldwide outbreaks of EHV-1-associated myeloencephalopathy (EHM) occur rarely but cause disproportionately damage to the equine industry. The percentage of horses with neurological disease during natural outbreaks of EHM in horse operations is usually 10–20%. This study aims to elucidate why four minor outbreaks became a massive media hype and raised great concerns in the general public opinion. Two EHM outbreaks occurred in two premises in the same period (February 2012). There appeared to be a connection between both premises (horses stabled in premise A were visiting premise B weekly for indoor riding lessons). A third case of EHM (C) was confirmed in a geographically divergent location, with a history of indirect contact with one of the diseased horses of premises B. Some weeks later a fourth individual case of EHM was confirmed in another premise without an apparent connection to premises A, B or C. In premises A and B most of the horses developed fever, and in the course of the outbreak about 25% of the horses developed neurological signs consistent with EHM. In both premises several horses had to be euthanized. Case C had to be euthanized within a few days but no other horses on this premise became febrile or showed neurology. Premise D was already closed for 3 weeks before the first horse became febrile and here one horse showed ataxia (recovered) and one other aborted. During the onset of the outbreaks nasal swabs and EDTA blood samples were scored EHV-1 positive by real-time PCR. The EHV-1 strain responsible for these outbreaks was characterised by allele-specific PCR as a classical N752 strain. Although the limited number of outbreaks was not significantly different from the normal EHM incidence in the Netherlands, a media hype developed on radio, television, Facebook, twitter, and several website discussion platforms. Even in parliament the question was raised whether EHM should become a notifiable disease. The Dutch National Equestrian Federation advised to transport horses only for emergencies, and regional and national competitions were cancelled. The impact of social media was enormous e.g. the topic 'herpes virus neurology' on the largest equine websites of the Netherlands was visited >90,000 times/day. Retrospectively only a limited number of horses on four premises were involved. However, as result of the 'voluntary' standstill and the cancellation of horse shows and meetings the additional costs of the outbreak were very high. For future outbreak management the role of social media platforms for knowledge sharing and demystification should be considered as an important component of a communication strategy.

Reproduction Diseases

Outbreak of *Salmonella abortus equi* abortion in embryo recipient polo mares

S. Buigues [1], A. Ivanissevich [2], M.A. Vissani [3], V. Viglierchio [4], L. Minatel [5], F. Crespo [4], M. Herrera [6], P. Timoney [7], and M.E. Barrandeguy [3]
[1] Las Liebres Polo Breeding Farm, [2] Cresal Veterinaria SA, [3] Instituto de Virología, CICVyA, INTA Castelar, [4] Biopolo SA, Embryo transfer center, [5] Cátedra de Patología General, Facultad de Ciencias Veterinarias, Universidad de Buenos Aires, [6] Dirección de Laboratorios y Control Técnico (DILAB), SENASA. Buenos Aires, Argentina, [7] Gluck Equine Research Center, University of Kentucky, Lexington, Kentucky, USA

Salmonella enterica serovar *abortus equi* is a host-adapted salmonella historically recognized as a cause of abortion in mares and a range of clinical conditions in foals. The bacterium is spread on breeding farms through direct or indirect contact with contaminated pasture, food or water. The features of an extensive outbreak of abortion caused by this organism on a polo pony breeding farm located in Buenos Aires Province, Argentina, are described. The at-risk population were 116 pregnant mares that were managed in two groups of equal size, according to stage of gestation. The mares were embryo transfer recipients involving four different stallions. They were allowed to graze freely at pasture during the daytime and at nights, were confined together in a small paddock when they had access to a commercial equine feed. No premonitory signs of impending abortion or other clinical signs were observed. During the abortion storm, which occurred between October 2nd and October 30th, 24 of 58 (41.3%) mares aborted in the group which were due to foal between November and February. Only a single abortion took place in the other group. A total of 7 aborted foetuses were submitted for laboratory diagnosis. Fetuses were fresh at time of expulsion, frequently enclosed in their respective membranes. With the exception of congestion of the liver and hyperplasia of the splenic lymphoid follicles, gross lesions were variable. Bacteria were observed in all tissues examined, with evidence of bacterial emboli in ischemic

areas of the adrenal glands. Tissues were negative for equine herpesviruses 1 and 4 and equine arteritis virus. A gram-negative motile bacterium was isolated in pure culture from a range of tissues which was identified as *Salmonella abortus equi* (4,12:-:e, n, x). Isolates of the bacterium were sensitive to a broad range of antibiotics. Remaining in-foal mares were treated with trimethoprim and gentamicin. After initiation of antibiotic treatment only two further abortions occurred, on November 10th and February 10th. The source of *S. abortus equi* was not identified. However, since recipient mares came from a range of farms, one or more mares could have been a carrier that recommenced shedding *Salmonella* resulting from the intercurrent stress associated with relocation. There is a need for greater awareness of *S. abortus equi* as a potential cause of widespread abortion in the mare and of the importance of breeding farms having in place appropriate biosecurity and preventive measures including vaccination, to minimize the risk of future occurrences of abortion due to this pathogen.

Development of a multilocus sequence typing method for analysis of *Taylorella* genus

F. Duquesne, L. Hébert, M.F. Breuil, C. Laugier, and S. Petry
ANSES, Dozulé Laboratory for equine diseases, Unit Bacteriology and Parasitology, 14430 Goustranville, France

The *Taylorella* genus is composed of two species: *Taylorella equigenitalis*, the causative agent of the contagious equine metritis (a sexually-transmitted infection of horses, first reported in 1977 and notified to the World Organisation for Animal Health), and *Taylorella asinigenitalis*, considered as a nonpathogenic bacterium despite clinical signs of metritis and cervicitis on mares following an experimental intrauterine infection [1]. Using the recently published genome sequences [2], we developed a multilocus sequence typing (MLST) method for future epidemiological and phylogenetic studies of the *Taylorella* genus. We selected twenty housekeeping genes within *T. equigenitalis* MCE9 and *T. asinigenitalis* MCE3 genomes. Among them, seven were validated by the analysis of an internal sequence of ≈450-bp, to define our MLST scheme. We performed PCR amplifications and sequencing on DNA extracted from 166 isolates (115 *T. equigenitalis* and 51 *T. asinigenitalis*) of diverse origins (Australia, France, United Kingdom, USA, Japan, United Arab Emirates) from 1977 to 2011. The sequences of each locus of each strain were aligned and compared (MEGA5 software). First, allele numbers were assigned to each unique sequence and then, a sequence type (ST) was attributed to each strain with a single combination of allele numbers. MLST data were analyzed by the eBURST algorithm. Our MLST scheme with seven loci shows 32 and 14 STs for *T. equigenitalis* and *T. asinigenitalis*, respectively. No ST was common between the two species despite the presence of common alleles for one of the six loci, reflecting potential allelic exchanges in the evolution of the *Taylorella* genus. We found one predominant ST for each species (ST1 and ST25), comprising 23% and 53% of isolates per ST respectively. The 32 *T. equigenitalis*-STs were resolved into five clonal complexes and four single clones. The founding genotype (ST1) of the dominant clonal complex, grouping 18 STs, is composed of isolates from the first outbreaks late 70s in United Kingdom, France, Australia and USA. The 11 *T. asinigenitalis*-STs were resolved into tree clonal complexes and five single clones. The challenge of developing a single MLST scheme for *T. equigenitalis* and *T. asinigenitalis* was achieved despite ≈20% genetic differences between their genome [2]. Our first results show an organization into clonal complexes confirming the relevance of this method for future epidemiological and phylogenetic studies of the *Taylorella* genus. Its simplicity of implementation, its robustness and the use of a common MLST database (under development) will allow the comparison and the pooling of results between laboratories.

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Evidence of *T. equiperdum* infection in the Italian Dourine outbreaks

R. Lelli, P. Calistri, A. Giovannini, and V. Caporale
Istituto "G. Caporale", Teramo, Italy

Dourine is a sexually transmitted parasitic disease of equids caused by flagellate protozoa of the species *Trypanosoma equiperdum.* Dourine is endemic in many areas of Asia, Africa, Russia, part of Middle East, South America and south-eastern Europe. In Italy, it was first eradicated in the late-40s and again in the 70s of last century following an epidemic due to the import of infected animals from former USSR. A stallion undergoing routine serological testing for stud purposes was found positive for dourine in May 2011, in Sicilia and the following tracing back detected four further outbreaks. A surveillance plan based on the serological examination of all stallions and mares older than two years of age was implemented following to these events and two further outbreaks were detected in 2011. The Authors describe the epidemiological, clinical, pathological and laboratory observations in horses infected in the 2011 dourine epidemic in Italy. Two stallions and five mares showing clinical signs were transferred to the Istituto "G. Caporale", Teramo (ICT), to both monitor the evolution of the disease and perform additional tests. Four of the five mares were euthanized and necropsied. Organs and tissues were sampled for histopathology. The cerebrospinal fluid (CSF) was tested for both anti-*T. equiperdum* antibodies and evidence of parasite presence. Blood, CSF, spleen, kidney, liver, udder, mammary secretion, lymph nodes, lung, skin wheals, genital organs, CNS, cranial nerves, joint fluid, urine were tested by a Real-Time PCR specific for the *Trypanosoma* subgenus. Serum samples were tested by two different

Complement Fixation Tests (CFT) procedures and by immuno fluorescent antibody test (IFAT). Clinical signs observed were: weight loss, labial ptosis, swollen joint, urticarial plaque-like skin lesions, udder, scrotal and ventral edema, congestion of the genital mucosa, neuritis of facial and lingual nerves. The lymphatic organs showed secondary reactive hyperplasia. Blood, CSF, Mammary tissue and secretion, lymph nodes, lung, skin wheals; genital tissues, cranial nerves, intra-articular fluid and urine tested positive to RealTime PCR in at least one of the diseased animal tested. Live *T. equiperdum* was found in the mammary secretion of a naturally infected mare and subsequently isolated by adult rabbit inoculation. Diseased animals showed high CFT positive titers consistently. A total of 25,862 animals were tested by CFT and 140 were found positive. Nine of the latter (0.5%) were confirmed as dourine cases. In 2011, dourine re-occurred in Italy and seven outbreaks were observed. The Italian veterinary authority implemented a nationwide surveillance program, following the first confirmed cases. The performances of the serological methods available suggested that test results should be interpreted with care before defining an animal testing positive as a case. Observations and test results in diseased animals were conducive to the improvement of the reliability of the overall dourine diagnostic procedure.

The possibility of aneurism of the anterior mesenteric artery with parasitic infection as a site of *Salmonella* Abortusequi carriage

H. Niwa, S. Hobo, Y. Kinoshita, M. Muranaka, Y. Katayama, K. Oku, and K. Hariu
Epizootic Research Center, Equine Research Institute, Japan Racing Association, Shimotsuke, Tochigi 3290412

Equine paratyphoid is an infectious disease specific to animals in the horse family, which sets up contagious abortions and multiple abscesses caused by *Salmonella* Abortusequi. It is known that some horses infected with *S.* Abortusequi become long-term carriers even after their recovery, and it has been suggested that these horses pose the risk as new sources of infection. We report interesting results that suggest a novel site of *S.* Abortusequi carriage in horses, as a result of pathological and etiological investigation of horses in outbreaks of equine paratyphoid occurring in Japan in 2007-2008. The investigation targeted eight horses suspected as carriers of equine paratyphoid due to their high antibody level against *S.* Abortusequi in two farms where outbreaks had occurred (four horses from farm A and four from farm B). A tube agglutination test using horse serum was carried out, and bacterial isolations from samples including blood, rectal swab, and sternal bone marrow fluid of the horses were attempted as antemortem inspections. At the time of the autopsy, general pathohistological and immunohistchemical investigations were carried out. Isolations of *S.* Abortusequi were also carried out on samples including the principal organs, lymph nodes, thymus, and lesions observed at the time of the autopsy. In antemortem inspections, all of the horses had high levels of antibodies, but all appeared outwardly healthy. No *S.* Abortusequi isolates were obtained. Although no difference was found between farm A and farm B in antemortem inspections, the autopsy observations of the horses in the two farms were significantly different from each other. Namely, systemic enlargement of lymph nodes, heavy infection with several kinds of intestinal parasites, and aneurism of the anterior mesenteric artery with infection by many *Strongylus vulgaris* larvae were observed in all horses from farm A. A large quantity of suppurative thrombus had formed in the aneurism in the horses, and a large number of *S.* Abortusequi organisms were detected from the thrombus. Many leukocytes phagocytosing *S.* Abortusequi were detected in the thrombus by immunohistchemical investigation using anti-O4 *Salmonella* antibody (Figure 1). In contrast, infection by *Parescaris equorum* was only observed in some of the horses from farm B, but no clear lesions were observed, and no *S.* Abortusequi organisms were detected in the horses. It has been reported that *S.* Abortusequi were detected from sternal bone marrow in horses remains for the long time, and this region has been considered as a site of *S.* Abortusequi carriage. However, the results of this investigation strongly suggests the possibility that aneurism of the anterior mesenteric artery with a parasitic infection is an important long-term site of *S.* Abortusequi carriage.

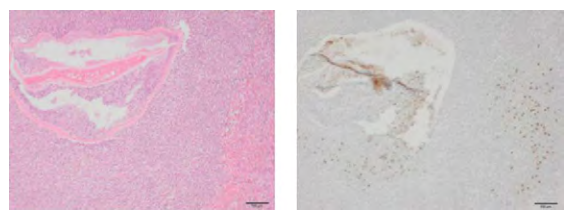

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Figure 1. Suppurative thrombus including *S. vulgaris* larvae stained with H&E (left) and immunohistchemical staining for O4-*Salmonella* somatic antigen (right).

Re-introduction of Equine Arteritis Virus into Argentina in 2010 and its consequences for the equine industry

C. Olguin Perglione [1], M.A. Vissani [1], M.S. Tordoya [1], F. Darqui [1], S. Miño [1], L. Becerra [1], P. Timoney [2], and M.E. Barrandeguy [1]
[1] Instituto de Virología, INTA Castelar, Buenos Aires, Argentina, [2] Gluck Equine Research Center, University of Kentucky, Lexington, Kentucky

Until 2010, the prevalence of Equine Arteritis Virus (EAV) infection in Argentina was low and restricted to the sport-horse breed, and the virus had not been involved in respiratory disease, abortion or foal deaths in the country. However, in mid-March 2010, an outbreak of abortion associated with EAV infection occurred on a Thoroughbred horse farm. Epidemiological and virological investigations revealed that the source of infection was virus-infective frozen semen imported from the Netherlands, which had been used to inseminate jumping mares, commingled with pregnant Thoroughbred mares on that premises. The semen had also been used to inseminate mares on four additional premises, from which the infection subsequently

spread via respiratory route through animal movements to seven other premises (identified after an extensive sero-surveillance investigation of epidemiologically linked facilities). The aim of the present study was to assess the long-term consequences of this series of disease events on the equine industry and the current EAV status of Argentina. A total of 38 stallions (belonging to the "Silla Argentina" breed) became seropositive during this occurrence. Twenty-nine were castrated without additional testing to determine whether any of them were EAV carriers and semen shedders. Semen samples for virological determination were obtained from the remaining nine stallions, and EAV was isolated from 4 of them. Sequencing and phylogenetic analysis of ORF5 of each isolate confirmed that they were identical to each other and to isolates from the cases of abortion and foal death, as well as virus isolated from the imported frozen semen. All clustered within the European subgroup 1 lineage of EAV. Three of the four EAV shedding stallions were castrated; one remains intact but is not allowed to be used for breeding because of legal restrictions imposed by the owner. A program of non-mandatory vaccination against equine viral arteritis (EVA) in stallions of different breeds was instituted; 317 stallions were vaccinated with a commercial modified-live virus vaccine (ARVAC®, Pfizer Animal Health) in 2010 and with an inactivated virus vaccine (Artervac®, Pfizer Animal Health) in 2011. All of the vaccinated stallions must be re-vaccinated every 6 months, or tested for EAV in semen before each breeding season because of their seropositive status. Seroepidemiological surveillance performed after the outbreak revealed 46 out of 2805 (August to December 2010), 320 out of 7618 (2011) and 29 out of 2087 (January to April 2012) samples were positive. Based on their respective histories, seropositive horses had either been previously vaccinated against EVA, or were animals involved in the series of outbreaks of EVA in 2010. No new seropositive horses were found since August 2010. The reintroduction of EAV in 2010 has had significant economic consequences for the Argentinean horse industry, including establishment of the carrier state in highly valuable stallions and the need for implementation of vaccination against the disease.

