REPORT OF THE COMMITTEE ON INFECTIOUS DISEASES OF HORSES

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The Committee met on October 22, 2012 at the Greensboro Sheraton Hotel, Greensboro, North Carolina, from 1:00 to 6:00 p.m. There were 29 members and 50 guests present. The meeting was chaired by Dr. W. Kent Fowler. The mission statement was reviewed and the committee decided no changes were necessary at this time. The monthly National Equine Conference Call was discussed and reported by Fowler to have an average of 56 call-ins on each monthly call. There are two proposed resolutions to be discussed in the business session.

Time-Specific Papers

Dr. Udeni Balasuriya, Gluck Equine Research Center, University of Kentucky, presented a time-specific paper on "Laboratory Diagnosis of Equine Herpesvirus-1 Infection in Horses: Advances and Challenges". The paper, in its' entirety, is included at the end of this report.

Dr. Massaro Ueti, USDA-ARS-Animal Disease Research Unit and Department of Veterinary Microbiology and Pathology, Washington State University, presented a time-specific paper on "Chemotherapeutic Elimination of Tick-borne Apicomplexan *Theileria equi* in Naturally Infected Horses". The paper, in its' entirety, is included at the end of this report.

Presentations and Reports

Equine Passports

Andy Schwartz
Texas Animal Health Commission

A number of states have signed agreements establishing interstate movement permits or interstate event permits, called equine passports here. These equine passports allow the movement of equine between states for a six-month period of time, if certain criteria are met. The passports take the place of a 45-day Certificate of Veterinary Inspection (CVI).

The number of Southern District states participating in a passport program is increasing, with 14 signatures on the current agreement. Six Western District states have had an agreement for a number of years. Producer participation in the passport program is high in both districts. Other multi-state agreements may exist.

In general, application must be made to the state of residence that includes proof of a current negative EIA test and veterinary inspection. Individual states can add their own requirements as the agreements are established. Some states use a paper certificate, while others use laminated certificates or wallet sized cards. Digital images of the equine are accepted by most states. If non-standard microchips are used, some states require the transporter to provide an appropriate microchip reader. An itinerary is kept on each equine should tracing is necessary. States may cancel passports in disease outbreak situations.

There is interest among some regulatory officials and industry representatives for the establishment of an equine passport that would be accepted by all states.

EIA Subcommittee Update

Andy Schwartz
Texas Animal Health Commission

The effort to bring about change in the USDA rules pertaining to Equine Infectious Anemia (EIA) began in 2004. A USAHA resolution that year asked the United States Department of Agriculture (USDA), Veterinary Services (VS) to update the Code of Federal Regulations (CFR) to create a national EIA control program. In 2006, another USAHA

resolution asked the USDA to place the current equine infectious anemia (EIA) Uniform Methods and Rules (UMR) in the CFR. The CFR was not changed.

In 2008 another USAHA resolution asked VS to fund a national EIA program and eradication efforts in high prevalence states. The resolution also asked that a working group be established to do a census on the horse population in the US, and determine the prevalence of EIA in the horse population. The resolution went on to ask that a three-tiered laboratory system be established, that information be shared by laboratories in an electronic format, and again asked that the UMR be added to the CFR. The VS response indicated that funds were not available for a national program, but there was support for sharing laboratory data electronically. The CFR was not changed, but work was initiated on a proposed rule.

The current EIA regulations outlined in 9 CFR 75.4 pertain to reactor equines and laboratory requirements. The CFR does not cover EIA testing requirements for interstate movements or handling of exposed equine. Updated EIA regulations are needed to codify current VS activities not supported by the CFR, such as requiring that a State/Federal animal health official or an accredited veterinarian collect and submit EIA samples as stated on the VS 10-11 form.

VS has drafted the proposed EIA rule, and has informed stakeholders of the concepts in the rule at USAHA, American Association of Equine Practitioners (AAEP), American Horse Council (AHC), and National Institute for Animal Agriculture (NIAA) meetings, and on conference calls sponsored by USAHA, AHC, and National Assembly of State Animal Health Officials (NASAHO). These meetings and calls were held between October 2011 and March 2012. Stakeholder responses to the concepts of the proposed rule were mixed. Many supported the rule, at least as a starting point for change, while others opposed it.

In June 2012, a Decision Memorandum for the Deputy Administrator of VS was prepared. The memo presented two options: continue the rulemaking process and publish the proposed rule, or discontinue the current EIA proposed rule activities. The Deputy Administrator elected to pursue the first option, that of continuing the rulemaking process and publishing the proposed rule. At this writing, the proposed rule has not been published.

Equine Piroplasmosis Subcommittee Update

Mike Short
Florida Department of Agriculture and Consumer Services

There continues to be significant efforts in equine piroplasmosis (EP) surveillance and research. EP testing of horses continues to be driven primarily by industry but some regulatory testing is occurring as well. Industry testing continues to occur thorough multiple routes including, sanctioned race tracks and breed sponsored events and sales. The majority of regulatory testing is being done though disease investigations and international export with some interstate testing.

According to the National EP Situation Report, there have been 190,085 horses tested since November 2009, with more than 42,000 tested in the past year. Since 2009 there have been 189 horses determined to be positive for EP, of those 11 were detected in the past 12 months (excludes the horses detected as positive during the investigation of the 2009 Texas ranch outbreak). All of the positive EP horses, except one with ongoing investigation, have been in one of two high risk categories; horses imported prior to August 2005 using the CF test and those involved in racing, primarily Quarter Horse racing.