A PCR-based screening program to assess the prevalence of *Taylorella equigenitalis* in breeding stallions in South Africa

M.L. Schulman [1], C.E. May [1], C. Joone [1], M. Monyai [1], C. Gerstenberg [2], R. Naidoo [2], J. Pienaar [2], and A.J. Guthrie [1]
[1] Faculty of Veterinary Science, University of Pretoria, Onderstepoort, South Africa, [2] Directorate Animal Health, Department Agriculture, Forestry and Fisheries, Pretoria, Gauteng, South Africa

The first outbreak of Contagious Equine Metritis (CEM) due to *Taylorella equigenitalis* in South Africa was reported to the OIE in May 2011 subsequent to importation of a stallion, the index case. Two additional positive stallions were identified on an initial trace-back. The outbreak-response prompted determination of the national prevalence and distribution of CEM. A nation-wide PCR-based screening of all breeding stallions motivated by a previous outbreak report [1] was implemented *via* a mandatory CEM-negative clearance certificate prior to use for natural breeding or semen collection. Compliance from breeders was facilitated by developing a web-based system providing an easily-accessed, rapid and cost-effective sampling, testing and reporting process on www.cemsa.co.za. A submission form, information, a breed-indexed list of stallions achieving CEM-clearance and a method for obtaining and submitting two sets of swabs (with an interval $>$ 7d) from the external genitalia were accessible on the website. A duplex PCR was chosen as the assay method due to potential for submission of samples with minimal restrictions on transit time and temperature criteria and rapid, high throughput, cost-efficiency and reported sensitivity [1,2]. A clearance certificate was issued *via* the website after negative results from both sets of samples. Data recording stallion identity, breed, country of origin, location, sample dates and PCR results were analysed from samples received in the interval 15 August 2011-31 March 2012. 1718 Stallions representing 35 breeds from all nine provinces of South Africa were screened. The breed representation ranged from 1 to 493. The screening identified 33 suspected positive stallions subsequently confirmed positive on bacteriology. All were located in Gauteng except for one (Western Cape) and all linked to a single artificial breeding facility in Gauteng. Two, missed by the initial trace-back investigation, had been present at this facility before the index stallion, confirming the presence of CEM in the country prior to this outbreak's detection. The innovative web-based screening provided a representative survey of the CEM status of the national population, unique demographic data and established the presence of CEM prior to importation of the index case. PCR, demonstrated as sensitive, enhanced sample submission criteria, turn-around times, reporting and cost-efficiency.

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Changes in semen quality in stallions challenged with the Kentucky 84 (KY84) strain of equine arteritis virus

J.R. Campos [1], M.H.T. Troedsson [1], P. Breheny [2], R.R. Araujo [3], E.L. Squires [1], P.J. Timoney [1], and U.B.R. Balasuriya [1]
[1] Maxwell H. Gluck Equine Research Center, Department of Veterinary Science, [2] Department of Biostatistics, University of Kentucky, Lexington, Kentucky, USA, [3] Department of Basic Sciences, University of Sao Paulo, Brazil

Equine arteritis virus (EAV) is the causal agent of equine viral arteritis (EVA), a respiratory and reproductive disease of horses. Following EAV infection, virus persists

in the male reproductive tract of 30-70% of stallions despite the presence of high titer neutralizing antibodies in serum. The objective of this study was to evaluate semen quality of stallions experimentally infected with EAV. We hypothesized there would be a significant effect on sperm quality due to high fever and edema of the scrotum/prepuce observed during the acute phase of the infection. Seven stallions of mixed breed were intranasally inoculated with the KY84 strain of EAV. Stallions were monitored for clinical signs of EVA (fever, dependent edema including the scrotum/prepuce) until 42 days post infection (dpi). Semen was collected every other day for the first 15 days and then twice weekly up to 79 dpi. Following collection, the total number of spermatozoa in the gel-free ejaculate was calculated. Semen samples from each stallion were immediately evaluated by using a computer-assisted sperm analysis (CASA) system to determine the percent of total and progressively motile spermatozoa. An aliquot of each ejaculate was fixed in 10% buffered formalin and sperm morphology was assessed by differential interference contrast microscopy. Isolation of EAV from samples was attempted in RK-13 (KY) cells according to the OIE described protocol. All stallions developed fever (38.7 to 40.8 °C) from 1 to 9 dpi. Virus was isolated from the semen of all stallions from 3 dpi until at least 79 dpi. Virus titers in semen ranged from 1×10^1 to 1.88×10^7 pfu/ml. There were significant decreases in sperm motility, concentration and percentage of normal spermatozoa from 9 to 79 dpi. Loess curves for each horse were fit and integrated to quantify for fever, virus and edema over the 60 day sperm cycle prior to each ejaculation. Linear mixed models were then fit to isolate the effects of each factor on semen quality. These models demonstrated strong evidence ($p \leq 0.002$) that edema and fever exert independent effects on all the semen quality parameters, but virus seems to exert little to no direct effect, as virus concentrations remained high after semen quality returned to baseline by 79 dpi. These data support the hypothesis that reduced semen quality resulted from the combined effect of elevated body temperature and edema of the scrotum/prepuce but not as a direct consequence of the presence of the virus in the stallion reproductive tract.

Diagnostic markers for experimentally induced ascending placentitis in mares

I.F. Canisso [1], B.A. Ball [1], M.H.T. Troedsson [1], C. Klein [1], and S.D. Stanley [2]
[1] The Maxwell H. Gluck Equine Research Center, Department of Veterinary Science, University of Kentucky, Lexington, KY 40546, USA, [2] California Animal Health and Food Safety Laboratory; University of California; Davis, CA 95616, USA

Ascending placentitis is a common cause of late-term abortion and the most frequent cause of premature delivery in mares. In many cases, ascending placentitis may be very advanced before clinical signs such as premature mammary development or vulvar discharge become evident. Therefore, improved and earlier diagnostic methods might improve treatment outcomes in some of these cases. To date, transrectal ultrasonography is the most commonly used technique for diagnosis of equine placentitis. Unfortunately, this technique is prone to a significant number of false positive diagnoses, which may lead to unnecessary treatment of pregnant mares; while later stages of placentitis are easily diagnosed via ultrasonography, earlier stages and subclinical cases of placentitis can be missed. Additional diagnostic tools are therefore needed to accurately identify cases of placentitis before visible changes in placental integrity become evident upon ultrasonography. Therefore, the objectives of this study were to evaluate plasma concentrations of a variety of potential diagnostic markers in mares: i) with experimentally induced placentitis with and without fetal fluid sampling; and ii) carrying normal pregnancies with and without fetal sampling. Mares carrying normal pregnancies (260-280 days of gestation) were assigned to the following experimental groups: i) control mares with (n=2) or without fetal fluid sampling (n=2) and ii) treated - mares with induced ascending placentitis with (n=4) or without fetal fluid sampling (n=6). Placentitis was induced via intracervical inoculation of *Streptococcus equi spp. zooepidemicus* ($5x10^6$ cfu). Fetal fluid sampling was performed with ultrasound guided transabdominal puncture (0, 5, and 12 days after inoculation or until abortion). Blood samples were obtained: pre-inoculation (-7 to -10d), inoculation/initial fetal fluid sampling (d=0), and then daily for 10 days or until abortion. The concentrations of activin-A, and macrophage migration inhibiting factor (MIF) were determined by EIA and concentrations of steroids were determined by LC-MS-MS in plasma. The data were analyzed via ANOVA (JMP 9, SAS Institute Cary NC). Treated mares (9/10) aborted within 7 days after inoculation, and one control mare aborted subsequent to fetal fluid sampling. The treated mare which failed to abort and the control mare that aborted were excluded from the analysis of diagnostic markers. Activin-A and MIF did not differ between control and inoculated mares. Analysis of steroids by LC-MS-MS indicated that 17b-estradiol sulphate decreased significantly in treated compared to control mares subsequent to intracervical inoculation. In conclusion, these data indicate that neither activin-A nor MIF were useful markers of acute placentitis in mares whereas 17b-estradiol sulphate decreased precipitously in mares subsequent to experimentally induced ascending placentitis due to *S. equi spp. zooepidemicus*.

Acknowledgments

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Infectious abortion caused by *Salmonella enterica* subsp *enterica* serovar Abortusequi in Argentina

E.E. Di Gennaro, N. Guida, P.G. Franco, E.V. Moras, and A.J. Muñoz
Cátedra de Enfermedades Infecciosas. Facultad de Ciencias Veterinarias, Universidad de Buenos Aires. Argentina.
UBACyT 20020100100149

The equine host-adapted serovar, *Salmonella enterica* subsp *enterica* (*See*) serovar Abortusequi (*SeeA*) cause a disease called equine paratyphoid. The disease is manifested as abortion with other signs, including fistulous withers, septicemia, and arthritis. *SeeA* is transmitted by the ingestion of contaminated food with uterine secretions of aborted mares [1].
Different episodes of reproductive failure have recently been reported at several farms in the Province of Buenos Aires. Some of these episodes were studied at the Laboratory-School of Diagnosis of Infectious Diseases at the Veterinary University, and in two of these cases, the isolation of the *SeeA* bacterium was posible. The recovery of this bacterium gives an alarm signal; therefore, we should reconsider former agents responsible for reproductive diseases which now re-emerge due to the lack or discontinuation of preventive immunizations or due to critical points that we should not dismiss when considering the epidemiology of the agent. The objective of this study is to identify the cause of abortions that showed clinical, anatomic and pathological features of the bacterial abortions reported at different farms. Placentas from aborted mares and aborted fetuses were examined. For the isolation of bacteria, heart blood, liver, spleen, lung, intestine and stomach content were cultured in pre enrichment and selective enrichment medium. The enrichment medium was streaked onto XLD and MacConkey agar plates. Salmonella like isolates were identified by biochemical analysis using primary and secondary test as described Cowan and Steel, 2003. [2] The serotype was verified at the Bacteriology Departament, INEI - ANLIS "Dr. Carlos G. Malbrán. Dra. María Inés Caffer, Three placentas from aborted mares and three aborted fetuses were examined. Fibrinonecrotic exudates or congestion were observed at the placentas. Liver and lung congestions and intestine petechial hemorrhages were observed at the fetuses. *See* was isolated from two fetuses from heart blood, liver, spleen and lung. UBA 874 *Salmonella* Abortusequi (4,12:-:e,n,x) and UBA 879 *Salmonella* Abortusequi (4,12:-:e,n,x) were identified from the two spleens.
The strains were used for developing formalin-inactivated bacterins in Tymerosal and OHAl adjuvant. The *SeeA* is a hard-to-isolate bacterium so in this study it was isolated in two samples only. Both isolated samples were enough to conclude that we are facing a re-emerging disease. It is known that animals with positive serologic response as well as vaccinated animals cannot be reinfected by the Salmonella bacterium. The complete aborted animal, fetal organs, placenta or even fecal material are adequate samples for cultivation since isolation and identifications provide the definitive diagnosis.

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Genome-wide association study (GWAS) among four horse breeds identifies a common haplotype in equine chromosome 11 (ECA11) associated with the *in vitro* $CD3^+$ T cell susceptibility/resistance to equine arteritis virus infection

Y.Y. Go [1], E. Bailey [1], F. Cook [1], P. Timoney [1], S. Coleman [1], J. MacLeod [1], K.C. Chen [2], D. Horohov [1], and U.B.R. Balasuriya [1]
[1] Maxwell H. Gluck Equine Research Center, [2] Department of Molecular and Biomedical Pharmacology, University of Kentucky, Lexington, KY

In a recent study, we demonstrated that the virulent Bucyrus strain (VBS) of equine arteritis virus (EAV) could infect *in vitro* a small population of $CD3^+$ T cells from some but not all horses. Thus, we hypothesized that the differences in *in vitro* susceptibility of $CD3^+$ T lymphocytes to EAV infection may have a genetic basis. The objective of this study was to investigate the possible hereditary basis for this trait and to further investigate whether the differences in the *in vitro* susceptibility or resistance of $CD3^+$ T cells would correlate with the outcome and severity of clinical signs *in vivo*. Peripheral blood mononuclear cells from 310 horses from four different breeds [Thoroughbred (TB), American Saddlebred (ASB), Standardbred (STB) and Quarter Horses (QH)] were infected with EAV VBS and subsequently subjected to dual-color flow cytometric analysis to identify horses with the $CD3^+$ T lymphocyte-susceptible or -resistant phenotype to EAV infection. Subsequently, 267 DNA samples from four horse breeds (TB, n=94; ASB, n=60; STB, n=60; and QH, n=53) that had a susceptible or a resistant $CD3^+$ T lymphocyte phenotype were tested using both Illumina Equine SNP50 BeadChip and Sequenom's MassARRAY® system. To investigate whether the differences in the susceptibility or resistance of $CD3^+$ T cells *in vitro* correlate with the outcome and severity of clinical signs *in vivo* the horses with $CD3^+$ T cell susceptible (n=4) or resistant (n=4) phenotype were experimentally infected with the recombinant VBS of EAV. Of the 310 horses, 167 horses had the $CD3^+$ T lymphocyte-susceptible phenotype and 143 horses had the $CD3^+$ T lymphocyte-resistant phenotype. Interestingly, there was a clear difference in prevalence of the susceptibility/resistance phenotype among breeds. GWAS identified a common, genetically dominant haplotype (GGGGAGGT) associated with the susceptible phenotype in a region of equine chromosome 11 (ECA11): 49572804-49643932. Biological pathways analysis revealed cellular genes within this region of ECA11 that

encode proteins previously associated with virus attachment and entry, cytoskeletal organization and NF-κB pathways. Following experimental infection, the clinical signs and viremia burdens are more severe in horses with $CD3^+$ T lymphocytes that are resistant compared with those that are susceptible to EAV infection in an *in vitro* experimental system. Furthermore, there was a significant difference between the two groups of horses experimentally infected with EAV in terms of cytokine mRNA expression and evidence of increased clinical signs in horses possessing the *in vitro* $CD3^+$ T cell resistant phenotype. The presence of a common haplotype indicates that the *in vitro* $CD3^+$ T cell susceptible/resistant to EAV infection trait occurred in a common ancestor of all four breeds suggesting that it may be segregated among other modern horse breeds. Furthermore, this study provided direct evidence for a correlation between variation in host genotype and phenotypic differences in terms of the extent of viral replication, severity of disease and cytokine gene expression caused by infection with virulent EAV.

In vitro susceptibility of $CD3^+$ T lymphocytes to EAV infection reflects genetic predisposition of stallions at risk of becoming carriers

Y.Y. Go, E. Bailey, P.J. Timoney, K.M. Shuck, and U.B.R. Balasuriya
Maxwell H. Gluck Equine Research Center, Department of Veterinary Science, University of Kentucky, Lexington, KY

Equine arteritis virus (EAV) is the causal agent of equine viral arteritis (EVA) which is a respiratory and reproductive disease of horses and other equid species. Following EAV infection, a variable proportion of stallions (30-70%) can become persistently infected and continuously shed the virus in their semen. Carrier stallions are the natural reservoir of EAV and ensure that the virus is maintained in equine populations. Recent studies in our laboratory have shown that an *in vitro* assay based on dual-color flow cytometry analysis of $CD3^+$ T cells could be used to divide the horses into susceptible and resistant groups. In this study, we have extended these previous studies and established a possible correlation between susceptibility of $CD3^+$ T lymphocytes to EAV infection and establishment of persistent infection in stallions. Peripheral blood mononuclear cells (PBMCs) were collected from carrier (n=7) and non-carrier (n=7) stallions. The susceptible or resistant phenotype of each animal was defined by dual-color flow cytometric analysis of *in vitro* EAV infected $CD3^+$ T lymphocytes. Subsequently, genomic DNA (gDNA) was isolated from PBMCs of each animal and was genotyped for single nucleotide polymorphisms (SNPs) using the Sequenom's MassARRAY® system (Sequenom Inc., San Diego, CA). The $CD3^+$ T lymphocytes from all EAV carrier stallions were susceptible to *in vitro* EAV infection. The percentage of $CD3^+$ T lymphocytes susceptible to EAV infection ranged from 4% to 17% among carrier stallions. In contrast, none of the EAV non-carrier stallions had the $CD3^+$ T lymphocyte susceptible phenotype. The data suggested that carrier stallions that have the susceptible $CD3^+$ T lymphocyte phenotype to EAV infection represent those at higher risk of becoming persistently infected compared to stallions that do not possess this phenotype. Subsequently, the distribution of SNPs was compared between carrier and non-carrier stallions using the option for association studies. The SNP data showed that five out of seven carrier stallions with the $CD3^+$ T lymphocyte susceptible phenotype had the "GGAGGT" haplotype previously reported to be highly associated with the trait. In conclusion, the data indicated that stallions that have the susceptible $CD3^+$ T lymphocyte phenotype to EAV infection may represent those at higher risk of becoming persistently infected compared to stallions that do not possess this phenotype. The data from this study clearly demonstrated that there is a genetic difference between stallions that become carriers after exposure to EAV and those that are able to clear the virus, non-carrier stallions. The trait could be determined by assessing the susceptibility of their $CD3^+$ T lymphocytes to *in vitro* EAV infection which is likely to be the most precise method. However, it is expensive, time consuming and cumbersome to run on a routine or large-scale basis. This study provides the possibility of development of a genetic test to identify stallions that are predisposed to persistent infection.