During the past year the EP Subcommittee held one meeting which took place via conference call. The primary discussion points and continued areas of interest and concern of the subcommittee are:

- Update on status of the EP Working Group long-term recommendations
  - The USDA has begun to implement some of the recommendations and the VS Management Team is continuing to review the other long term recommendations for potential implementation.
- EP Uniform Standards Document
  - The USDA is working on an EP Uniform Standards document to include the current guidance in VS Memo 555.20, the Long-term recommendations from the EP Working Group and the laboratory EP testing approval notice, in one comprehensive document.
- *B. caballi* suspect case testing protocol completed.
  - Since the USAHA meeting last year, the USDA has approved a *B. caballi* adjunct test, validated at ARS in Pullman, Washington. The new protocol for any horse testing positive for *B. caballi* on the cELISA test, is to automatically be tested on this new confirmatory test at National Veterinary Services Laboratory (NVSL). The complete results are being issued on the regular NVSL results report, including a statement of interpretation in the comments section of the report. The testing is being completed on most samples within 2-3 business days from the date of receipt at NVSL. The horses with a confirmatory negative test can be moved based on the final NVSL report with no further regulatory restrictions. A copy of the final NVSL report should be used as proof of the EP negative status for movement and event entry purposes.
  - A confirmatory test for *B. caballi* was needed as there have been a small number of *B. caballi*, cELISA positive results on horses that have had no epidemiological link to high risk disciplines or management and have been of low risk signalment and/or of low risk breed. These same cases have been yielding discrepant results on additional testing. In many of those cases, while the cELISA is at the 40% inhibition

level or higher it is believed that the result is a false positive and the test is reacting to a protein not originating from the *B. caballi* organism.

- Center for Epidemiology and Animal Health (CEAH) National Center for Risk Analysis is conducting a feasibility study for an *Amblyomma cajenennse* risk assessment.
    - In February of this year the Parasitic Disease Committee of the USAHA recommended that CEAH, National Center for Risk Analysis conduct a risk assessment on the potential for *Amblyomma cajenennse* to be transported via livestock or wildlife from Texas to other states and that CEAH determine the natural range and current known locations of *A. canjenennse*.
    - CEAH has stated three primary aims of the study,
        - Define the geographic distribution of the Cayenne tick in the US based on tick survey data.
        - Characterize the habitat associated with the presence of the Cayenne tick in the US.
        - Evaluate the interstate movements of horses, cattle, and feral swine within the defined tick habitat.
    - Part of the risk assessment will be based on wildlife tick surveillance being conducted by the Southeastern Cooperative Wildlife Disease Study (SCWDS), which began this summer. The SCWDS wildlife tick surveillance is being conducted in South Texas in an effort to better determine wildlife host range and geographic distribution of *A. cajenennse* and *Rhipicephalus (Boophilus) microplus.*

- Continued illegal movement of livestock across the Mexican/US border.
    - There is ongoing illegal movement of livestock from Mexico into the US. These livestock pose a continual risk to US livestock as they often carry disease and foreign ticks.
    - In May of this year, fourteen horses (ten adults and four foals) were seized while moving illegally from Mexico into the US. All ten adult horses tested positive for EP.
    - According to the USDA there were 440 animals (horses and cattle) seized while trying to move from Mexico into the US illegally in 2011.
- Ongoing EP surveillance
    - In the past year EP surveillance testing has decreased due to a reduction in both regulatory and industry testing requirements.
    - There is concern that needed EP surveillance will continue to drop due to multiple factors including:
        - Reduction in the perceived importance of testing as the number of domestic case falls.
        - Economic pressure reducing industry driven testing.
- Review of the USDA responses to the two EP Subcommittee resolutions passed during the IDOHC secession at last year's USAHA meeting.
    - The USDA responses to both resolutions were positive and implementation of both resolutions is expected in the near future.
        - Resolution 21, requested that a protocol be approved for the release of positive EP horses after treatment and testing negative via multiple test methods including the cELISA test.
        - Resolution 22, requested that import testing for horses include the complement fixation test (CFT) in addition to the cELISA test.

Update on African Horse Sickness in South Africa

Alan Guthrie
Equine Research Centre, Faculty of Veterinary Science, University of Pretoria, South Africa

The complete text of this presentation is included at the end of this report.

Equine Leptospirosis

Craig Carter, DVM, University of Kentucky, Veterinary Diagnostic Laboratory

The complete text of this presentation is included at the end of this report.

Panel Discussion on an Infectious Disease Outbreak at a Large Equine Event

Angela Pelzel McCluskey, USDA-APHIS-VS, Western Region Epidemiologist
Dee Ellis, Executive Director of Texas Animal Health Commission
Keith Roehr, Colorado State Veterinarian
Glenn Petty, Executive Vice President, Arabian Horse Association
Charles Vail, Senior Partner, Littleton Equine Medical Center
Moderator: Kent Fowler
Roles assumed:

- Federal Regulatory Dr. Angela Pelzel-McClusky
- State Regulatory Dr. Dee Ellis
- State Regulatory Dr. Keith Roehr

- Show Management Mr. Glenn Petty
- Show Veterinarian Dr. Charlie Vail

The panel members were to assume that they were responding to the scenario and provide input from their respective positions. The panel discussion was provided a scenario of an Equine Herpes Myeloencephalopathy incident at a large equine event. The 239 acre event venue had ten barns with 1,080 permanent stalls and 42 temporary tent barns. The venue had a great deal of movement of animals, people and commodities. An isolation stall/barn was present on the show premises.

Scenario:

The Horse Show Veterinarian Report – Sick horse on Sunday at 0700 on day two of competition.

A horse located in Barn 5 had a temperature 102.5º F and became acutely ataxic and recumbent. This horse had arrived at the show from a 150 horse stable three days earlier. The horse was purchased by an East Coast owner for $175,000 six weeks ago. There are eight more days of competition for this event. The horse show veterinarian contacted State regulatory officials of the possibility of EHV based on clinical signs. Nasal swab and blood test results are expected on Monday PM.

Moderator: What are your recommendations at this time for the index horse?

Show Veterinarian Vail: There are 20 horses in Barn 5. Most important at this time is that everyone should get a thermometer for every horse, monitor temperatures and there should be no movement in or out of the barn at this time.

State Regulatory Official Roehr: Recommend a hold order - a limitation of movement - since there are no laboratory results. If the affected horse is recumbent, the horse may be more effectively treated in medical facility, if possible to move, and not in the isolation facility. No decision would be made in a vacuum; I would talk with event organizers and the show veterinarians; an inventory of owners would be needed but not much contact at this time– need participant information from show managementat this time since contacts will likely be needed.

State Regulatory Official Ellis – It would be advantageous to isolate this horse and disinfect the area, but this horse is recumbent; it would be better off isolated…we had this same type of scenario happen in Texas, but we were able to get the affected horse moved. I would think of rabies and other things, not just EHV-1. There would not be any official action at this time. I would talk with the show veterinarian and show management. Texas has a plan for biosecurity and has recommendations; I would think about and talk about contingency plans.

Federal Regulatory Official Pelzel – There would be no federal involvement at this time. EHV-1 is not an immediate reportable disease to USDA. USDA reports EHV-1 on the OIE six month report and most states have some reportability for EHV-1. Colorado and Texas have plans. USDA appreciates receiving the information from the states and provides support upon request.

Show Management Petty – I would listen to the show veterinarian recommendations. It is important to not have knee jerk reactions. We have two areas - one isolation area for suspects and one quarantine area for affected horses. At the current Arabian National Show there are 3,400 stalls and we have a quarantine area; I follow show veterinarian recommendations. A few years ago we had a dead horse on a Saturday when horses were leaving and we had no contact information for exhibitors. We listen to the show veterinarian. When the rumor mill starts, this is where it is difficult for us to control movement of horses from the facility and manage approval for departure – we have a $0.5-1 million dollar liability in hotel expenses alone with large shows; we have $50-100 M worth of horses on the ground - we rely on the advice of veterinary professionals.

Scenario Inject #2: At 1500 on Sunday, a horse located in Barn 26 that was on the same plane as the index horse has a temperature of 104.1º F and slight hind limb ataxia and another horse in an adjacent stall to the index horse in Barn 5 has a temperature of 101.7º F and no other symptoms. Both horses were swabbed and bled and the samples were to be sent to the laboratory on Monday morning.

Moderator: Does this additional information impact your initial response?

Show Veterinarian Vail: With Barn 5 I would hold steady. I would contact the state veterinarian and the show management to move the Barn 26 horses to an isolation area; I would leave the recumbent horse where it is in Barn 5.

State Regulatory Official Roehr: I concur with the show veterinarian; my concern is increasing, there would be discussions ongoing with show management; we already have contact information for exhibitors.

State Regulatory Official Ellis: I would need contact information. I would have a conference call with other state veterinarians and continue monitoring of activities. In Texas we would allow movement of horses from the grounds on VS 1-27s.

Show Veterinarian Vail: I would continue the focus on Barn 5 and the Barn 26 single horse; in Barn 5 and 26 we would obtain BID temperatures on all horses.

Scenario Inject #3: Monday morning 0730, there are confirmed reports that 12 horses have left the show premises from Tent 25 at 2400 hrs due to owner/trainer concern of health risk and reportedly headed home (600 miles away).

Moderator: What are the concerns with movement of horses from the facilities? What messaging is taking place at this time prior to receiving test results?

State Regulatory Official Ellis: This changes my perception with evidence of disease spread. I would talk to show management to find out additional information on these horses and where they are from. I would have a conference call

with other state veterinarians that have horses from their state at this show. We have contingency plans in our state plans for movement of horses on VS 1-27s.

Show Management Petty: I would follow the recommendation of the state veterinarian. We have signage to limit movement in certain area. We would institute biosecurity measures with veterinarians, farriers, feed deliveries, etc.

Show Veterinarian Vail: I would defer to show management in terms of what happens on the grounds. I would communicate to exhibitors that biosecurity measures have been instituted and we are waiting for test results. Our state veterinarian is a great communicator and keeps us appraised of actions.

Moderator: Dr. Roehr, you are being contacted for a media interview.

State Regulatory Official Roehr: I would refer to our Public Information Officer. The key considerations are that some people have left and there are others who are thinking about leaving…transparency is important. We must talk through these issues with people. There are problems with doing too little and problems with doing too much…we are waiting for test results and it is difficult to say much more at this time. We need proper biosecurity from the beginning with affected barns and must remember that people are great fomites. There are questions where the recumbent horse would receive the best care if it can be moved.

Moderator: What about communication to all exhibitors?

State Regulatory Official Ellis: We have a situation where communication is critical. There is a need to talk to the show veterinarian and an epidemiologist. I would meet with show management on contingencies…arrange meeting with participants to assess feelings and provide information - for sure need interaction with participants on their desire to stay or leave the venue and a conference call with other state veterinarians…would meet with press for press conference. I would plan out the day…I may seek consultants…then give out focused information even before test results are back...if some exhibitors chose to head home then conversations need to take place with state veterinarian in state of origin.

Show Management Petty: I would follow guidance from veterinarians…I would meet with trainers since they are a smaller group of people and communicate the situation.

Scenario Inject #4: Monday 1400 - test results positive for neurotropic equine herpes virus (NEHV) are received on index horse - PCR CT value 31 on nasal swab indicating a high viral load. Monday 1600 - neurologic symptoms have progressed on index horse and the insurance company has approved the euthanasia request. Two additional horses at show (five total) which were on the same plane with the index horse are showing compatible EHM clinical signs.