Molecular epidemiology of Equine Infectious Anaemia Virus in France from 2007-2009

A. Hans [1], D. Gaudaire [1], E. Morilland [1], C. Laugier [1], S. Zientara [2], and C. Leroux [3]
[1] Anses, Laboratory for equine diseases, 14430, Goustranville, France, [2] Anses, Laboratory for Animal Health, UMR 1161 ANSES/INRA/ENVA, 94703 Maisons-Alfort, France, [3] UMR754 INRA UCBL University Lyon 1, Lyon, France

Equine infectious anaemia virus (EIAV) is the causative agent of equine infectious anaemia (EIA). It belongs to the *Retroviridae* family, genus *Lentivirus*. Clinical signs associated with the infection are mainly characterized by fever, anaemia, oedema and various signs of depression. Infected horses will never eliminate the virus and thus remain a reservoir for the virus even in absence of clinical symptoms. Viral transmission from one animal to another occurs mainly through blood, via insect bites and iatrogenic mode. Insects, mainly Stomoxys, serve as mechanical vectors (virus does not replicate in the insect) keeping the infectious virus in their mouthparts for few hours after biting. This mode of transmission promotes viral spread in horse population mainly during horse show. In 2007, 2008 and 2009, several EIA premises were reported in south east of France in two counties (Ardèche and Var). The aims of this study was to undertake the molecular characterization of viruses responsible of those EIA premises and compare them to strains described in the litterature. A total of 21 spleens coming from seropositive animals, tested by AGID test, were obtained (3 in 2007, 2 in 2008 and 16 in 2009). Total from DNA from 1 g of tissue was extracted. Those samples were tested by semi-nested

PCR assay. Phylogenetic analysis were performed by amplification and sequencing of the *Gag* gene (1 400 bp). In 2007 and 2008 only one premise has been declared in the Ardèche county with 4 equids infected (2 donkeys and 2 ponies), those equids did not exhibit any clinical sign. The 2009 episode in the Var county was far more important. Indeed, more than 500 horses were placed under surveillance including virus detection and restriction of movement. Ultimately, 16 seropositive mainly asymptomatic horses, with only one animal declaring clinical EIA, were slaughtered. Three samples 2007, one from 2008 and 12 samples from 2009 gave a positive signal by amplification by semi nested-PCR. Phylogenetic reconstructions based on the 1400 bp gag sequences from 2007-2009 French cases and others from GenBank grouped the viruses in different clusters consisting of virus originated from North America, China and Europe. All EIA sequences characterized from 2007 to 2009 were not related to any of the group described previously. France is not free of EIA but the cases diagnosed are isolated and are mainly asymptomatic horses. This study showed for the first time that different isolates of EIA viruses are circulating in France. Moreover, those isolates have never been described previously in the literature.

Comparison of two treatment methods for the elimination of Contagious Equine Metritis in stallions

B. Keys, M.L. Schulman, K. May, C. Joone, M. Monyai, D. Mpofu, H.J. Marais, and A.J. Guthrie
University of Pretoria, Onderstepoort, Gauteng, South Africa

An outbreak of contagious equine metritis (CEM), the aetiological agent being *Taylorella equigenitalis,* was confirmed in a group of stallions in Gauteng, South Africa during 2011. *T. equigenitalis* is sensitive to a wide range of antimicrobial drugs *in vitro* (Timoney 1996). Treatment regimes have been used with an antibiotic choice based on sensitivity, along with cleaning of the genitalia and application of a topical antimicrobial agent (Sellon & Long 2007). According to USDA (United States Agricultural Department) treatment protocol, nitrofuracin is the topical antimicrobial agent currently recommended. The objective of this study was to compare the efficacy of two topical antimicrobial agents- 0.2% nitrofurazone (Furex, Aspen Pharmacare, Woodmead, South Africa) and 1% silver sulphadiazine (Silbecor, Biotech Laboratories, Midrand, RSA), and to determine the treatment duration required in stallions. Twenty three out of 32 Lipizzaner stallions tested positive for *T. equigenitalis* on both bacterial culture and qPCR on swabs taken from the external genitalia. All stallions were removed from their usual place of residence and placed in an isolation facility under strict quarantine measures. The centre was kept free of horses for three weeks and all stables and equipment were thoroughly cleaned and disinfected. The 23 positive CEM stallions were randomly assigned to either of the two topical antimicrobial ointment groups: 0.2% nitrofurazone (Group 1: n= 12) or 1% silver sulphadiazine (Group2: n=11). Stallions were sedated to allow effective extrusion of the penis. The treatment protocol consisted of topical treatment of the external genitalia without inclusion of systemic antimicrobials. The first two consecutive days consisted of topical cleansing of the penis and prepuce with a surfactant solution- 5 % docusate sodium (Docusol, Kyron Laboratories, Benrose, RSA) once daily to remove all smegma and external debris. Thereafter, treatment consisted of topical cleansing of the external genitalia with a 5% docusate sodium and irrigation with a disinfectant solution-4% chlorhexidine gluconate (Dismed Bioscrub, Dismed Pharma, Halfway House, RSA) before drying of the area and application of either one of the two topical antimicrobial ointments for a minimum of seven days or until testing negative on qPCR swabs taken daily. Positive stallions remained at the isolation facility until confirmation of negative status in individual's $\geq$21 days post-treatment by swabs submitted for bacterial culture and qPCR, whereupon they were allowed to return to the centre. Sellon, D.C., Long, M.T., 2007, *Equine Infectious Diseases*, Saunders Elsevier, St. Louis, Missouri. Timoney, P.J., 1996, 'Contagious Equine Metritis', *Comm Immun Microbiology of Infectious diseases*, 19 (3) 199-204.

Chimeric viruses containing the N-terminal ectodomains of GP5 and M proteins of porcine reproductive and respiratory syndrome virus do not change the cellular tropism of equine arteritis virus

Zhengchun Lu [1], Jianqiang Zhang [1], Chengjin M. Huang [2], Yun Young Go [1], Kay S. Faaberg [3], Raymond R.R. Rowland [4], Peter J. Timoney [1], and Udeni B.R. Balasuriya [1]
[1] Maxwell H. Gluck Equine Research Center, Department of Veterinary Science, University of Kentucky, Lexington, KY 40546, [2] Fort Dodge Animal Health, Overland Park, KS 66210, [3] Virus and Prion Research Unit, National Animal Disease Center, USDA, Agricultural Research Service, Ames, IA 50010, [4] Department of Diagnostic Medicine and Pathobiology, College of Veterinary Medicine, Kansas State University, Manhattan, KS 66506

Equine arteritis virus (EAV) and porcine reproductive and respiratory syndrome virus (PRRSV) are members of the family *Arteriviridae*; they share many biological properties but differ significantly in cellular tropism. EAV can replicate in a variety of primary cells while PRRSV can only replicate in a very limited number of cell types. The glycosylated GP5 and the unglycosylated M protein (encoded by ORF5 and ORF6, respectively) are the major envelope proteins of EAV and PRRSV; they form a disulfide-linked heterodimer in the mature virus particles. Formation of the GP5/M heterodimer is essential for the expression of neutralization epitopes of both viruses. The GP5/M heterodimer is also presumed to play important roles in attachment to the host cell receptor and in cell entry. In this study, we used an infectious cDNA clone of EAV and reverse genetic techniques to characterize the role of the major envelope proteins (GP5 and M) in the cellular tropism of EAV. Using an infectious cDNA clone of the

modified live virus vaccine strain of EAV, we engineered a panel of five chimeric viruses by exchanging the N-terminal ectodomains and/or full-lengths of the two major envelope proteins (GP5 and M) from PRRSV. The growth characteristics and plaque morphology of EAV/PRRSV chimeric viruses were studied using equine endothelial cells (EECs). The cellular tropism of EAV/PRRSV chimeric viruses were tested in different EAV-susceptible cell lines (EEC, RK-13, BHK-21, and MARC-145), as well as in PRRSV-susceptible cells (porcine alveolar macrophages [PAM] and MARC-145) to confirm the cellular tropism and infectivity of the progeny virus. Virus replication in various cell lines was determined by IFA staining with MAb 3E2 (α-N of EAV) and polyclonal pig anti-PRRSV sera. Of the five chimeric viruses, the recombinant viruses expressing the N-terminal ectodomain of PRRSV GP5 alone or M alone or together (GP5ecto, Mecto and GP5&Mecto, respectively) in the EAV backbone were viable and genetically stable. Compared to the parental virus, these three chimeric viruses produced lower titers and smaller plaque sizes indicating that they have a compromised phenotype. The three chimeric viruses could only infect EAV-susceptible cell lines but not PRRSV-susceptible cells. The two major envelope proteins may not be determining factors in the cellular tropism of EAV and other arteriviruses. These findings further support the recent findings that the minor envelope proteins GP2, GP3, GP4 and E are the critical proteins in mediating cellular tropism.

Confirmation of the first outbreak of contagious equine metritis in South Africa

C.E.. May [1], M.L. Schulman [1], C. Gerstenberg [2], A. Grobler [3], A. Mphele [2], and A.J. Guthrie [1]
[1] University of Pretoria, South Africa, [2] Directorate Animal Health, Pretoria, South Africa, [3] Veterinary Services, Department: Agriculture and Rural Development, Gauteng, South Africa

Historically, South Africa was considered to be free of Contagious Equine Metritis (CEM), caused by the bacterium *Taylorella equigenitalis*. The disease was first identified in the country in April 2011 in Gauteng Province and confirmed and reported to the OIE on the 9th May 2011. The index case, an imported Warmblood stallion, had been transferred to an Artificial Insemination (AI) Centre in Gauteng following his release from post-arrival quarantine in South Africa. The outbreak involved the index stallion and 9 mares that had been AI-ed with his semen. Initially, semen collected from the stallion succeeded in obtaining embryos for transfer in 3 "in contact" mares, but subsequent AI attempts in 4 mares resulted in mucopurulent endometritis post-AI. An additional "test" mare was then inseminated, with resultant endometritis, and swabs obtained from the genitalia of this mare and the stallion were submitted for culture. The resultant isolates were suspicious for *T. equigenitalis*. Samples were sent to a World Organisation for Animal Health (OIE) CEM reference laboratory, the Animal Health and Veterinary Laboratories Agency (AHVLA) in Bury St Edmunds, Suffolk and qPCR confirmed *T.equigenitalis*. All animals at the facility were state quarantined and a trace–back implemented. All horses on the index property were classified as high-risk or low-risk for further testing. All stallions collected on the same phantom on the same day as the index case were classified "high risk" and stallions collected on separate days were classified "low risk". A complete epidemiological investigation, including trace-back and trace-forward, was performed on all "high risk" stallions and potential direct contact animals. The index case and 9 in-contact mares were quarantined and treated for CEM until they tested negative on qPCR and culture. Vertical transmission was only confirmed between the index case and the test mare. Testing of the "high risk" horses yielded one additional positive stallion, and subsequently, an additional stallion resident at his home property also tested positive. The suspected horizontal transmission to the "high risk" stallion appears to have been via contact with a contaminated breeding phantom at the AI facility, and to the additional stallion via fomite transmission from him at their home property. Based on these data it was impossible to determine if the index case was the source of the outbreak, or if he had been infected by a horse already resident in South Africa. A nationwide qPCR screening of all breeding stallions was implemented. This outbreak highlighted the importance of fomite transmission which appears to be an underestimated feature of this infection. The widespread recognition of this particular organism as posing a primarily venereally-transmitted threat underplays the risk of its transmission by fomites which is apparently common when infected animals are inadvertently used in artificial breeding systems.

Equine coïtal exanthema in France: evidence of EHV-3 infection by real time PCR

S. Pronost [1,2], S. Brau [1,3], L. Legrand [1,2], and J.L. Cadoré [3]
[1] Frank Duncombe Laboratory, Animal Health Department, 14053 Caen, France, [2] University of Caen Lower Normandy EA 4655 U2RM 14000 CAEN France, [3] UMR 754 INRA-ENVL-UCBL Rétrovirus et pathologie comparée IFR 128, Université Lyon 11 avenue Bourgelat – 69280 MARCY L'ETOILE, France

Equine coital exanthema (ECE) is an acute viral infection of horses caused by equid herpesvirus 3 (EHV-3). The negative impact of ECE on equine industry concerning the forced disruption of mating activities was recently reported in particular in Argentina. ECE is highly contagious and animals are infected via direct cutaneous contact during the act of coitus although there is evidence supporting the possibility of non-coital spreading. Lesions are commonly found on the vulva and perineum of the mare and on the penis, prepuce and testicles of the stallion. A presumptive diagnosis of ECE may be performed on the basis of typical lesions. Confirmation can be made on the basis of paired serology tested for EHV-3 neutralising antibody or with direct

virus detection by cell culture or PCR. While most of the recent studies were described in Argentina, the need to improve the presence of such virus in other countries still exists. In France, between March 2002 and March 2012, thirteen swabs were collected from the genital area of 4 stallions and 9 mares for EHV-3 investigation after an ECE suspicion. DNA was extracted using the QIAamp DNA mini kit. We used consensual herpes PCR method associated with sequencing from 2002 to 2005 and had developed a qPCR method (using SybrGreen) for samples tested from 2006 to 2012. The real-time PCR was tested against EHV-1, EHV-4, EHV-2, EHV-5 and AHV-5 and a range of pathogens often isolated from the equine reproductive tract. These included Equine viral arteritis virus, *Taylorella equigenitalis*, *Klebsiella pneumoniae* and *Streptococcus zooepidemicus*. The analytical sensitivity of the real-time PCR was compared to $TCID_{50}$. The qPCR was validated for specificity and presented a sensitivity of 3.2 $TCID_{50}$/50μl. Ten cases were confirmed by PCR analysis between March 2002 and March 2012 in France out of thirteen examinations. Six mares and four stallions were declared positive after confirmation of the presence of EHV-3 DNA by classical PCR (3 cases) and real-time PCR (7 cases). Different horse breeds were concerned by the disease (Comtois, Welsh Pony, Shetland pony, French Saddle horse, Thoroughbred) and were all between 3 and 27 years old. All cases are from sporadic infections that were observed in different French departments (35, 71, 72, 69, and 54) except that occurred in May 2005 which involved seven horses; even if only one stallion and one mare were confirmed positive by PCR. During this episode there is evidence of contamination of mare by a stallion without noticeable lesions. Our study presents a new efficient real-time PCR test which could be used for confirmation of ECE diagnosis and demonstrate that the virus circulate in France.

Bacterial superinfection with venereal pathogens subsequent to topical therapy for *Taylorella equigenitalis* in stallions

M.L. Schulman, B. Keys, C.E. May, and A.J. Guthrie
Faculty of Veterinary Science, University of Pretoria, Onderstepoort, South Africa

Venereal pathogens including *Klebsiella pneumoniae* and *Pseudomonas aeruginosa* may establish asymptomatic carrier status and associated subfertility after colonisation of the external genitalia of stallions due to a disturbance of normal commensal microflora subsequent to antimicrobial and disinfectant therapy [1]. An outbreak of *Taylorella equigenitalis* among the resident stallions at the Lipizzaner Centre in South Africa was managed by a currently-accepted treatment protocol of topical antibacterials and disinfectants [2, 3]. A predisposition to infection has been reported following similar therapy for *T. equigenitalis* [4]. A controlled study aimed to observe the effects of treatment on the bacterial microflora of the external genitalia of the *T. equigenitalis*-infected stallions. The protocol consisted of topical treatment of the penis and prepuce of positive stallions with surfactant (5% docusate sodium solution), disinfectant (4% chlorhexidine gluconate solution) and either one of two topical antimicrobials (0.2% nitrofurazone or 1% silver sulphadiazine). Twenty one positive stallions randomly allocated to either of two treatment groups, Group 1 (nitrofurazone, n= 11) or Group 2 (silver sulphadiazine, n= 10), were treated daily for > 9d until swabbing negative for *T. equigenitalis* on daily qPCR, confirmed by bacteriology after 3 weeks. The mean interval (and range) for successful treatment overall was 15.1 d (9-28 d). Nine negative stallions quarantined without treatment served as controls. Four weeks after the last treatment, three sites (distal urethra, urethral fossa including sinus and *lamina interna*) were swabbed in all 30 stallions for bacteriology. The effects of treatment and antibacterial choice on bacterial cultures and predilection sites for bacterial pathogens were compared using standard statistical methods. Statistical significance in all cases was set at $P < 0.05$. There was a significant effect on the colonisation by bacterial pathogens following either treatment with a 54.5% and 80.0% occurrence in the stallions of Group 1 and 2 respectively compared with 11.1% in the Controls. The culture sites showed a similar distribution between the two organisms and with an occurrence of 36.0% (urethral fossa and sinus), 22.0% (*lamina*) and 11.1% (urethra). Current treatment protocols for *T. equigenitalis* significantly increase the probability for subsequent colonisation of the external genitalia by potential bacterial pathogens.