Moderator: What current actions based on positive EHM?

State Regulatory Official Roehr – You should never issue a quarantine without knowing how you are going to release it. It is very difficult to quarantine all horses on the show premises for any length of time for many reasons. We would likely send horses to home premises and isolate horses. We have plan in Colorado for large events. Many people will want to leave; may use 1-27 sealed shipments; media communications would be in full swing …one unified message, well-coordinated.

Show Veterinarian Vail: I would meet with show management and the state veterinarian; if show is going to continue and people are still coming in they need to know the situation.

Show Management Petty: I would rely on state and federal officials since this is getting beyond show management; we are very concerned about the progression of this disease incident.

Moderator: Any quarantine actions at this time? What is the mechanism to allow horses to move off the show premises?

State Regulatory Official Ellis: I would go to show management - does entire show need to be cancelled? Can we clean and disinfect and continue on in other areas? This would be the time when we would get USDA involved because of potential interstate movements; an epidemiology team is needed to put the pieces together …complicating factors are trainers…state veterinarians and receiving states need to know the situation.

Federal Regulatory Official Pelzel: From an epidemiological standpoint we are asked for testing schemes etc. Field epidemiologist - to create scenario plans that fit with state plans and also higher epidemiological evaluation and analysis; USDA could provide support locally (area veterinarian in charge (AVIC)and federal VMOs will provide support to state personnel, ie., writing VS 1-27 permits, sealing trailers and receiving sealed trailers…sealed trailers on long trips - must have right kind of trailer for the load.

State Regulatory Official Roehr: EHV-1 is regulated in Colorado; need informed consultation and participation (ICP) to coordinate management of the event and manage movements; can be effective without creating undo concern; unique thing about EHV-1 is that once neurologic, the outcome is usually not good; cancellation of events for the well-being of horses has been seen previously.

Show Management Petty: Panic starts with additional horses being diagnosed; many shows cancel from fear; in the past some local veterinarians were giving bad advice and inciting fear – we saw cancellation of 16 Arabian shows following Ogden incident; this is an area where good communication with state officials is essential; social media increases panic so news releases can counteract this inaccurate information; we have to contract and guarantee hotel rooms and golf carts, etc…large shows have real economic problems with cancellations.

Moderator: Dr. Vail, would you be recommending additional testing?

Show Veterinarian Vail: No. Temperature charts and monitoring becomes the passport out of the facility…ultimately it is the State Animal Health Officials (SAHO) decision. Do the State Animal Health Officials determine where the horses can go?

State Regulatory Official Ellis: We would go back to the IC to evaluate before departing…we will evaluate movement to locations other than home after evaluation…in Texas I was asked repeatedly if we should cancel our show. It would be of big help to State Animal Health Officials to have criteria to evaluate just this...maybe we can develop something to help – a State Animal Health Official toolkit.

Show Management Petty: We have moved horses to race tracks during off season…vacant horse farms are also an option and fair facilities that are not in use.

Comments:

Nick Striegel: A spreadsheet of criteria to determine risk of going on with the show was developed in Colorado…would be useful to ask organizers what the risk would be?

Carl Heckendorf: There are benefits of the Biosecurity Toolkit developed in California; it is absolutely imperative that event managers proactively address these things; also American Association of Equine Practitioners (AAEP) website has valuable information on the same subject.

Marilyn Siminuch: Do participants sign an agreement that they will comply with biosecurity and pay for quarantine area?

Show Management Petty: Participants sign agreement that they will abide by United States Equestrian Federation (USEF) and show rules; we have veterinary lists with phone numbers, etc.

Kent Fowler: Following the Ogden event, contacting owners was difficult so we encourage event managers to obtain correct contact information from participants at registration.

Show Management Petty: For emergency purposes, we asked for hotel information from participants.

Ellis: We need to do more outreach, but we have limited staff; we would like to meet with all large event managers to discuss these things in advance; at the Appaloosa show last year, the show veterinarian was the key to solving a great number of the problems.

Ellis: Social media is part of the problem and part of the solution; expectations are to chat with them but we learned quickly what we weren't communicating effectively and we were able to bring misinformation to a halt by using social media.

Roehr: We also gave daily updates and many questions were answered in this manner. An option is to have a trainers meeting about moving horses early on, so that movement off premises could be coordinated.

Petty: Our public relations department was put under pressure to write things, we linked to state vet website and USDA website and then did e-blast of links to members which cut down on misinformation.

Committee Business:

Following conclusion of the scientific program, the Committee went into Business Session. Two resolutions were considered, approved and forwarded to the Committee on Nominations and Resolutions for approval by the general membership. One of these resolutions requests that USDA-APHIS-VS-NVSL proceed with the nEHV-1 ring trial and make every effort to standardize testing methodology for nEHV-1 PCR testing at diagnostic facilities in the United States. The other resolution urges USDA-APHIS-VS to reevaluate the dourine and glanders testing policy change for US domestic equids and allow this NCIE recommended testing upon request at the owner's expense. This testing provides US owners exporting horses the opportunity to pre-test domestic horses and possibly avoid a domestic horse returning home from being denied entry into the United States. The meeting was adjourned at 6:00 p.m.