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Effect of acyclovir, ganciclovir and cidofovir on equid herpesvirus 3 *in vitro*

A. Vissani [1], S. Tordoya [1], L. Becerra [1], E. Thiry [2], and M. Barrandeguy [1]
[1] Instituto de Virología, CICVyA, INTA-Castelar, CC25, 1712 Castelar, Buenos Aires, Argentina, [2] Veterinary Virology and Animal Viral Diseases, Department of Infectious and Parasitic Diseases, Faculty of Veterinary Medicine, University of Liege, B-4000 Liege, Belgium

Equine Coital Exanthema (ECE), which is caused by equid herpesvirus 3 (EHV-3), is a venereal, highly contagious disease, characterized by the formation of papules, vesicles, pustules and ulcers on the external genitalia of mares and stallions. EHV-3 infection is endemic in horses

worldwide. Episodes of EHV-3 reactivation and re-excretion in latently infected horses also serve as a source of virus. Although ECE has no direct effect on fertility or pregnancy, its economic impact on the equine industry resides in the need of temporarily withdrawing the stallions and mares from the reproductive activity. Prevention is based on segregating affected mares from reproduction. However, regarding that there are latently infected animals, in which the virus is reactivated and generally re-excreted subclinically, and that this phenomenon is not predictable, the prevention approach previously described does not eliminate the risk of contagion. Although it has not been fully explored, field observations and scarce experimental data are promising about the use of acyclovir and ganciclovir as topical medication on ECE lesion. The aim of this study was to compare the activity of selected antivirals compounds to inhibit or reduce the replication of EHV-3 *in vitro*. Monolayers of EDerm cells were infected with 500 μl of the virus, containing up to 30 pfu/well, as it was previously determined by a plaque titration method. Overlay medium with carboxymethyl cellulose 0.75%, supplemented with the different concentrations of each antiviral compound (acyclovir, ganciclovir and cidofovir), were added. Cells infected with the virus without antiviral treatment were used as a control. After incubation, cells were fixed and stained with 0.1% formalin-buffered crystal violet solution. The parameters to assess the efficacy of the antiviral compounds were plaque number and plaque size, expressed as percentage of inhibition. The concentration of each compound required to inhibit plaque number and plaque size by 50% (IC50%), was obtained from the dose-response (percentage of inhibition) curves generated from the data. The three compounds tested were able to reduce plaque number and plaque size induced by EHV-3 infection in EDerm cells. Ganciclovir proved most potent to reduce the number (IC50%: 0.04-1 μg/ml) and the size (IC50%: 0-0.1 μg/ml) of plaques of EHV-3 *in vitro*. Cidofovir was intermediately potent to reduce plaque number and plaque size with an IC50% of 1-10 μg/ml and IC50% of 0.4-4 μg/ml respectively. Lastly, acyclovir was the least effective compound to reduce plaque number (IC50%: 8-20 μg/ml) and plaque size (IC50%: 4-10 μg/ml). As preliminary conclusions, ganciclovir displays the best overall inhibitory activity on EHV-3 *in vitro*. Further *in vitro* researchs are ongoing and *in vivo* experiments are also scheduled.

Working Horse

Efficacy of alpha-cypermethrin treated mesh against African horse sickness virus vectors

P.C. Page [1], K. Labuschagne [2], G.J. Venter [2], J.P. Schoeman [1], and A.J. Guthrie [1]
[1] Faculty of Veterinary Science, University of Pretoria, Onderstepoort, South Africa, [2] Agricultural Research Council - Onderstepoort Veterinary Institute, Onderstepoort, South Africa

Culicoides biting midges have been implicated as vectors of African horse sickness virus. Recommended control measures for African horse sickness include vaccination, stabling at night, screening of stables with mesh and the use of repellents or insecticides. The efficacy of repellent impregnated mesh applied to light traps against *Culicoides* as well as the insecticidal effect of hair from horses treated with cypermethrin has been reported. The objectives of this study were to determine the field efficacy of alpha-cypermethrin treated high density polyethylene (HDPE) mesh applied to light traps against *Culicoides* and the insecticidal efficacy of the mesh against *Culicoides imicola*. Two randomized, controlled, observer-blinded experiments were conducted. In Experiment 1 the efficacy of HDPE mesh (Alnet, South Africa) treated with alpha-cypermethrin (Fendona®6, BASF, Switzerland), untreated HDPE mesh, and control polyester mesh (standard Onderstepoort light trap mesh, ARC-OVI, South Africa) was compared. The meshes were applied to three Onderstepoort UV light traps operated for 12 nights in a Latin square design. The total number of *Culicoides* and *C. imicola* collected per night was analysed. In Experiment 2 the *in vitro* insecticidal efficacy of meshes against field collected, nulliparous female *C. imicola* was assessed. Midges were exposed in petri dishes to alpha-cypermethrin treated HDPE mesh for a period of 1 or 3 min, or untreated control HDPE mesh for a period of 3 min. Mortalities in the three groups were assessed at 5, 10, 30, 60 min and 24 h post exposure. Thirty replicates of each exposure period were conducted. Statistical analyses were done using SigmaPlot 12.0 (Systat Software Inc, USA). The mean (± SE) number of *C. imicola* collected with the control light trap (2,156 ± 743) was significantly ($P < .05$) higher than the untreated HDPE mesh (523 ± 214) and the alpha-cypermethrin treated HDPE mesh (303 ± 99). *Culicoides imicola* mortality at the 5, 10, 30, 60 min and 24 h time points in the 1 min and 3 min alpha-cypermethrin treated HDPE mesh exposure groups was 63.1, 87.7, 94.9, 99.5, 100 and 78.1, 90.2, 99.0, 99.6 and 99.6%, respectively. *Culicoides imicola* mortality was significantly ($P < .001$) higher in the two groups exposed to the alpha-cypermethrin treated HDPE mesh compared to the untreated control HDPE mesh at all time points. Mortality was only significantly higher ($P = .017$) in the 3 min alpha-cypermethrin exposure group compared to the 1 min exposure group at the 5 min time point. Alpha-cypermethrin treated HDPE mesh applied to stables or jet stalls could be used to effectively protect horses against *Culicoides*. Further studies on the field efficacy of alpha-cypermethrin treated HDPE mesh applied to stalls housing horses as well as the effect of the mesh on stall ventilation are required.

Macrophages and lymphocytes support the development of *Theileria equi* pre-erythrocytic stages, but neither B nor T lymphocytes are required to establish infection *In vivo*

J.D. Ramsay [1], M.W. Ueti [2], W.C. Johnson [2], G.A. Scoles [2], D.P. Knowles [2], and R.H. Mealey [1]
[1] Washington State University, Pullman, WA, [2] Animal Disease Research Unit, USDA-ARS, Pullman, WA

Theileria equi is a tick-transmitted apicomplexan hemoprotozoan parasite that causes acute hemolytic disease (equine piroplasmosis) and persistent infection of wild and domestic equids worldwide. The life cycle of *Theileria equi* is biphasic in the horse, with a period of intraleukocyte development (pre-erythrocytic phase) followed by patent erythrocytic parasitemia. The specific phenotype of schizont-infected leukocytes is not defined for *T. equi* and represents a significant gap in our understanding of its pathogenesis. In closely related apicomplexan parasites of cattle (*Theileria annulata* and *T. parva*) the phenotype of infected leukocytes is well described and known to impact pathogenesis, adaptive immunity, and strain virulence. To resolve this gap in *T. equi* pathogenesis, PBMC were isolated from three immunocompetent horses and co-cultured with *T. equi* sporozoites. On day 9-14 post-inoculation (DPI) the immunophenotype of infected leukocytes was assayed by flow cytometry and IFA using a panel of three mAb for cell surface labeling [anti-IgM, anti-CD3, or anti-CD172a] and a second mAb to detect infection [anti-equine merozoite antigen (EMA) 1/2]. Previous work has shown anti-EMA 1/2 mAb specifically labels the pre-erythrocytic stages of *T. equi*. To determine if B and T-lymphocytes are required to establish infection *in vivo*, two SCID foals were infected with *T. equi* sporozoites by intravenous inoculation and monitored for parasitemia by real time PCR. Flow cytometric analysis of *in vitro* infected PBMC showed a subset of cells in each culture were dual labeled with a leukocyte-specific mAb and anti-EMA 1/2 mAb. Further analysis demonstrated that a relatively high proportion of the B-lymphocytes and macrophages were infected (37% and 30%, respectively), as compared to the low proportion of T-lymphocytes (9%), suggesting that *T. equi* preferentially infects B-lymphocytes and macrophages. IFA performed on the same cultures verified that the dual labeled cells were infected with schizonts. IFA also demonstrated that signal generated by leukocyte-specific mAb was unevenly distributed and less abundant on infected cells. Infection of SCID foals with *T. equi* sporozoites resulted in patent merozoite parasitemia 11 and 13 DPI, and demonstrated that B and T-lymphocytes are not required to establish infection *in vivo*. The results of *in vitro* experiments demonstrate that the leukocyte tropism of *T. equi* is not limited to lymphocytes, as previously reported, but extends to included monocytes/macrophages. The establishment of infection in foals lacking B and T-lymphocytes (SCID foals) with sporozoites provides strong *in vivo* support of this finding. *T. annulata* has a similar host cell range, suggesting that the immune responses that control pre-erythrocytic *T. annulata* (i.e. cytostatic macrophages and cytotoxic T-cells) may also be important for controlling *T. equi*. This information will therefore help focus the search for host immune responses that can stop *T. equi* infection from progressing to the persistent erythrocytic stage.

Trypanosoma brucei central nervous system infection in working equidae in West Africa: an emerging disease

D.G.M. Sutton [1], L.J. Morrison [2], P.J. Pollock [1], C. Hahn [3], P.E. Johnston [1], S. Sharpe [1], J. Rodgers [2], and M. Murray [2]
[1] School of Veterinary Medicine, College of Medical, Veterinary and Life Sciences, University of Glasgow, 464 Bearsden Road, Glasgow G61 1QH, [2] Wellcome Centre for Molecular Parasitology, Glasgow Biomedical Research Centre, University of Glasgow, 464 Bearsden Road, Glasgow G61 1QH, [3] Royal (Dick) School of Veterinary Studies, University of Edinburgh, Easter Bush, Midlothian EH25 9RG

Working equidae are essential to agriculture and socio-economic development in The Gambia, in a region where trypanosomiasis is hyperendemic. Seroconversion to *Trypanosoma brucei* exceeded 68% in a population of working equidae in this country in 2010 (43/72 donkeys; 24/29 horses), higher than estimated in 2001 and 2006. Coincident with increases in *T.brucei* prevalence has been the emergence of a fatal neurological syndrome among equidae in the same geographical area. Identification of the causal agent of this devastating syndrome and its clinicopathological features was the aim of this prospective study. Sixteen equidae with characteristic neurological abnormalities were evaluated in the Central River District of The Gambia in 2010. Buffy coat microscopy was used for field trypanosome identification. Indirect fluorescent antibody testing (IFAT) for *T.brucei*/*T.evansi*/*T.equiperdum*, and WNV serology was performed subsequently from frozen serum, and trypanosome oligonucleotide PCR performed from FTA card samples. PCV, TSP and white cell differential counts were recorded. Lumbosacral CSF was collected in 8 individuals and subjected to similar evaluation. In 5 individuals that deteriorated despite treatment, *post mortem* examination was performed with histopathological evaluation of brain, spinal cord and selected tissues. Neuropathological evaluation was performed in 8 further specimens for which historical and/or video footage was available. Immunohistochemical evaluation of 10 brain sections was performed using specific antibody to *T. evansi*, incorporating both negative (equine, mouse) and positive (mouse brain infected with *T.brucei)* controls. Donkeys and horses were equally represented, with equal sex distribution, and age range (6 months - 12 years). Duration of neurological signs ranged from 2 days to months, with recrudescence in some individuals, and history of previous trypanocidal treatment. Abnormalities included marked hindlimb ataxia, coupled with forelimb proprioceptive deficits, and variable forebrain signs, such as reduced mentation, central blindness, and unilateral cranial nerve deficits. Wasting and lethargy were present. PCV was not below expected range, and peripheral trypanosomes were seen in only 1 individual, after intensive screening. Dourine IFAT was positive in 13/16 blood samples, and 3/8 CSF specimens, while CSF PCR

for *T.brucei* was positive in 3/6 cases. All CSF specimens were negative for WNV by nested RT-PCR. Severe CNS histopathological changes included a lymphoplasmacytic meningoencephalitis, with perivascular infiltrates primarily in the white matter. Pathology was diffuse, affecting cord tissue also. IHC using monoclonal *T.evansi* antibody was positive in all specimens, and negative in all control specimens. Central nervous system trypanosomiasis was confirmed in working equidae in The Gambia, and is increasing for reasons unknown. Equine neurological trypanosomiasis is usually progressive and fatal, and is of great welfare and economic importance in this region. Further work is required to determine whether *T. brucei brucei*, *T. b. evansi* or *T. b. equiperdum* is the causative agent, which will require novel PCR techniques. This is of fundamental importance in developing a control strategy for the syndrome, as *T. b. brucei* has the tsetse fly as vector, while *T. b. equiperdum* is dependent on sexual transmission. Treatment of the neurological syndrome with available trypanocidal agents is not successful.

Glanders in the Middle East

U. Wernery, and R. Wernery
Central Veterinary Research Laboratory, P.O. Box 597, Dubai, UAE

Glanders is caused by *Burkholderia (B.) mallei*, a Gram-negative, non-motile, non-encapsulated non-sporforming bacteria which is evolutionary related to *B. pseudomallei*, the agent of melioidosis. Many different animal species including humans are susceptible to glanders to varying degrees. Infections are usually fatal. The disease is endemic in many countries of the Middle East but also has re-emerged in other countries due to a tremendous increase of equine trade over the last decades. In the Middle East, which consists of 16 countries, more than half of them are affected by the disease. In many of them the disease emerged only recently due to a lack of veterinary services including quarantine facilities, lack of awareness among equine owners, lack of transparency and poor control measurements. Glanders was detected for the first time in the UAE in 2004, occurring in an official UAE quarantine facility in Dubai. In 2009 the disease was detected in Kuwait, followed by Bahrain in 2010 and Lebanon in 2011. The Central Veterinary Research Laboratory (CVRL) in Dubai diagnosed these outbreaks through serological testing. Research that followed the outbreaks of glanders resulted in the designation of CVRL by the OIE as an OIE reference laboratory for glanders in 2008. From a scientific point of view, disease events over the past several years and the experimental infection of feral donkeys with glanders have significantly increased our knowledge and concern about glanders and the risk of a more global distribution of the disease. This presentation explains the emergence and re-emergence of glanders in the Middle East and presents a collection of digital images of the different forms of glanders in different animal species. At the end of the presentation, the various diagnostic tools and their interpretations are also explained.

The *ema* family of *Theileria equi*: a proposed source for antigenic variation

L.N. Wise, and D.P. Knowles
Washington State University & USDA ARS, Pullman WA 99163

Theileria equi is a tick-borne globally distributed apicomplexan parasite of equids. Infection results in hemolytic anemia and associated systemic illness. Resolution of infection invariably results in life-long persistence and transmission risk. Global equine commerce is curtailed due to restricted movement of infected horses. The U.S. equine population was considered free of *T. equi* infection and disease as of 1988. However, re-emergence of *T. equi* in the U.S. coupled with identification of novel tick vectors has revealed limitations of current infection and disease control strategies. Life-long persistence of *T. equi* in the absence of clinical disease is a critical component of pathogenesis due to the continual risk of transmission. The mechanisms by which *T. equi* persists remain unclear due to voids in knowledge concerning pathogenesis, ability to undergo antigenic variation and host immune responses. DNA from *T. equi* USDA (Florida) strain was used to construct a small and large insert plasmid library as well as a bacterial artificial chromosome library. The libraries were end sequenced to generate 130,858 sequence reads that were assembled with the Celera assembler. Annotation was facilitated by development of an EST library for gene model prediction. The genome sequence has been deposited in GenBank under accession number ACOU0000000. Completion of the *T. equi* genome identified 10 *ema* genes (8 full-length, 1 truncated and 1 with insertions). These *ema* genes encode for merozoite surface antigens, termed equi merozoite antigens (EMAs). Infected horses consistently respond with high antibody titers to these proteins, specifically to EMA1 and 2. The antibody response to these EMA proteins is correlated with protection yet is unable to eliminate infection. With the unexpected discovery that genes encoding EMA1 and 2 belong to the multi-gene family, additional analysis was performed. Pairwise comparison of predicted *ema* gene products revealed shared amino acid identities of the full-length members range from 33 to 59% and the predicted peptide length of each range from 273-286 amino acids. In non-clonal culture of *T. equi* merozoites, all 10 *ema* genes were found to be transcribed. Analysis of the annotated *T. equi* genome failed to detect a gene family with characteristics consistent with an ability to contribute to antigenic variation. However, while the *ema* gene family products are structurally very similar to each other, their collective variability suggests a potential role in antigenic variation. Therefore a hypothesis concerning differential expression of EMA proteins at the merozoite surface was developed. Support for this hypothesis includes recent data that indicates the antigenic profile of *T. equi* as recognized by the infected horses' immune system may be different between individual horses. The inconsistent results obtained when these antigens were compared to those predicted by the genome, brings to question what EMA proteins are being

expressed during the course of infection and how this differential expression may play a role in antigenic variation and immune evasion.

Pathogenesis of glanders in experimentally infected feral donkeys using different infection routes and doses

D. Altemann [1,2], R. Bauerfeind [2], and U. Wernery [1]
[1] Central Veterinary Research Laboratory, PO Box 597, Dubai, United Arab Emirates, [2] Justus-Liebig-Universität, Institut für Hygiene und Infektionskrankheiten der Tiere, Frankfurter Strasse 85-89, 35392 Giessen, Germany

A glanders infection trial was carried out in ten feral donkeys which were caught in the northern mountains of the United Arab Emirates (UAE). The donkeys which were of different gender and age were infected with the *Burkholderia (B.) mallei* strain Dubai 7 which was isolated from a glanderous horse in 2004. One donkey was kept as a control. The strain was stored at -80°C in PBS[1] as a first passage. The donkeys were housed in the quarantine facility of the Ministry of Environment and Water and the entire experiment which lasted 7 months was conducted according to the Welfare Federal Law no. 16/ 2007 of the UAE under the supervision of a Welfare Committee consisting of four veterinarians. The results of this experiment are summarized in Table 1 and were as follows:

- donkeys were slightly susceptible to the challenge strain because very high infectious doses were necessary to induce disease
- the clinical signs and pathological lesions observed were the same as known from equine glanders with fever, decreased appetite, farcy, severe rhinitis and granulomatous lesions in the nose and lungs
- at the onset of the disease, which occurred in some cases as early as 3 days p.i.[2], blood values of fibrinogen, WBC[3], neutrophils as well as rectal temperature were increased and remained elevated until death
- seroconversion was observed as early as 7 days p.i.[2] with positive cELISA[4] first, followed by complement fixation test. The OIE acknowledged CFT[5] could only be performed with satisfaction when sera were diluted 1:2 with veronal buffer and heat inactivated at 63°C for 30 min
- in all infected donkeys, the intradermopalpebral and intradermal mallein test at the neck were positive, whereas the negative control remained negative.

Development of a species specific PCR for the differentiation of *Trypanosoma equiperdum* and *Trypanosoma evansi:* application to the 2011 outbreak of dourine in Italy

J. Cauchard [1], A. Madeline [1], F. Duquesne [1], S. Petry [1], I. Pascucci [2], and P. Büscher [3]
[1] Laboratory for Equine Diseases, Anses, Goustranville, Dozulé, France, [2] Istituto Zooprofilattico Sperimentale dell'Abruzzo e del Molise G. Caporale, Teramo, Italy, [3] Department of Parasitology, Institute of Tropical Medicine Antwerp, Antwerpen, Belgium

Trypanosoma equiperdum and Trypanosoma evansi are respectively the causal organisms of dourine and surra. The clinical signs of dourine and chronic surra are very similar and prohibit correct differential diagnosis. Depending on the diagnosis established, the OIE terrestrial code imposes a stamping-out policy for dourine free countries whereas a treatment for horses infected by surra is possible. The goal of this study is, by analyzing different *Trypanosoma equiperdum* and *Trypanosoma evansi* "supposed" strains, to provide molecular tools for

Table 1
Summarized results of glanders infection trial in feral donkeys using different routes and doses

ID no.		8	7	11	3	4	1	2	6	5	12
Infection dose cfu/ ml[6]		2,0 x 10^2	1,0 x 10^8	1,7 x 10^8	1,0 x 10^8	1,0 x 10^8	1,0 x 10^9	1,0 x 10^9	4,0 x 10^8	3,0 x 10^8	control
Infection route		s.c.[7]	nasal	nasal	oral tube	oral tube	oral tube	oral tube	feed	water	
First clinical signs at day p.i.[2]		4	4	3	15	3	3	8	7	5	
Euthanized on day p.i.[2]		16	8	10	23	12	16	16	27	17	
Temperature (°C) Mean with SD[8]	before infection	37,6 ± 0,0	37,4 ± 0,4	36,8 ± 0,9	37,2 ± 0,6	37,1 ± 0,5	37 ± 0,6	37,1 ± 0,6	37,1 ± 0,5	37 ± 0,4	37,5 ± 0,8
	after infection	39,6 ± 0,8	39,3 ± 1	39,2 ± 0,4	39,2 ± 0,3	38,6 ± 0,9	38,8 ± 0,4	38,6 ± 0,6	38,9 ± 0,8	38,7 ± 0,4	
Fibrinogen (mg/ dl) Mean with SD[8]	before infection	213 ± 50	226 ± 44	208 ± 32	216 ± 48	231 ± 56	222 ± 54	189 ± 38	197 ± 46	234 ± 68	214 ± 54
	after infection	626 ± 105	848 ± 0,0	572 ± 148	633 ± 170	638 ± 130	637 ± 118	535 ± 125	358 ± 88	526 ± 184	
WBC[3] (10^9/ L) Mean with SD[8]	before infection	13 ± 2	10 ± 4	8 ± 1	8 ± 2	9 ± 2	9 ± 2	14 ± 2	10 ± 1	8 ± 1	8 ± 2
	after infection	15 ± 5	11 ± 5	17 ± 6	15 ± 5	11 ± 5	10 ± 2	12 ± 2	20 ± 13	11 ± 3	
Neutrophils (%) Mean with SD[8]	before infection	70 ± 12	59 ± 12	64 ± 7	49 ± 8	49 ± 7	42 ± 12	29 ± 8	50 ± 8	49 ± 5	55 ± 11
	after infection	75 ± 13	78 ± 7	85 ± 5	71 ± 10	68 ± 14	65 ± 7	44 ± 11	65 ± 11	63 ± 9	
Seroconversion (day p.i.[2])	CFT[5]	12	neg.	neg.	9	7	9	7	8	9	neg.
	cELISA[4]	10	8	7	9	7	9	7	8	7	neg.

Abbreviations: [1]PBS: Phosphate buffered saline, [2]p.i.: Post infection, [3]WBC: White blood cells, [4]cELISA: Competitive enzyme- linked immunosorbent assay, [5]CFT: Complement fixation test, [6]cfu/ ml: Colony forming units per milliliter, [7]s.c.: subcutaneous, [8]SD: Standard deviation

distinguishing the two species. Thirteen random primers were used to amplify fragments of genomic DNA extracted from seven *Trypanosoma equiperdum* strains, including two recent isolates, nine *Trypanosoma evansi* strains and one *Trypanosoma evansi* Type B strain. After successful repetition of the RAPD method, sixty three reproducible fragments with sizes between 0,2 and 0,9 Kb were selected. Profile analysis showed that three putative *Trypanosoma equiperdum* strains were grouped with the *Trypanosoma evansi* strains. These results are adding more information on the problem of the classification of *Trypanosoma equiperdum*. The fragments of interest have been used to determine primer candidates for the identification and discrimination of the two species using polymerase chain reaction. In the context of the 2011 dourine outbreak in Italy, PCR candidate has been used to confirm the species of the trypanosomes associated to the clinical manifestations of the diseases.

Variation in *Theileria equi* drug susceptibility *in vitro* and the potential role of ABC transporters as mediators of parasitic drug resistance

S.A. Hines, L.S. Kappmeyer, K.L. Mealey, S.R. Leib, W.C. Johnson, M.W. Ueti, D.P. Knowles, and R.H. Mealey
Washington State University, College of Veterinary Medicine, Pullman, WA 99163

Theileria equi is a tick-borne apicomplexan parasite and an agent of equine piroplasmosis (EP). Although the United States was declared free of EP in 1988, multiple recent U.S. outbreaks emphasize the ineffectiveness of current control programs and the critical need for effective chemotherapeutic strategies for parasite clearance in infected horses. Evolving drug resistance is commonly observed in many apicomplexans, most notably the related human malarial agent *Plasmodium falciparum*, in which genetic variations of certain members of the ATP-binding cassette (ABC) family of transporter proteins have been identified as contributors to resistance. In *T. equi*, the recently annotated genome has revealed 45 ABC transporter family members, more than any other hemoparasite sequenced to date. The purpose of the present research was to determine variation in drug susceptibility between *T. equi* isolates, including those from clinical cases in which treatment failed to clear the parasite. Furthermore, genetic and functional evaluation of specific ABC transporters in these isolates is underway to assess their potential effects on drug susceptibility. The initial stages of this research involved optimization of *T. equi* culture conditions and development of an *in vitro* susceptibility assay for imidocarb dipropionate, the anti-protozoal drug used most commonly worldwide to treat equine piroplasmosis. An isolate from a recent *T. equi* outbreak in Texas was successfully adapted into culture and, along with the USDA laboratory Florida strain, evaluated for *in vitro* susceptibility to imidocarb. An initial concentration of 0.5 – 1 percent parasitized erythrocytes (PPE) was achieved for each isolate at the start of the assay, and cultures were maintained in a 96-well plate at 37° C in a 5% CO_2 atmosphere. Imidocarb was diluted in culture media to concentrations ranging from 0.00015 to 0.33 mg/mL and applied at the time the cultures were inoculated, with triplicate samples for each concentration. Media was changed at 24 hours, and parasite growth was assessed at 48 hours using hydroethidine staining with flow cytometry analysis for enumeration of parasitized red blood cells. This revealed an inhibitory concentration (IC_{50}) of 0.075 mg/mL for the Florida strain, and 0.004 mg/mL for the isolate from Texas, indicating an appreciable difference in susceptibility even between these two isolates which have previously been clinically susceptible to treatment with this drug. Evaluation of other isolates and drugs using this cultures system, as well as the ongoing characterization of the role of specific ABC transporters in this variation of drug susceptibility, will allow development of alternative treatment strategies and potential testing of clinical isolates for genetic markers of drug resistance.

How does the ecology of *Culicoides* biting midges influence the risk of an epidemic of African Horse Sickness?

G. Lo Iacono [1], C. Robin [2], S. Gubbins [3], R. Newton [2], and J. Wood [1]
[1] University of Cambridge, Cambridge, UK, [2] Animal Health Trust, Newmarket, UK, [3] Institute for Animal Health, Pirbright, UK

A more profound knowledge of the ecology of *Culicoides* biting midges is of fundamental importance for risk assessment and efficacy of control strategies for African Horse Sickness (AHS). In the light of current knowledge, the influence of non-susceptible host (*e.g.* ruminants) on the risk of an epidemic is rather ambiguous because of two possible, contrasting effects: i) dilution effect occurring when non-susceptible host, compared to equides, exerts a larger attractiveness to Culicoides species; ii) strengthening effect arising when the local abundance of *Culicoides* increases with the availability of the host. We developed a mathematical model to assess the risk of an outbreak of AHS by calculating the basic reproductive number R_0 [1] under two hypothetical scenarios on: i) *Culicoides* abundance and its relationship with host densities ii) *Culicoides* preference for host species. The theoretical framework was then applied to: a) identify the conditions ensuring that the presence of non-susceptible vertebrate hosts results in a reduction of R_0 b) estimate the number of non-susceptible-hosts to horse ratio required to reduce R_0 below one (under a dilution effect) and thus to control the disease. Figure 1 shows the ratio of R_0 calculated in presence of both hosts (susceptible and non-susceptible) relative to that in presence of horses only, as function of the vector feeding preference and non-susceptible-host to horse ratio. When this relative value is less than one then the influence of non-susceptible-hosts is advantageous. Figure 2 shows the proportion of non-susceptible-host relative to the number of

horses leading to $R_0 < 1$ as function of R_0 in presence of horses only and the vector feeding preference. The abundance of *Culicoides* is assumed to linearly increase with the local availability of hosts. By identifying clear hypotheses on the ecology of *Culicoides,* the modelling framework removes some of the ambiguities on the role of alternative hosts kept in proximity to equine hosts in increasing or decreasing the risk of epidemic of AHS. This is particularly important considered the paucity of relevant experiments on vector abundance and feeding preference.

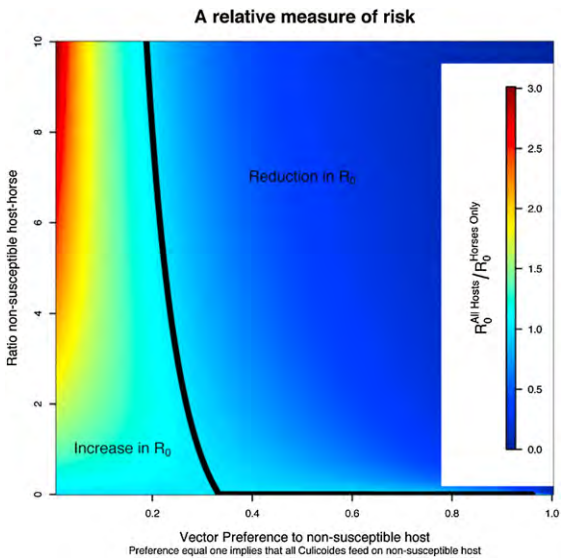

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Figure 1. Ratio of R_0 in presence of all hosts relative to that in presence of horses only, as function of the vector feeding preference and non-susceptible host to horse ratio.

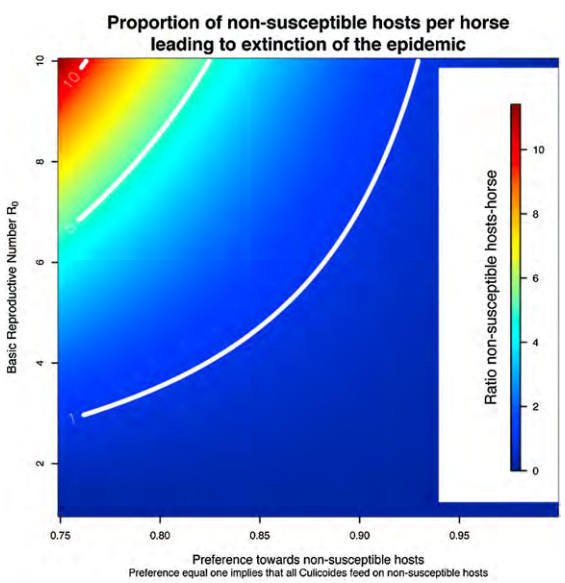

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Figure 2. Proportion of non-susceptible host relative to the number of horses leading to $R_0 < 1$.

Reference

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Persistence of inflammatory skin syndrome in polo and pleasure horses in northern Nigeria

W.P. Mshelia [1], P.H. Mamman [2], I.I. Onoja [1], and E.I. Fadimu [2]
[1] Department of Veterinary Medicine, Faculty of Veterinary Medicine, Ahmadu Bello University, Zaria, Nigeria, [2] Department of Veterinary Microbiology, Faculty of Veterinary Medicine, Ahmadu Bello University, Zaria, Nigeria

The continuing prevalence of inflammatory skin syndrome in Polo and Pleasure horses in northern Nigeria has been a constant risk to the health and welfare of horses. Corynebacterium *spp* are gram-positive pleomorphic rods that are associated with two closely related inflammatory skin syndromes in horses: ulcerative lymphangitis and abscesses. In this outbreak 40 clinically infected horses within an age range of 6 to 15-years of age in 3 states were sampled between May 2008 and June 2011. The animals showed various clinical signs which include multiple abscesses, fever, depression, lameness. While single large abscesses measuring about 10 cm in diameter was observed in the pectoral region of two horses out of the 40 sampled. All the horses sampled showed chains of nodules with or without ulceration on the pectoral region. There was no lesion found in the hock except for one horse that showed generalized nodules and ulcers. No age, sex or breed predisposition is known to exist but in this study the Nigerian, West African dongola, Sudan country-bred and locally crossed-ponies are mostly affected. While among the exotic it's commonly seen in polo horses of South African origin than the Argentine polo ponies. A complete history was taken for each of the horses encountered. The abscesses where sampled aseptically. Bacterial cultures were followed by biochemical tests and confirmed the presence of *Corynebacteruim spp, Staphylococcus spp and streptococcus spp* . Fungal spp. isolated include *Aspergillus fumigatus, Aspergillus niger,Trichophyton spp.,Mucor spp.* From the biochemical test, all the Corynebacteria isolates were catalase and urease positive but nitrate negative. The antimicrobial susceptibility test showed that staphylococcus is susceptible to ciprofloxacin and amoxillin, while gentamycin, zinnacef, streptomycin and septrin are resistant. Staphylococcus *spp.* showed resistance to ampiclox, rocephin and erythromycin. Streptococcus *spp* were susceptible to amoxillin. Ciprofloxacin, streptomycin and erythromycin was intermediate. Streptococcus *spp*. was resistant to ampiclox, rocephin and septrin. Corynebacterium *spp.* was susceptible to ciprofloxacin but resistant to ampiclox. Considering availability and the fact that no fluoroquinolones are approved for use in horses, penicillin G was the drug of choice. Lesions were surgically drained and cauterized and 50,000 UI/kg of penicillin G was administered

intramuscularly every 12 hours for a minimum of 30 days. Phenylbutazone at 4.4 mg/kg repeated after 24 hours and continued at 2.2 mg/kg 12 hourly for 12 days intramuscularly. Hydrotherapy and exercise was carried out at regular intervals. The persistence of this skin infection is an indication that it circles on an endemic bases in the horse population in northern Nigeria.