CHEMOTHERAPEUTIC ELIMINATION OF TICK-BORNE APICOMPLEXAN *THEILERIA EQUI* IN NATURALLY INFECTED HORSES

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Equine piroplasmosis caused by hemoprotozoan parasites, *Theileria equi* and *Babesia caballi*, is a tick-borne disease that affects equids [1]. Infection and disease is of economic importance due to the significant impact on the international trade [1]. Once horses are infected with *T. equi*, they remain carriers for life [2]. Persistently infected horses are therefore a continuous source for transmission by tick-vectors [3,4]. While feeding on infected horses, ticks ingest infected erythrocytes. Then the parasite sexual reproduction occurs in the midgut resulting zygotes [5]. Zygotes invade tick midgut epithelial cells and transform into kinetes. Kinetes are then released into the tick hemolymph and invade salivary glands, where parasite transform into sporozoites [6]. During the second feeding on naïve horse, sporozoites are inoculated into the host via tick saliva and infect lymphocytes. Following replication of schizonts, the lymphocytes are lysed and merozoites invade erythrocytes [7]. Asexual replication occurs within erythrocytes causing acute infection characterized by a hemolytic disease of varying severity. Humoral and cellular immune responses control acute infection and recovered horses are persistently infected [8].

Theileria equi is an exotic pathogen for the Unites States. However, the discovery of ticks within the US that are capable of transmission and recent re-emergence have increased concerns that equine piroplasmosis could become endemic in the US [9,10]. Current control strategies in the US include permanent quarantine or euthanasia [9]. These methods are expensive to owners and therefore an alternative control strategy for *T. equi* is necessary. In this presentation, the ability of imidocarb dipropionate in eliminating *T. equi* from naturally infected horses is discussed.

Natural infection of horses with *T. equi* was determined by competitive ELISA and nested PCR targeting *ema*-1 [11,12]. These naturally infected horses were treated with imidocarb dipropionate (Imizol®; Schering Plough Animal Health) using a dose of 4.0 mg/kg at 72 hour intervals for a total of four intramuscular injections [13]. Following the treatment, elimination of *T. equi* was determined by nested PCR and cELISA at multiple time points, and blood transfer from treated horses into naïve splenectomized horses.

After treatment, the majority of horses were tested nested PCR negative at multiple time points and blood transfer from these horses (nested PCR negative) into naïve splenectomized horses failed to transmit *T. equi*. Although the findings are consistent with elimination of parasite, the remarkable outcome of this study was the long-term persistence of specific antibody against *T. equi*. We discuss these findings in the context of disease control and eradication with an impact to the equine movement by removing equine babesial infection as a global animal restriction.

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LABORATORY DIAGNOSIS OF EQUINE HERPESVIRUS-1 INFECTION IN HORSES: ADVANCES AND CHALLENGES

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Background:

Equine herpesvirus-1 (EHV-1) has a global distribution and almost all domesticated horses will become infected/reinfected with this virus during their lives, an event that may have significant clinical consequences. EHV-1 can cause acute upper respiratory tract disease, abortion, neonatal death and neurological disease that may lead to paralysis in horses[4]. In a high percentage of infected animals, EHV-1 establishes life-long latent infections in long-lived cells such as the neurons in the trigeminal ganglia and lympho-reticular tissues associated with the respiratory tract[2,4]. Reactivation of latent virus can lead to recrudescence of disease and result in virus transmission to additional susceptible hosts. Outbreaks of neurologic disease are thought to be initiated by viral reactivation followed by nasal shedding of the mutant EHV-1 strain by latently infected carrier horses[6]. The severity of clinical disease resulting from EHV-1 infection is determined by a number of host related factors including age, physical condition, immune status and whether the infection is the result of a primary exposure, reinfection or the reactivation of latent virus[3,4]. Although it appears that all EHV-1 strains can induce abortion in pregnant mares, only certain strains (neuropathogenic or mutant) have the ability to cause wide scale outbreaks of equine herpesvirus myeloencephalopathy (EHM) or neurologic disease in horses[17]. The earliest recorded outbreak of paralytic disease directly linked to EHV-1 was in 1966[28]. This was originally thought to be a comparatively rare event limited to a handful of cases each year. Unfortunately, the neurologic form of EHV-1 infection appears to be becoming more prevalent with an increasing number of outbreaks characterized by a high case-fatality rate among affected horses on farms, at racetracks and at riding schools since 2000[5,27]. This has led to increased industry awareness of the problem and prompted the United States Department of Agriculture (USDA) to designate EHV-1 myeloencephalopathy as a "potentially emerging disease of the horse"[7]. It has been reported that the neuropathogenic phenotype of EHV-1 can result from a single non-synonymous nucleotide (nt) A to G substitution at position 2254 ($A \rightarrow G_{2254}$), leading to a change from neutral asparagine to negatively charged aspartic acid at amino acid position 752 ($N \rightarrow D_{752}$) [13,17,31]. EHV-1 isolates with the A_{2254} genotype have been linked principally with non-neurologic infections, while viruses possessing the G_{2254} genotype are frequently but not invariably associated with neuropathogenic disease [31].

Laboratory Diagnosis of EHV-1 Infection:

Rapid, accurate and timely diagnosis of equine herpesvirus-1 (EHV-1) infection in horses is important for equine practitioners, horse owners, breeders and trainers. The clinical signs of EHV-1 related respiratory disease can mimic those caused by other equine viral respiratory pathogens such as EHV-4, equine influenza virus, equine arteritis virus (EAV), equine rhinitis virus A and equine adenovirus-1[20]. Similarly, EHV-1 induced abortions and neurologic disease must be differentiated from those caused by other infectious (EAV, EHV-4 abortions, equine protozoal myeloencephalitis, eastern equine encephalitis and West Nile virus encephalitis) and non-infectious causes. When confronted with a disease outbreak, confirmation of a provisional clinical diagnosis by means of a rapid, sensitive and specific laboratory diagnostic test(s) is essential to ensure that appropriate biosecurity and quarantine measures are implemented without unnecessary delay.