Electrocardiography, echocardiography and cardiac troponin I evaluation in horses suffering from natural AHS infection

M.G. Sanz, U. Kafka, M.N. Saulez, P. Page, N. Villarino, and A. Carstens
Department of Companion Animal Clinical Studies, Faculty of Veterinary Science, University of Pretoria, South Africa

African horse sickness (AHS) is a severe, non-contagious, insect-borne disease that causes high mortality in horses. Little information is available on the effects of AHS on clinical parameters. The objective of this study was to evaluate the cardiac system to aid on treatment and prognosis of AHS. Horses hospitalized at the Onderstepoort Veterinary Academic Hospital with confirmed AHS were included in the study after owner's consent. Physical examination, base-apex ECG, echocardiography and serum cardiac troponin I (cTnI) were evaluated at different times until horses were either discharged or euthanized. A total of 11 horses were included, 6 survived and 5 were euthanized. Mean (range) heart rate at arrival was 59 (42-76) beats per minute. Tachycardia was present in 81% of the horses at arrival, 5/6 horses at discharge and in 4/5 horses at the time of euthanasia. cTnI was elevated at arrival in 2/6 survivors and 4/5 of the euthanized horses; however it increased above normal values (>0.03 ng/ml) in 10/11 horses as the disease progressed. Electrocardiography and echocardiography were normal in all horses at all times. This is the first report of cardiac evaluation in horses with AHS. Tachycardia and myocardial damage were common findings even at discharge. Serial cTnI measurements may be useful to evaluate disease progression and prognosis. cTnI needs to be evaluated before return to exercise is considered. Drugs that may affect the cardiac system function should be avoided. Abnormalities were not detected by ECG or echocardiography even when performed frequently.

EIA

Dynamics of equine infectious anemia virus (EIAV) infection in naturally infected mules

G.L. Autorino [1], C.J. Issel [2], R.F. Cook [2], G. Manna [1], A. Cersini [1], F. Rosone [1], R. Frontoso [1], A. Caprioli [1], and M.T. Scicluna [1]
[1] Istituto Zooprofilattico Lazio e Toscana, Roma,
[2] Department of Veterinary Science, Gluck Equine Research Center, Lexington (KY)

During the Italian surveillance program for EIA, mules were discovered to have a relatively high rate (10%) of seroprevalence. To determine if inapparent carrier status in this hybrid species is immune mediated, a group of 10 seropositive mules all without overt clinical signs, were treated with dexamethasone. In addition, plasma-associated EIAV RNA concentrations were determined prior and post immune suppression to investigate if mules established constant viral load set-points and if these varied between individuals. Ten seropositive inapparent carrier mules (2 to 30 years), obtained from 5 independent outbreaks, were kept under observation for a four-month period and monitored for febrile responses and decreases in platelet counts. Dexamethasone was administered for 9 days starting at 2 months. Serum and plasma samples were collected daily during dexamethasone treatment and at regular intervals pre- and post-immunosuppression. Humoral responses were investigated by C-ELISA, agar gel immunodiffusion (AGID) test and immunoblotting (IB). Plasma-associated viral-RNA loads were determined with a TaqMan® based RT-PCR, directed against exon 1 of *tat*, using a quantified internal standard. The EIAV strains infecting the mules were similar to ones previously identified in Europe, with six possessing nucleotide sequence identity to EIAVRom-4 (GU060662.1), two to EIAVIta-1(EU240733.1) and two to EIAVIta-90 (HQ888862.1). Regardless of strain, some mules did not establish constant viral-load set-points but, instead, showed considerable temporal variation with amounts of viral RNA differing by 30,000-fold in one subject. However, variation was less (95-fold) in some animals while, in others, viral RNA was undetectable prior to immune suppression suggesting very significant differences between individuals in their ability to control EIAV replication. All mules had increases in viral RNA loads, following treatment with dexamethasone, and five exhibited clinical signs consistent with EIA. Although all animals were reactive in IB, humoral responses as measured by AGID and C-ELISA were variable, with two mules remaining AGID test-negative (one of which was also C-ELISA negative) throughout the period. At least three subjects had anamnestic responses post-immunosuppression, as defined by a four-fold change in C-ELISA endpoint titer. There are significant individual differences between mules in their ability to control EIAV. Therefore, the infected mule population does not comprise a uniform reduced risk for EIAV transmission. In addition, maintenance of viral loads and, in at least 50% of mules, the absence of clinical signs is dependent on active immune responses as evidenced by dexamethasone treatment. Furthermore, there was no correlation between age, sex or virus strain in terms of clinical signs, viral RNA loads or humoral responses. For example, of the two subjects consistently negative in AGID, one had viral RNA detectable in just one sample post-immunosuppression whereas in the other it was measurable at all time-points (average value $4.9x10^3$ copies/ml).

Challenges and proposed solutions for more accurate serological diagnosis of equine infectious anemia

C.J. Issel [1], M.T. Scicluna [2], S.J. Cook [1], R.F. Cook [1], A. Caprioli [2], I. Ricci [2], F. Rosone [2], J.K. Craigo [3], R.C. Montelaro [3], and G.L. Autorino [2]
[1] Department of Veterinary Science, Gluck Equine Research Center, University of Kentucky, Lexington, KY, [2] Istituto Zooprofilattico Sperimentale delle Regioni Lazio e Toscana, Rome, Italy, [3] Center for Vaccine Research, University of Pittsburgh, Pittsburgh, PA

The serologic diagnosis of persistent infections with the equine lentivirus, equine infectious anemia virus (EIAV), is possible because equids produce antibodies against the major EIAV proteins when exposed to the virus. Since 1972, control programs for EIA based on serology have depended on the agar gel immunodiffusion (AGID) test which tests for antibodies against the major core protein (p26) of EIAV and, since the mid-1980s, a variety of enzyme linked immunosorbent assay (ELISA) test kits which also detect antibodies to the p26 antigen. This study documents the presence of EIAV genetic sequences in a number of persistently-infected horses and mules whose serums were interpreted as negative or equivocal on AGID tests but positive on ELISA tests and in immunoblot tests. The immunoblot test included antigens from a cell-adapted Wyoming strain of EIAV and has thus far proven effective in detecting antibodies against the 3 major proteins of EIAV (the surface unit gp90, the transmembrane gp45 and the major core p26) in samples from the across the US, Italy, and South American and Asian nations. Strategies designed to take advantage of the combined strengths of the ELISA and AGID tests were shown to be effective in limited studies in the US and are shown here to be effective in a national surveillance program for EIA in Italy. In the survey, 17% (25/149) of the equids considered to be infected with EIAV on combined comparative serologic data had reactions in the AGID test that were interpreted as negative or equivocal. These data document the benefits of using the three tiered laboratory system for the diagnosis of EIA, originally promulgated by the Committee on Infectious Diseases of Horses of the US Animal Health Association. The three tiers include ELISA-first testing, followed by confirmation of positives by AGID testing and, when needed, further testing by immunoblot for recognition of multiple EIAV antigens. Although the ELISA-first strategy introduces some confusing results, the discovery of up to 20% more cases of EIA makes it compelling. In our opinion, it is better and more defensible to find two samples in a thousand with resolvable but falsely-positive ELISA tests for EIA than to release two to three horses in ten thousand with falsely-negative test results for EIA (the rates seen in the Italian surveillance presented here). The data also illuminate the challenges for accurate diagnosis of EIA infections based on detection of virus/viral genetic sequences (RNA or DNA) or antibody detection alone.

EIAV: News on the vaccine

C. Leroux
UMR754 INRA University Lyon 1, Retrovirus and Comparative Pathology, Lyon 1 University, Lyon, France

EIAV (Equine Infectious Anemia Virus) is a lentivirus, member of the retrovirus family and is related to HIV (Human Immunodeficiency Virus). EIAV infects horses and donkeys worldwide and is responsible for a persistent infection leading to a recurring disease classically characterized by clinical episodes associating fevers and thrombopenia. The recurring episodes are clearly associated with the emergence of new viral populations generated by mutation of the viral genomes able to temporary escape the host immune response. As with other retroviruses in humans and animals, the individuals remain infected for life and there is no cure available. Over the past three decades, efforts have been made to develop an efficient protective immune response by prophylactic vaccines able to protect horses against infection by homologous as well as heterologous EIAV strains. This review will focus on the "Chinese" and "American" strategies using either viral vaccine strains attenuated by successive passages in donkeys and donkey cells or genetically attenuated vaccines by suppression of viral genes. Their efficiencies go from exacerbation of clinical signs in vaccinated animals to protection against disease. Protection from heterologous strains remains a challenge. These developments are of importance in the context of animal health as well as models for HIV vaccines or against other retroviral infections.

Stability differences of envelope-specific T cells responses between newly EIAV infected and inaparent carrier horses

C. Liu [1], S.J. Cook [1], J.K. Craigo [2], R.F. Cook [1], C.J. Issel [1], R.C. Montelaro [2], and D.W. Horohov [1]
[1] Maxwell Gluck Equine Research Center, University of Kentucky, Lexington, KY, [2] Department of Molecular Genetics and Biochemistry, University of Pittsburgh, Pittsburgh, Pennsylvania

Unlike other lentiviruses, EIAV replication can be eventually controlled in most infected horses leading to an inapparent carrier state free of overt clinical signs. Maintenance of this carrier state is absolutely dependent on active immune responses as evidenced by the fact that immunosuppressive drugs can induce the recurrence of disease. However, the immune mechanisms that are responsible for this control of infection are not yet identified. As the resolution of the initial infection is correlated with the appearance of the virus specific CTL, it appears that cellular immune responses play an important role. However, most studies into this protective mechanism have been limited to the identification of specific epitopes, usually at a single time point in the infection. Few studies have examined the cellular immune responses to the viral antigens throughout

the infection period. Since the virus undergoes rapid mutation following infection, the adaptive immune response must also evolve to meet this challenge. In order to fully understand the evolution of the T cell response during infection, we synthesized forty-four peptides, spanning the entire surface unit protein (gp90) of EIAV, and monitored the peptide-specific T cells responses both *in vivo* and *in vitro* over a six month period following challenge. One inapparent carrier (D64) and four recent EIAV infected ponies were included in this study. Peripheral blood mononuclear cells (PBMC) were isolated and stimulated separately with all 44 peptides. The EIAV gp90 epitopes-specific immune responses *in vitro* were determined by ELISPOT-IFNg assay. In parallel, all peptides were injected intradermally and punch biopsies were collected for real-time PCR to monitor the cellular immune responses *in vivo*. As shown by both *in vivo* and *in vitro* assays, two of the four recently infected ponies recognized gp90 peptides three weeks post challenge. Similar to the CMI response to HIV infection, peptide-specific T cell recognition patterns changed over time. While some peptides were recognized throughout the sampling period, other peptides were only recognized at the later time points. Also, the response to some specific peptides disappeared after 6 months post infection. By contrast, peptide recognition by the inapparent carrier (D64) was more stable. The mechanisms responsible for this change remain unclear, but this dynamic shift in the immunodominant epitopes hierarchy in the newly infected horses may be the result of mutations in specific epitopes leading to an escape from T cell recognition. In the inapparent carrier, persistent recognition focusing on the more conserved peptides results in a more effective T cell response where virus replication is tightly controlled. These results indicate that T cell responses evolve during the early stage of EIAV infection. This interaction between virus mutation and T cell evolution needs to be considered when designing vaccines.

What feedback after five years from the implementation of the Italian National Surveillance Programme (NSP) for Equine Infectious Anemia (EIA)

M. Sala [1], G. Ferri [2], M.T. Scicluna [1], P. Scaramozzino [1], I. Ricci [1], R. Nardini [1], L. Ruocco [2], M. Paglialunga [2], R. Forletta [1], and G.L. Autorino [1]
[1] Istituto Zooprofilattico Lazio e Toscana, Via Appia Nuova 1411, 00178, Rome, Italy, [2] Italian Ministry of Health, Viale Giorgio Ribotta 5, 00144, Rome, Italy

Introduction: since 2007, the Italian NSP for EIA imposes the annual sero-testing of the equine population, older than 6 months. Aims of the NSP are to: i. identify EIA outbreaks; ii. control infection spread through adoption of biosecurity measures; iii. evaluate EIA spatial and temporal trends; iv. identify infection clusters; v. provide evidence for further interventions/regulations. The objective of this study was to evaluate the trends of EIA prevalence in Italy since its adoption and estimate the risk for EIA infection for each equid species. Materials and Methods: the NSP data were analysed to estimate annual EIA seroprevalence by Region and equid species. Seroprevalence trends were evaluated using the Chi-Squared test for linear trends in proportion, (LC 95%; $p < 0.05$). Odds Ratios were calculated to estimate the risk for EIA infection for each equid species. Case definition: equid testing ELISA or AGID-positive, confirmed in AGID or Immunoblotting. Outbreak definition: holding with at least one confirmed case. Results and Discussion: on average, about 228,000 equids were tested annually for EIA; 10,500 donkeys, 2,350 mules and 215,500 horses. Positivity in donkeys was ocasionally detected while, a significant seroprevalence decrease was registered in horses: from 0.21% to 0.07%. Although the seroprevalence in mules was significantly higher than in horses, the decreasing trend was throughout the period, passing from 10.3% to 1.8%. However, from the data, the mule resulted to be about 50 times more at risk of resulting EIA positive compared to the horse (e.g. 2010: OR=51.4; IC95% 41- 64.4). Outbreaks dropped from 235 to 96 and in 2011, 80% were found to be incident and/or originating from previously not tested equids. Conclusions: clusters of infection were identified in Central Italy were EIA is endemic in draught animals, making them at a higher risk of infection while, EIA is sporadic in Northern Italy and Sardinia. In Southern Italy, while EIA is also considered as sporadic, a large population of equids has yet to be enrolled in the NSP. In general, the sport and racing horses are free from infection. The low incidence occurring in different Regions justifies the reduced frequency of testing introduced in the NSP in 2011. The control measures included in the surveillance programme were effective in lowering the seroprevalence and also the number of outbreaks especially in Central Italy. Nevertheless, focus must be put on the equine population living in rural conditions as well as horses not yet enrolled in the NSP, since they still might represent a risk for EIA spread. An additional risk, not to be underestimated, is the low sensitivity of the still recommended test, which is the agar gel immunodiffusion.

Genomic and Immunologic analysis of the Chinese attenuated EIAV vaccine

J.H. Zhou, Y.Z. Lin, X.F. Wang, J. Ma, C. Du, and R.X. Shen
Harbin Veterinary Research Institute, Chinese Academy of Agricultural Sciences, Harbin 150001, China

The development of lentiviral vaccines is extremely difficult due to the broad genomic variation and fast in vivo evolution of the viruses. An attenuated vaccine against equine infectious anemia virus (EIAV) was developed by Shen et al in China in 1970's by serially passing a pathogenic wild-type strain in donkeys for 110 generations followed by 121 in vitro passages in donkey monocyte-derived macrophages. Laboratory experiments and country-wide vaccination confirmed that this attenuated EIAV vaccine effectively protected vaccinates from infection of pathogenic strains. To understand the mechanism that the EIAV vaccine effectively protects the infection of pathogenic strains, the genomic and immunologic characteristics of this vaccine were analyzed. Sequencing data of viral genomes revealed that the attenuating process of the

vaccine significantly extended the diversity of viral quasispecies and resulted in multiple consensus mutations in all viral genes with different frequencies. In addition, the vaccine strain showed an in vivo evolution pattern similar to pathogenic strains and developed several pathogenic strain-specific sites in the putative envelope protein sequence. Further studies revealed that the vaccine induced significantly different humoral and cell-mediated immune responses, primarily the increased neutralizing and conformation-dependent antibodies, elevated CD4+ T cell proliferation and INF-γ-secreting CD8+ T cells from that induced by its parental pathogenic strain. Importantly, the cell-mediated immunity appeared to be responsible for the sterilizing immunity to EIAV. Furthermore, the enhanced ability to elicit the expression of multiple cytokines and chemokines, as well as to induce apoptosis of the target cells, is considered one of the major characteristics of the vaccine that determines the strength of immune responses. Finally, experimental data indicated that consensus mutations at multiple sites of multiple genes generated during attenuating process of the vaccine enabled the strain keeping avirulent in vivo, even under the treatment of immune suppression. Based on the above results, we proposed the Dynamic and Broad Immune Induction hypothesis to underline the mechanism of the EIAV vaccine. The hypothesis regards the antigenic variation at the primary vaccinating point and the in vivo evolution similar to that of the pathogenic strains as key points of the attenuated EIAV vaccine to induce protective immunity, which blocks the immune evasion of pathogenic strains. Further studies will be performed to test the contributions of vaccine diversity and divergence to the induced immune protection, which will provide references for developing the new generation of EIAV vaccines.