Laboratory diagnosis of various forms of EHV-1 infection is done either by direct demonstration of virus (virus isolation or viral antigen) or viral nucleic acid detection (polymerase chain reaction [PCR]) or indirectly through serologic evidence of recent infection. The OIE Manual of Diagnostic Tests and Vaccines for Terrestrial Animals (2008) recommends any of these methods for the diagnosis of EHV-1 infection[18]. Different diagnostic laboratories will select the test(s) or assay(s) for diagnosis of EHV-1 infection based on available facilities and expertise. However, the clinician or the practitioner still needs to be aware of the inherent advantages and limitations of any given test in order to be able to interpret the results of a test appropriately. The primary objective of this article is to provide an update on advances that have been made in the recent years in diagnostic testing of samples for EHV-1 and some caveats when using molecular tests for its detection in clinical specimens.

Sample collection:

It is imperative to collect the appropriate clinical sample(s) at the correct time by selecting the most suitable case(s) within an affected group of horses. Virus shedding from the respiratory tract is generally short lived (<10 days post infection [dpi]), may be intermittent and is most reliable during the acute phase of the disease or early, febrile phase of respiratory infection (1-5 dpi). Nasal/nasopharyngeal swab samples collected 10 days or later after the onset of first clinical signs are less likely to yield positive results on attempted virus isolation. Nasopharyngeal swabs (16") are preferred over regular nasal swabs[24]. They should be transported to the laboratory in viral transport medium containing

antibiotics for testing. During outbreaks, it is important to collect samples from in-contact horses that are febrile and which may not show any other clinical signs at the time because clinical signs appear later in the course of the disease. It is especially important to collect nasopharyngeal swabs in the early stages of disease since in suspected cases of EHM, neurological signs appear towards the end of the viremic phase of infection, by which time virus shedding from the respiratory tract is waning or may have ceased. Furthermore, during the viremic phase of the disease (4-10 dpi), before the appearance of neurologic signs, virus also can be detected in peripheral blood mononuclear cells (PBMCs [buffy coat fraction]). Approximately 10-20 ml of sterile blood samples should be collected into EDTA (ethylene diamine tetra-acetic acid [purple top tubes]; the preferred anticoagulant). The blood samples should be transported directly to the laboratory on ice (4°C), but not frozen. On occasion, the virus can also be detected in the cerebrospinal fluid (CSF) in parallel with the appearance of neurological signs (7-16 dpi). However, while brain and spinal cord samples collected at necropsy are not usually suitable for virus isolation, they may be useful for confirmation of viral DNA by PCR. The placenta, lung, liver, spleen and thymus should be collected aseptically in suspected cases of EHV-1 abortion for virus detection. Portions of these tissues can also be collected in 10% buffered formalin along with spinal cord from cases of EHM for histopathological (hematoxylin and eosin [H & E]) and immunohistochemical (IHC) examinations[10].

Serological diagnosis:

Serological diagnosis of EHV-1 infection can be achieved by demonstration of a four-fold rise in antibody titers in paired sera taken during acute and convalescent stages of the disease (sera collected at onset and two-four weeks later). It should be kept in mind that serum from mares that aborted or from horses with EHV-1 neurologic disease may already contain peak levels of antibodies, and no increase in titers will be detectable in subsequently collected sera. Serum antibody levels to EHV-1 can be determined by virus neutralization, ELISA or complement fixation tests. However, there are no internationally recognized reagents or standardized laboratory protocols for performing these tests and as a result, there is frequent variation in serological results among laboratories. Furthermore, all these serological tests detect antibodies that are cross-reactive between EHV-1 and EHV-4. However, the demonstration of a four-fold or greater rise in antibody titers to EHV-1 or EHV-4 by any of these tests between paired sera is serological confirmation of recent infection with one of the viruses. A glycoprotein G (gG) specific ELISA that can distinguish EHV-1 and EHV-4 is commercially available.

Traditional Methods of Detection of Virus and Viral Antigens:

Virus isolation (VI) - EHV-1 isolation can be attempted from nasal/nasopharyngeal swabs, tissues of aborted fetuses (placenta, lung, liver, thymus and splf

een), PBMCs and central nervous system material from cases of neurological disease in continuous cell lines (rabbit kidney-13 [RK-13], equine dermis [ED], and baby hamster kidney-21 [BHK-21], Madin-Darby bovine kidney [MDBK], pig kidney-15 [PK-15] and primary or diploid equine cells (equine lung or kidney cells and equine endothelial cells)18. Unlike EHV-4 which requires equine derived cell cultures for isolation, EHV-1 can be isolated in a variety of non-equine derived cell types. However, the sensitivity of different cells and cell lines can vary significantly and this can lead to false negative results. Tissue samples for VI should be kept at 4°C, not frozen at -20°C, until inoculated into cell culture. Samples that cannot be processed within a few hours after collection should be stored at -70°C. Blood samples collected for buffy coat separation should not be frozen. The identity of virus isolates recovered from clinical specimens must be confirmed by PCR, indirect immunofluorescent or neutralization assays using EHV-1 specific antisera or monoclonal antibodies. PCR has largely superseded VI in most diagnostic laboratories, which test specimens for EHV-1 and -4.

Immunofluorescence and immunohistochemical staining **-** Frozen sections of postmortem material can be stained with conjugated or unconjugated monoclonal antibody (MAb) or polyclonal antiserum to EHV-1[18]. FITC conjugated polyclonal swine antiserum to EHV-1 can be obtained from the National Veterinary Services Laboratories of the United Stated Department of Agriculture, Ames, IA. Immunohistochemical (IHC) staining methods to detect viral antigen in paraffin-embedded tissues of aborted equine fetuses or neurologic cases have been described. IHC staining along with H & E examination is particularly useful for the simultaneous evaluation of histopathological lesions and identification of the infectious agent in affected tissues. Furthermore, both assays can be performed on infected cell monolayers, which can be used to confirm isolation of EHV-1 in the laboratory. Both IFA and IHC staining must include a positive and negative control consisting of sections from known EHV-1 infected and uninfected tissues.