An outbreak of equine infectious anemia at a horse riding center in Argentina underscores the limitations of serological testing

D. Duclós [1], M.A. Vissani [2], I. Alvarez [2], C Olguín Perglione [2], S.J. Cook [3], F. Cook [3], and M.E. Barrandeguy [2]
[1] Private veterinarian, [2] Instituto de Virología, CICVyA, INTA-Castelar, Buenos Aires, Argentina, [3] Gluck Equine Research Center, University of Kentucky, Lexington, Kentucky, USA

Although Equine Infectious Anemia (EIA) is endemic in Northern Argentina the disease has a low prevalence elsewhere in the country. This report documents an EIA outbreak at a horse riding center in San Nicolas, 260 km northwest of Buenos Aires. Horses at this center are tested for EIA by AGID every 60 days and on tests conducted on February 7th, 2012 no seropositive animals were detected. On March 5th 2012, a new horse with a negative EIA-test certificate became a resident at the facility. Twenty days later it developed signs of depression, apathy, anorexia and weight loss with death occurring 3 days after the onset. As EIA was not suspected there was no necropsy or samples collected. On April 9th the horses were again subjected to routine mandatory EIA testing and all were seronegative. The riding center comprises over 18 hectares with a permanent population of 21 horses and ponies. EIA virus (EIAV) specific antibodies were detected by AGID and Western Blotting. Plasma and blood cells were collected for PCR-based detection of EIAV nucleic acids.Two horses presented on April 13th and April 25th with very similar clinical signs to those outlined above and in tests conducted to identify a cause, both were seropositive to EIA. These horses were isolated immediately, the Animal Health Authorities (SENASA) notified and the riding center quarantined. On May 2nd, a third, clinical case tested positive in AGID. This animal died on May 5th. Subsequently, all remaining horses were subjected to daily clinical monitoring and to date, febrile responses have been observed three additional horses with one testing positive in AGID and some in the group giving weak positive reactions in more sensitive western blot assays. The time line of events coupled with the fact that a stable population of horses/ponies had previously, repeatedly tested negative for EIA strongly suggests the riding center outbreak was caused by the horse introduced on March 5th. The fact this horse possessed a negative EIA certificate illustrates a deficiency of indirect serological testing namely, the delay between pathogen exposure and production of detectable antibodies. Therefore, horses can be infected with EIAV and pose a considerable threat for transmission long before the production of antibodies measurable by AGID. Unfortunately, in this case transmission was facilitated by high horsefly vector numbers in the summer months (December to March) caused by proximity of the riding center to water. Molecular detection and genetic characterization of this highly pathogenic EIAV strain is ongoing. This outbreak of EIA in Argentina's main horse-breeding region is an infrequent event and is of great concern for the industry in general.

Report of the surveillance of Equine Infectious Anaemia in France in 2010

A. Hans [1], D. Gaudaire [1], E. Morilland [1], F. Lecouturier [1], C. Laugier [1], N. Ponçon [2], and S. Zientara [3]
[1] Anses, Laboratory for equine diseases, 14430, Goustranville, France, [2] Direction Générale de l'alimentation, Bureau de la santé Animale, Paris, France, [3] Anses, Laboratory for Animal Health, UMR 1161 ANSES/INRA/ENVA, 94703 Maisons-Alfort, France

Equine Infectious Anaemia (EIA) is caused by Equine Infectious Anaemia Virus (EIAV). EIAV is a retrovirus infecting only equids, its genome consists of a positive single stranded ribonucleic acid (RNA) of 7500 base pairs. Clinical signs associated with the infection are mainly characterized by fever, anaemia, oedema and various signs of depression. In France EIA is a notifiable disease, in 2010 the surveillance program leads to 14 429 testings performed by the network of approved laboratories using the Agar Gel Immuno-Diffusion (AGID) test. Among those analyses 26 were positive for EIA and came from 10 horses. None of the 10 positive horses exhibited any

clinical signs. Five were from France and five came from Romania. Positive horses identified in 2010 were from 7 different premises divided in two different episodes. The first episode detected in South West of France consisted of 4 related premises where 5 French Trotters were tested positives and euthanized. The index case has been tested as part of a control before exportation. Primary premise located in the Dordogne County was a breeding farm where 2 mares were tested positive for EIA. Others premises, where two positive horses were detected, were located in the Lot-et-Garonne and Gironde County. All Infected animals (5) came from the breeding farm in Dordogne and have been kept over there several years where they have been probably infected since it is the only source of infection identified. Epidemiological investigations lead to test more than 400 horses, in 38 different Counties, which have been trained in the Dordogne Farm between 1990 and 2010. No other positive animals have been identified during this seroepidemiological study. The second EIA episode identified in 2010 was from horses which have been imported from Romania. Indeed, following EIA cases identified in Belgium and in UK at the end of 2009, French Ministry of Agriculture decided to test horses that came from Romania since January 2007. Investigation detected 80 equids, among those horses 38 were slaughtered or re-exported. Only 35 horses were still on the French territory at the date of testing and five of them exhibited a positive result for EIA using AGID test. Horses kept at the vicinity of those positive horses were tested but no transmission was seen. In this context the European commission (EU) has modified in June 2010 the regulation regarding importation of horses from Romania in other European member states. EU enforced a quarantine and a testing at destination for horses coming from Romania.

Molecular epidemiology of Equine Arteritis Virus in France following the 2007 outbreak

A. Hans [1], D. Gaudaire [1], S. Pronost [2], B. Ferry-Abitbol [3], C. Laugier [1], and S. Zientara [4]
[1] Anses, Laboratory for equine diseases, 14430, Goustranville, France, [2] Frank Duncombe laboratory, EA 4655 U2RM Caen University Lower Normandy, Caen, France, [3] Institut Français du Cheval et de l'Equitation (IFCE), 49411 Saumur, France, [4] Anses, Laboratory for Animal Health, UMR 1161 ANSES/INRA/ENVA, 94703 Maisons-Alfort, France

Equine Viral Arteritis (EVA) is characterized by a broad range of clinical signs such as hyperthermia, anorexia, oedema and depression. It may cause abortion in mares and a temporary infertility in stallions. The virus, Equine arteritis virus (EAV) that belongs to the *Arteriviridae* family, can be transmitted either by respiratory or veneral route. Following the initial infection, stallions may become asymptomatic carriers and can shed the virus in their semen. Those stallions are reservoir for EVA and have to be properly handled to prevent any viral spread in horse population. During the summer of 2007, an outbreak of EVA occurred in Normandy, France. Only draught and saddle horses were affected. This 2007 outbreak occurred following the used of semen contaminated by EAV for insemination. The objectives of this study were to undertake a thorough epidemiological investigation to identify shedders stallions following the 2007 EVA outbreak as well as the molecular characterization of EAV isolates encountered in France. Positive semen samples were coming from stallions collected in 2007 and 2008. Those samples were tested by Quantitative RT-PCR and by Virus isolation on cell culture as describe in the OIE manual Chapter 2.5.10. Phylogenetic analysis were performed by amplification and sequencing open reading frames 2a-7 (ORFs 2a-7) encoding the viral structural proteins. The new governmental directive leads to the testing of nearly 4500 stallions in 2008 compared to the 450 tested in 2007. Among them 39 stallions were found positive and shed EAV in their semen. Phylogenetic analysis using the ORFs2a-7 sequences and others from GenBank grouped the viruses in two groups: the North American and the European which is divided in subgroups 1 and 2. The majority of viruses (20) isolated and characterized in France were members of the European subgroup 2, six of them belong to the European subgroup 1 and one isolate belongs to the American lineage. Moreover, isolates from the 2007 outbreaks grouped in a distinct cluster, named Normandy cluster, inside the European subgroup 2. The EVA strain responsible of the 2007 outbreak grouped, inside the European subgroup 2, in a new distinct cluster and seems to be different to those used to be isolated in France previously. Appearance of this new virus might explain the speed of the viral spread in horse population observed as well as the severity of clinical signs associated with the infection as shown by the death of 5 foals recorded during this outbreak.

The soluble form of EIAV receptor encoded by an alternative splicing variant inhibits EIAV infection on target cells

Yue-Zhi Lin [1], Fei Yang [1], Shu-Qin Zhang [2], Cheng Du [1], and Jian-Hua Zhou [1]
[1] Key Laboratory of Veterinary Biotechnology, Harbin Veterinary Research Institute, Chinese Academy of Agricultural Science, Harbin 150001, China, [2] Institute of Special Wild Economic Animal and Plant Science, Chinese Academy of Agricultural Science, Changchun 130112, China

Equine lentivirus receptor 1 (ELR1) was found as the solo receptor for equine infectious anemia virus (EIAV) and identified as a member of the tumor necrosis factor receptor (TNFR) superfamily. In addition to the previously published membrane-binding form of ELR1, two other major alternative splicing variants of ELR1 were identified from mRNAs of equine macrophages. One spliced species (ELR1-IN) contained an insertion of 153 nt between the sites of 786-787 nt of the published

ELR1 cDNA sequence, which resulted in a premature stop signal 813 codons downstream. This translational termination was predicted truncating the receptor at the C-terminus of the transmembrane domain. The other species (ELR1-DE) had a deletion of 109 nt and caused a shift of the open reading frame and an appearance of a stop codon 312 nt downstream. Because ELR1-DE presumably encoded a peptide of mere 23 residues, only ELR1-IN was further analyzed for potential functions on EIAV infection of target cells. Firstly, the expression of the soluble form ELR1 (sELR1) by ELR1-IN was confirmed by Western-blot and immunofluorescence analysis. Like ELR1, the transcription level of ELR1-IN varied in different individuals of horse and at different time points in same individuals. The radio of ELR1-IN mRNA species to that of ELR1 was approximate 1:2. However, the expressions of both forms of the receptor were significantly regulated by the infection of EIAV. Additionally, pre-incubation of the recombinant sELR1 with EIAV significantly inhibited the infection of EIAV on equine macrophages, which is the primary in vivo target cell of the virus. Fetal horse dermal (FHD) cells are susceptible to EIAV in vitro. The replication of EIAV in FHD cells transiently transfected with ELR1-IN was markedly reduced when compared with the replication in cells transfected with the empty vector. Taken together, our data implicate that sELR1 is an important cellular factor that inhibits the infection of EIAV on host cells.

Equine Infectious Anemia Virus (EIAV) *gag* gene evolution *in vivo*

Z.A. Willand [1], X. Yu [2], S.J. Cook [1], J.K. Craigo [3,4], R.C. Montelaro [3,4], C.J. Issel [1], and R.F. Cook [1]
[1] Department of Veterinary Science, Gluck Equine Research Center, University of Kentucky, Lexington, KY 40546, USA, [2] Department of Statistics, University of Kentucky, Lexington, KY 40506, USA, [3] Center for Vaccine Research, [4] Department of Microbiology and Molecular Genetics, University of Pittsburgh, Pittsburgh, PA15261, USA

Introduction: There is less than 26% predicted amino acid identity in Gag p9 sequences between different EIAV isolates. This heterogeneity is probably associated with the unstructured configuration adopted by regions of this protein that may broaden the range of permissible amino acid substitutions at specific sites. Therefore, p9 coding sequences are hypothesized to evolve more rapidly in EIAV infected equids than those specifying Gag antigens where there is significantly more conservation between isolates. Material and methods: The complete viral *gag* gene from two ponies (564, 567) infected with a laboratory adapted ($EIAV_{PV}$) strain and two horses (98-07, E72) infected with different field isolates ($EIAV_{PA}$, $EIAV_{FL}$) of EIAV were amplified using a PCR-based technique and sequenced with overlapping primers. All sequences were assembled using ContigExpress (Vector NTI Advance 11, Invitrogen Corporation, Carlsbad, CA) and aligned using AlignX (Vector NTI Advance 11). Results and Discussion: Nucleotide (nt) substitution rates in *gag* as a whole varied between subjects from 0.143 in 567 to 2.062 substitutions per year per 100 nt in 98-07 with the ratio of synonymous to non-synonymous changes for most animals being $>2:1$. The nucleotide substitution rate in p9 was not significantly different from the other gag coding sequences in $EIAV_{PV}$ or $EIAV_{FL}$ infected animals but did show significant differences from p26 ($p = 0.0018$) and p11 ($p = 0.003$) but not p15 ($p = 0.5153$) in the $EIAV_{PA}$ infected horse 98-07. Conclusions: Host and or viral strain factors are important determinants of *gag* gene mutation rates in EIAV infected equids with the rate of non-synonymous changes being 10-fold higher in 98-07 than in all other subjects. With the exception of 98-07, *gag* gene sequences including those encoding p9 were relatively conserved over time.

Biosecurity

Biosecurity at Equine Events

K.A. Flynn [1], E.M. Wilson [1], and J.L. Traub-Dargatz [2]
[1] California Department of Food and Agriculture, Animal Health Branch, Sacramento, CA 95816, [2] Animal Population Health Institute, College of Veterinary Medicine, Colorado State University, Fort Collins, CO 80523 and USDA:APHIS:VS Centers for Epidemiology and Animal Health, Fort Collins, CO, 80525

The May 2011 Equine Herpesvirus-1 outbreak associated with the Western National Cutting Horse Event in Ogden, UT increased awareness and need for biosecurity measures at equine events. During the outbreak, the California Department of Food and Agriculture, Animal Health Branch (CDFA AHB), received numerous requests from equine industry stakeholders in the state for guidance on keeping horses healthy at equine events. The California Equine Medication Monitoring Program (EMMP) Advisory Committee, representing a broad range of equine disciplines regulated by the program, is responsible for addressing concerns of the California horse show industry. With more than 1600 shows that register with the EMMP each year, the EMMP Advisory Committee made a formal request for CDFA AHB development of a toolkit to help event organizers identify infectious disease risks for their event venue and determine the best mitigation measures. This industry request was key in the initiation of toolkit development to enhance biosecurity at equine events in

California. An initial conference call with the EMMP advisory committee resulted in an outline of subjects for inclusion in the toolkit. Since a critical step to evoke change is the involvement of the end users, ten (10) California equine event venues were visited by a CDFA AHB veterinarian to observe current biosecurity and management practices and insight was sought from six (6) California equine event mangers on the type of resource materials they thought would be useful in the toolkit. In March 2012, the "Biosecurity Toolkit for Equine Events" was completed and posted on the CDFA website (http://www.cdfa.ca.gov/ahfss/animal_health/equine_biosecurity.html). The document contains three sections. "Section 1- Basic Biosecurity" has recommendations for horse entry requirements, horse stabling, manure handling and disposal, feed and hay storage, equipment handling, cleaning and disinfection procedures, horse-to-horse contact, horse-to other species contact, isolation of sick horses, wildlife and vector control, visitor access to barns, traffic control and record keeping. "Section 2 - Enhanced Biosecurity and Infectious Disease Control" has recommendations for response to recognized disease to include prompt on-site isolation of suspect horses and the role of regulatory veterinarians in reportable disease outbreaks. "Section 3 – Appendix" provides valuable resource documents and event document templates. Since each equine event and venue is unique, the toolkit encourages equine event organizer consultation with a veterinarian for development of a biosecurity and infectious disease control plan tailored to their event. The toolkit provides guidance for the assessment and development of event-specific plans that address the specific disease risks identified for the event and venue. The availability of a biosecurity toolkit provides equine event organizers the opportunity to assess and make decisions on equine infectious disease risk management for their venue.

Exploration of issues surrounding equine disease transmission in Ireland, in particular at unregulated horse gatherings

J.P. Johnson [1], S.J. More [2], J.A. Collins [1], and V.E. Duggan [1]
[1] School of Veterinary Medicine, [2] Center for Veterinary Epidemiology and Risk Analysis University College Dublin, Ireland

Thousands of equine events take place in Ireland every year some of which are tightly regulated with excellent biosecurity but many of which are poorly regulated or unregulated with little or no regard for biosecurity. The potential for contagious disease transmission both at these events, and when horses return from these events to their farm of origin, is immense. Maintaining the health of the horse is an integral part of protecting its welfare. Control of infectious diseases should be seen not only in the important context of individual animal welfare but also with regard to the broader implications for the viability of the equine industry itself, particularly were an exotic disease to be introduced. The objective of this study was to provide early indication that different standards exist in different equine facilities in Ireland with regard to the prevention of disease transmission. Measures (risk factors) for the occurrence of disease transmission for a number of equine contagious diseases were defined. The frequency of occurrence of these measures was examined at a number of equine facilities, regulated and unregulated, in Ireland over the summer/autumn months. Frequency was measured as a direct yes/no or by intensity i.e. on a scale. The frequency of occurrence of measures was examined in total and in different subsets of facilities for example regulated and unregulated events. A high percentage of events in all degrees of regulation were considered high risk for contact with fomites, public access and control of wild birds and vermin. A high percentage of partially regulated and unregulated events also were considered high risk in the areas of contact between horses and sanitation of stables, where relevant. Different standards exist relating to prevention of disease transmission among horses, between the types of equine facilities in Ireland; however, certain categories of risk need to be addressed across the entire industry.