Contemporary Molecular Biologic Methods of Viral Nucleic Acid Detection:

Polymerase chain reaction (PCR) based assays **-** Several reports have documented the use of PCR-based assays, both standard and real-time, for the detection of EHV-1 in clinical specimens[8,9,12,13]. The PCR based techniques are rapid, sensitive and do not require the presence of infectious virus in the clinical samples. These techniques can be used for diagnostic detection of EHV-1 nucleic acid in clinical specimens, tissue culture fluid (TCF) from inoculated cultures and paraffin-embedded archived tissues. A variety of type-specific PCR primers and probes have been published which can distinguish between EHV-1 and EHV-4, as well as other EHVs (EHV-2, EHV-3 and EHV-5). A multiplex PCR assay for simultaneous detection of both EHV-1 and EHV-4 nucleic acids has been described. The OIE manual described a more sensitive nested PCR assay targeting the glycoprotein B genes (gB) of EHV-1 and EHV-4, which allows identification, and discrimination of these two EHVs. However, it should be borne in mind that nested PCR assays are prone to give a higher number of false positive results due to sample carryover and cross contamination. Therefore, the use of a nested PCR assay should be avoided in the routine laboratory diagnosis of EHV-1. The PCR assays (standard, nested and real-

time) target a number of EHV-1 genes (gB, gC, gD and DNA polymerase) but some of these genes are highly variable among EHV-1 field isolates and consequently, not all primers and probes may have similar or equivalent sensitivity. To avoid false negative test results, the primers and probes should be designed based on highly conserved regions of these genes and assays should be properly developed and validated based on testing a significant number of clinical samples before they are recommended for routine diagnostic purposes.

Hemoglobin and lactoferrin have been identified as PCR inhibitor components in erythrocytes and leukocytes, respectively. In addition, some anticoagulants (e.g. heparin) are reported to interfere with the PCR assay. These PCR inhibitors act primarily by inactivating the Taq DNA polymerase used in PCR assays. Various protocols and DNA extraction procedures are available to purify DNA and eliminate these inhibitors prior to performing the assay. However, these extra steps are time-consuming, may not completely remove inhibitors or may lead to a loss of target DNA resulting in decreased sensitivity of the real-time PCR assay. Consequently, the detection of EHV-1 DNA in blood or buffy coat cells may be less sensitive and this may result in significant variation in sensitivity of PCR assays among diagnostic laboratories.

Real-time quantitative PCR assays - A number of real-time PCR assays targeting various EHV-1 genes (gB, gD and viral DNA polymerase [open reading frame 30; ORF30]) have been described in the literature[11,15,23,25]. However, very few of these assays are properly designed and validated. Real-time PCR assays targeting gB and gD are used for measuring the viral copy number ("viral load") in clinical samples by some laboratories[25]. Two methods are used to quantify the viral DNA copy number in clinical specimens: relative quantification using cellular housekeeping genes (e.g. β-actin) or absolute quantification using a standard curve based on plasmid dilutions of the target gene. However, the quantification of viral DNA copy number in a clinical sample is challenging for the following reasons: 1). The sample collection method is not uniform due to various amounts of mucous, nasal discharge, debris and epithelial cells in the clinical specimens, and the method of swab collection (swab length, variation in contact time with the mucosa [2-20 seconds], together with type and volume of transport medium [0.1-7 ml]); 2). Nasal/nasopharyngeal swabs are mostly acellular (very few epithelial cells and leukocytes). Thus cellular genes should not be used for correcting DNA purification efficacy and calculating the viral DNA copy number in nasal secretions. Absolute quantification based on a standard curve should be used to quantitate virus in nasal secretions; this method should provide a more accurate assessment of viral DNA copy number in clinical specimens.

Real-time allelic discrimination PCR assays - Although numerous studies have examined the validity and efficiency of EHV-1 real-time PCR assays as diagnostic and research tools, there remains an urgent need for an assay that would enable reliable and simultaneous detection of EHV-1 and at the same time, discriminate between neuropathogenic and non-neuropathogenic strains in clinical specimens[11,14-16,22,26]. EHV-1 isolates with the A_{2254} genotype have been linked principally but not invariably with non-neuropathogenic infections, while viruses possessing the G_{2254} genotype are primarily but not invariably associated with neurologic disease characterized by a high clinical-attack rate and a high case-fatality rate[31]. This single nt polymorphism in ORF30 led to the development of an allelic discrimination, real-time PCR (rPCR) assay to distinguish between potential neuropathogenic and non-neuropathogenic EHV-1 strains[1]. The first allelic discrimination rPCR assay (E_2) described by Allen (2007) had a distinct advantage over existing PCR assays in that it could simultaneously detect and genotype EHV-1 strains[1]. However, subsequent evaluation of clinical samples using this allelic discrimination PCR assay in several diagnostic laboratories demonstrated that this assay lacks adequate sensitivity for routine diagnostic applications and may also generate false dual positive (A_{2254}+G_{2254}) results, seriously compromising its usefulness for A_{2254}/G_{2254} genotype differentiation. Additionally, false negative results are produced in this assay by the presence of a single, additional nt substitution within ORF30, at position 2258[29]. Recently, we have developed a new allelic discrimination EHV-1 rPCR assay (E_1)[30] and compared its sensitivity and specificity with the original assay (E_2) described by Allen[1]. The new assay has the following key features: 1). The new E_1 assay has been validated with a large number of archived tissue culture fluid samples (TCF; n=76) and clinical samples (n=433); 2). The new assay was ten times more sensitive than the original E_2 assay, with a lower detection limit of ten infectious virus particles; 3). The new assay was able to accurately discriminate between A_{2254} and G_{2254} genotypes, whereas E_2 produced 20 false dual positive results with only one actual mixed A_{2254}+G_{2254} genotype confirmed; and 4). The new assay offers greater sensitivity and accuracy for the detection and A/G_{2254} genotyping of EHV-1, making this improved real-time PCR assay a very valuable diagnostic tool for investigating outbreaks of EHV-1 infection.