Acknowledgments

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Development of communication plan for an equine infectious disease outbreak

N.A. White DVM, MS, DACVS [1], and
J.L. Traub-Dargatz DVM, MS, DACVIM [2]
[1] Marion duPont Scott Equine Medical Center, Virginia Tech, Leesburg, Virginia, [2] Colorado State University, College of Veterinary Medicine and Biomedical Sciences, Fort Collins, Colorado

The response to an equine infectious disease outbreak incorporates risk management, resource management, and horse management and is unique to each situation. The American Association of Equine Practitioners (AAEP) in 2006 developed guidelines for infection control to assist their veterinary members with planning for and responding to equine infectious disease outbreaks. Based on recent multi-state outbreaks of equine contagious disease there was a recognized need to have the ability to provide communication of factual information related to outbreak situations. In situations where the sharing of factual information about an outbreak is delayed unexposed horses could be put at risk. In addition, where there is a lack of sharing of factual information, the concern by equine owners, trainers, event organizers and veterinary practitioners can lead to decisions that could have an unwarranted negative impact on the industry as a whole due to cancellation of events and other equine activities. In the fall of 2011 an AAEP task force, made up of AAEP members with experience in outbreak investigation, veterinary hospital administration, western performance horse practice, racetrack practice and diagnostic laboratory testing was charged with development of a communication plan to be implemented during disease outbreaks. Because an equine veterinarian would be the first medical professional to

examine a sick horse(s) and subsequently engage in a diagnostic and containment plan, the AAEP is a logical group to initiate recommendations for a communication plan. The proposed communication plan and user-friendly decision trees for communication during a disease outbreak have been created. A separate decision tree for the different outbreak venues (farm, racetrack, another type of equine event venue such as a show or fair or sale) were deemed necessary because some communication steps or the groups involved are different. Key components of the proposed communication plan are the creation of an Equine Disease Communication Center to be shared by the AAEP and the American Horse Council (AHC) and creation of an equine website where information can be posted once received and reviewed by the communication center staff. At the time of submission of this abstract the task force presented recommendations, which were approved by the AAEP board of directors. Subsequently the recommendations were submitted to the AHC Health and Regulatory committee with final approval by the AHC board of trustees. Effective communication minimizes speculation, clearly identifies a spokesperson, allows end users to base their decisions on the most up to date factual information and establishes expectations and timeframes for the various steps in outbreak mitigation. We would like to acknowledge the other members of the AAEP task force including Drs. Linda Mittel, Jerry Black, Peter Timoney, and Rick Arthur.

A review of twenty years of equine infectious disease monitoring in Switzerland: past, present and future

F.D. Wohlfender [1], G. Schüpbach [1], V. Gerber [2], M. Wehrli Eser [3], R. Hauser [4], and H.P. Meier [2]
[1] Veterinary Public Health Institute, Vetsuisse Faculty, University of Berne, Switzerland, [2] Swiss institute of equine medicine, Vetsuisse Faculty, University of Berne, Switzerland, [3] Equine clinic, Vetsuisse Faculty, University of Zurich, Switzerland, [4] Swiss Federal Veterinary Office, Liebefeld-Berne, Switzerland

In 1990 a voluntary sentinel reporting system for equine infectious diseases called “Equinella” was initiated by the Equine Clinic of the University of Berne, the Swiss Association of Equine Practitioners and the Swiss Federal Veterinary Office (FVO). The objective of our study was to analyze the data from that system as a basis for future improvements in equine disease monitoring.Initially, approximately 42 institutions distributed throughout the country (practitioners, clinics, university hospitals, laboratories, the national stud and the Swiss army) reported fortnightly clinical or/and laboratory diagnosis of specific equine infectious diseases. These institutions covered about 20% of the Swiss equine population which consisted at that time of approximately 50’000 animals. Until 2009 the data were disseminated every fortnight by means of the disease bulletin of the FVO. Until 1998 an additional annual report with a summary of results and background information was published. Since 2010 data are published weekly in an online disease database. Initially, most reports referred to infections of the respiratory tract such as strangles, EHV-4 and influenza, but over the past 20 years reports of Piroplasmosis (*Theileria equi, Babesia caballi*) cases (mostly imported horses) gradually increased. Cases of EHV-1 and Ehrlichiosis were reported almost every year. Within the last 10 years Borna disease outbreaks seemed to have decreased (28 cases from 1989-1999 in comparison to 4 cases from 2000-2011). Borreliosis and Rhodococcosis were reported for the first time in Switzerland through Equinella (1992 and 1995 respectively). Cases of Salmonellosis, CEM and EVA were found occasionally, while EIA was never reported. Over the first ten years of collecting disease data, the numbers of reporting institutions as well as case numbers remained relatively stable. Since 1990 the situation has changed. The Swiss equine population increased to currently approximately 100’000 animals, but at the same time the number of reported disease cases and the number of reporting institutions declined to around 20 cases and 10 reporting institutions in 2011. The most significant drop of reported cases was noticed after the abandonment of detailed feedback (i.e. the annual report) to the reporting institutions. We therefore presume that Equinella currently does not represent the true disease situation in Swiss equids very well and that improvements are needed to increase its value. The elaboration of a new concept for a re-launch of Equinella is necessary. This work is currently in progress.

Identifying infectious respiratory disease risk factors at equine events

K.A. Flynn, W.K. Fowler, and E.M. Wilson
California Department of Food and Agriculture, Animal Health Branch, Sacramento, CA 95814

Epidemics of equine influenza virus respiratory disease in competition horse populations in Hong Kong, South Africa and Australia had notable health and economic impact on the equine industry. The most common route for transmission of respiratory pathogens is direct contact with aerosolized droplets of respiratory tract secretions. Indirect fomite transmission of pathogens is another effective mechanism for disease transmission. Many factors contribute to risks for respiratory disease outbreaks at equine events for all disciplines of equine athletes locally, nationally and internationally: stress of frequent and long distance transportation, comingling of horses of varying health status, the close stabling of horses, the concentration and number of animals and humans on the event premises, the frequency of movements on the premises and the routine practices of the event facility. An asymptomatic horse excreting respiratory pathogens can potentially expose numerous horses directly and indirectly in equine event settings. Exposure risks vary depending on the type of event and the level of horse commingling. Contamination of stalls, equipment and personnel may occur when an asymptomatic infected horse or a sick horse is not isolated. Without strong biosecurity protocols and isolation areas for sick animals, the challenges of infectious respiratory disease control increase. In 2011, ten (10) California equine event premises were assessed for on-site practices with

potential for direct and indirect transmission of respiratory pathogens by a California Department of Food and Agriculture (CDFA) Animal Health Branch (AHB) veterinarian. Observations of potential for direct horse contact were made in the stabling areas, exhibition areas, points of entry to the premises and communal areas at different events. Common practices and behaviors of horses and personnel were also documented. Practices observed in certain disciplines may contribute to an increase risk of disease transmission; for example, tying of cutting horses from multiple states to a fence with direct nose-to-nose contact or exercising jumpers from multiple states in small, covered warm-up arenas. The potential exists for direct horse-to-horse contact at all equine events in the stabling areas, communal wash stalls and exercise areas. Many other common practices at equine events including, but not limited to, permitting the public direct contact with horses, bit inspection procedures, use of communal water troughs and the sharing of hoses, cross ties, feed tubs, water buckets and wipe rags, increase the risks for inadvertent disease transmission. Evaluation of event-specific risk factors is a critical first step in respiratory disease prevention at equine events. To assist equine event organizers assess venues and implement equine infectious respiratory disease risk management strategies, a biosecurity toolkit was developed. The CDFA "Biosecurity Toolkit for Equine Events" is available at http://www.cdfa.ca.gov/ahfss/animal_health/equine_biosecurity.html. Implementation and effective communication of mitigation measures and biosecurity practices for an equine event and monitoring of compliance during an event are essential for minimizing risks of an equine respiratory disease outbreak at an event.

Surgical site infections - to sample or not to sample?

U. Grönlund Andersson, and K. Bergström
National Veterinary Institute, Uppsala, SWEDEN

Surgical site infections (SSI) are one of the more common hospital-acquired infections in equine medicine. High profile antimicrobial resistant bacteria like methicillin resistant *Staphylococcus aureus* (MRSA) and intestinal bacteria belonging to the family of *Enterobacteriaceae* producing extended spectrum betalactamases (ESBL) that are resistant to third generation's cephalosporins are often encountered in SSI. These infections in human medicine cause an increasing degree of morbidity and mortality, as well as economic cost. In human medicine there has been an increased attention to monitor the incidence of these infections in order to raise the awareness and address inventions to control them. However, in equine medicine, data on incidence of SSI and causing bacterial agents and resistance pattern are scarce. The aim of this study was to identify and fill these knowledge gaps for equine hospitals in Sweden. Three equine hospitals voluntarily joined the project. The hospitals monitored the number of SSI for a given period including checking patients approximately a week after surgery by calling the owners during a period of one year. All SSI was sampled and analysed using routine bacteriology including susceptibility testing. Only two could deliver total data on number of surgeries per year and the number of surgical site infections that was encountered. For these two hospitals recorded 456 and 460 surgeries per year and the incidence of SSI was reported as median 2.6% (range 0-7.5%) ad median 3.6% (range 0-8.3%). In total 25 horses from the three hospitals had a positive bacterial growth and 41 bacterial species were found: 13 coagulase positive staphylococci including five MRSA isolates, 11 betahemolytic streptococci, 7 *Escherichia coli*, 3 *Actinobacillus* spp., 2 *Enterobacter cloacae* both ESBL-producing, and five samples with different bacterial species In nine horses two species were isolated and from SSI in one horse five bacterial species were isolated. The MRSA isolates were susceptible to only ciprofloxacin and chloramphenicol. The ESBL-producing isolates were susceptible to only ciprofloxacin and florfenicol. The susceptibility results of the other isolates will be presented at the conference together with species identification of the coagulase positive staphylococci and betahemolytic streptococci using PCR and genetic relationships using *spa*-type and PFGE.These data show the issue of retrieving accurate incidence data of SSI and the complexity for laboratories to find the causative agent. Nearly 50% of the horses were reported with more than one bacterial species which may persuade the veterinarian to use broad spectrum antimicrobial drugs. In addition, superficial SSI have shown to heal with appropriate wound treatment without systemic antimicrobials. However, it's crucial to sample SSI for bacteriology and susceptibility testing for monitoring the hospital's bacterial flora facilitating a more accurate surgical prohylaxis. The results can serve as an indicator of quality of the infection control program. An efficient infection control program is essential to minimize the spread of high profile antimicrobial resistant bacteria between horses, horses and humans.

New environmental disinfectant technology, a brief review

L.G. Pantaleon DVM, MS, DACVIM
Ogena Solutions, Versailles, KY 40383

Emergence of multi-drug resistant organisms are a constant threat to human and animal health. Among veterinary teaching hospitals, 82% reported outbreaks of hospital acquired infections, of which 52% required restricted admissions to control the outbreak. Outbreaks lead to great economic loss to the health care and veterinary industries. Furthermore the limited prospect for newer, safer and affordable antimicrobials along with the emergence of multi-resistant microbes; makes biosecurity strategies paramount. Disinfectants are the backbone of infection control and its use to prevent and control pathogenic microorganisms is a well-accepted practice. There is increasing concern regarding human and environmental safety for some commonly used disinfectants. Sodium hypochlorite, quats, chlorine gas and glutaraldehyde cause occupational illnesses. Quats can induce immunoglobulin-E sensitization to aeroallergens, thus predisposing to

occupational asthma. Residues found in surface and ground water is concerning due to the long term environmental impact. Additionally, developing countries have imposed restrictions on the use of phenolic disinfectants. If pathogens that develop resistance to disinfectants could also develop resistance to antibiotics is still debatable. It has been shown that exposure to quaternary ammonium significantly increases the chances for co-selection of antibiotic resistance within environmental bacteria. Disinfectants at the point of use are rapidly bactericidal, thus the risk of inducing antibiotic resistance is low. However it is downstream, where sub-lethal concentrations of disinfectants provide selective pressure. Therefore, the use of biodegradable disinfectants with high microbicidal action at the application point but lack of microbicidal activity downstream is justified. Hydrogen peroxide is a non-halogen oxidant, targeting multiple microorganism structures (lipids, proteins and DNA) ultimately causing death; its germicidal activity can vary depending on the formulation. Additionally, oxidation is less likely to induce resistance to the disinfectant. Accelerated hydrogen peroxide (AHP) is formulated with low levels of hydrogen peroxide in combination with anionic surfactants, thus increasing its germicidal potency, cleaning ability and material compatibility. Along with the hydrogen peroxide, the stabilizers and surfactants have a high safety and biodegradability profile and are free of aquatic toxicants. Contact time is a vital factor to consider for environmental disinfection. Most disinfectants require a minimum of 10 minutes contact time, which is often unrealistic in field conditions, thus generating a false sense of security and risking spreading pathogens over a wider area during the disinfection process. Figure-1. AHP disinfectants are broad spectrum and depending on its formulation, the microbicidal time varies between 30 seconds to 5 minutes, thus allowing for a more realistic contact time. Studies have also shown that AHP disinfectants are also able to kill high levels of *C. difficile* spores in 10 minutes. Biosecurity must be an integral part of veterinary patient care. The use of a disinfectant that is effective and safe, with attainable contact time is a fundamental part of any biosecurity protocol. Proper disinfectant selection must be followed by personnel education and compliance in order for it to be effective.

Other

Multicenter, retrospective study of vertebral osteomyelitis and diskospondylitis in adult horses

M.C. Coleman DVM, DACVIM [1],
M.K. Chaffin DVM, MS, DACVIM [1],
J. Griffin DVM, DACVR [1], W.V. Corapi DVM, PhD, DACVM [1],
T.E. Norman VMD, DACVIM [1], A.L. Johnson DVM, DACVIM [2],
and K.G. Magdesian DVM, DACVIM, DACVECC, DACVCP [3]
[1] Texas A&M College of Veterinary Medicine, [2] University of Pennsylvania New Bolton Center, [3] University of California School of Veterinary Medicine

Vertebral osteomyelitis and/or diskospondylitis are rare in adult horses. While there are several case reports of this disease in the literature, to the authors' knowledge there are no large studies evaluating the clinical features and outcome of this disease in adult horses. The objective of this study was to describe the signalment, clinical signs, results of clinicopathologic testing, results of diagnostic imaging and necropsy examination, results of microbiologic testing, location of lesions, treatment, and outcome of adult horses affected with vertebral osteomyelitis and/or diskospondylitis. Records from 19 adult horses, from 3 referral centers were retrospectively evaluated. Cases with radiographic and/or necropsy findings consistent with osteolysis were included. Cases consisted of 11 males and 8 females, ranging in age from 2-24 years. A variety of breeds were represented. The most common clinical signs included stiffness and poor performance. Ataxia was noted in 1/3 of cases. Clinicopathologic features were variable, with a normal white blood cell count in 50% of the cases. Lesions in the cervical vertebral body were most common. Lysis of adjacent vertebral endplates were noted in 2/3 of cases. Thirteen horses survived to discharge, with 7 returning to the previous level of performance. Vertebral osteomyelitis and/or diskospondylitis are uncommonly reported in horses. Clinical signs and clinicopathologic features are variable. Prognosis is fair to guarded.

Multicenter, retrospective study of vertebral osteomyelitis and diskospondylitis in foals

M.C. Coleman DVM, DACVIM [1],
M.K. Chaffin DVM, MS, DACVIM [1],
J. Griffin DVM, DACVR [1], W.V. Corapi DVM, PhD, DACVM [1],
T.E. Norman VMD, DACVIM [1], N.M. Slovis DVM, DACVIM [2],
A.L. Johnson DVM, DACVIM [3], K.G. Magdesian DVM, DACVIM,
DACVECC, DACVCP [4], and C.C. Clark DVM, DACVIM [5]
[1] Texas A&M College of Veterinary Medicine, [2] Hagyard Equine Medical Institute, [3] University of Pennsylvania New Bolton Center, [4] University of California School of Veterinary Medicine, [5] Peterson and Smith Equine Hospital

There are several cases reports in the literature of vertebral osteomyelitis and/or diskospondylitis in the foal, but there are no large studies evaluating the clinical features and outcome of this disease in foals. The objective of this study was to describe the signalment, clinical signs, results of clinicopathologic testing, results of diagnostic imaging and necropsy examination, results of microbiologic testing, location of lesions, treatment, and outcome of foals $\leq$ 1 year of age affected with vertebral osteomyelitis and/or diskospondylitis. Cases with radiographic and/or necropsy findings consistent with osteolysis were

included. Records from 15 juvenile horses, from 5 referral centers were retrospectively evaluated. Foals ranged in age from 2 weeks-4 months. There were 11 male, 4 females, and a variety of breeds represented. The most common clinical signs included fever, tachycardia, tachypnea, anorexia, and lethargy. Spinal ataxia and recumbency were noted in 50% and 20% of foals, respectively. Clinicopathologic features were variable, with an elevated white blood cell count in half of the cases. Lesions in the cervical vertebral body were most common. Evidence of a septic process of another body system was noted in less than half of the cases. Seven foals survived to discharge. In conclusion, vertebral osteomyelitis and diskospondylitis are rare in foals. Clinical signs and clinicopathologic features are variable. Prognosis is guarded for affected foals.