Finally, the new assay could detect both A_{2254} and G_{2254} genotypes in the same clinical specimen, which confirms previous findings and raises many questions about the impact that at least two simultaneously replicating virus strains can have on viral pathogenesis, latency and reactivation[22]. While the ability to identify multiple genotypes within clinical samples represents a significant step forward in our understanding of the dynamics of *in vivo* EHV-1 replication events, dual false positive results are very detrimental in any diagnostic situation.

Verification of the new E_1 assay for use in an accredited veterinary diagnostic laboratory – Since any new assay that has been published in a peer-reviewed journal can be accommodated for routine diagnostic purposes, accredited laboratories should be required to evaluate performance of the assay to demonstrate the assay performs up to expectation. With this in mind, the new E_1 assay was tested with 30 known EHV-1 positive and 30 known EHV-1 negative equine nasal swab samples that were submitted for routine diagnosis to the California Animal Health and Food Safety Laboratory, School of Veterinary Medicine, University of California, Davis, CA. Only one sample per animal was

considered in this evaluation. To cover the entire diagnostic range of the assay performance, samples were chosen to represent strong, moderate and weak positive cases. Specifically, 27% of the positive samples were considered strongly positive (n=8), 30% of the samples moderately positive (n=9) and 43% were considered to be weakly positive (n=13). A sample was considered a known positive if it tested positive by PCR using the E_2 assay, which was considered the gold standard prior to the development of E_1 assay. On the other hand, a sample was considered to be negative if it tested negative by the E_1 assay. Mismatches between the two assays were repeated and followed up by PCR sequence analysis (n=8). All of the mismatches had tested weakly positive in the E_2 assay but tested negative in the new E_1 assay. In the case of four of the eight positive samples, a band was detected by agarose gel electrophoresis. Bands were subjected to sequence analysis and in all 4 cases, the visible product was the result of nonspecific binding of the primer sets giving rise to false positive results. No PCR product was detectable in the remaining four mismatches. The same sample set was evaluated using a modified extraction method involving an automatic extraction platform (Bio sprint, 96, Qiagen, Valencia, California; Reagents were purchased from Life Technologies, Grand Island, New York; using MagMax 96 Viral RNA isolation kit and MagMAX™-96 AI/ND Viral RNA Isolation Kit) to determine whether modification in conducting the assay affected the assay performance. Analytic sensitivity including testing samples spiked with both viruses. Repeatability studies were performed in addition to diagnostic sensitivity and specificity evaluations and these confirmed the assay was not affected by the changes. High efficiency and precision of the new assay over a dynamic range of five logs of either virus genotype (A_{2254} or G_{2254}), in concert with the results of the field sample testing would indicate the new assay is highly reliable in a diagnostic setting.

Differences in Sensitivity of Real-Time PCR Assay and Virus Isolation and Interpretation of Results:

There is some confusion about interpretation of real-time PCR and VI results in the laboratory diagnosis of EHV-1 infection. The sensitivities of these two assays are significantly different and there is only one study that compared VI to quantitative real-time PCR (qPCR) using clinical specimens from experimentally inoculated horses[19]. This study demonstrated that VI could only detect infectious virus up to 5 dpi, whereas qPCR could detect EHV-1 DNA up to 21 dpi in the same nasal swab samples. However, the number of viral DNA copy numbers that could be detected by qPCR decreased significantly from 10^5-10^7 per ml (1-6 dpi) to 10^0-10^1 per ml between 14-21 dpi. Furthermore, the number of positive horses that could be detected by these two assays varied significantly during the course of the infection. The number of horses positive by VI dropped from 87% (1-2 dpi) to 20% by 5 dpi and virus could not be detected after 6 dpi. In contrast, the qPCR could detect viral DNA in 87% of horses up to 12 dpi following which the percentage rapidly dropped to 53% by 14 dpi and 13% by 21 dpi. The number of horses tested positive by qPCR dropped rapidly starting at 12 dpi. Taken together, these data clearly showed that the number of horses sampled during an outbreak and the type of diagnostic test performed can have a significant impact on confirming diagnosis of EHV-1 infection. Finally, the interpretation of real-time PCR and VI testing should be done with caution. The following points play an important role in interpretation of the respective laboratory test results: 1). Real-time PCR detects a very small number of viral DNA molecules in clinical specimens, whereas VI requires a higher level of viable virus particles (replication competent) to produce plaques in cell culture; 2). Real-time PCR detects both infectious virus and noninfectious viral DNA in clinical samples, and therefore has a very high sensitivity as compared to the traditional VI in cell culture; 3). A real-time PCR positive result does not mean there is infectious virus in a clinical sample, only VI can detect infectious virus; 4). Real-time PCR assay should be the first choice for rapid detection of EHV-1 nasal shedding during outbreaks and identifying horses and facilities that need to be placed under quarantine; 5). Additional to PCR testing, it is important to perform VI during outbreaks and archive the EHV-1 strain(s) for retrospective molecular characterization and molecular epidemiological studies.

Key Questions about Detecting A→G_{2254} Substitution (A/G_{2254} Genotype) in Clinical Specimens:

Some studies support an association between EHM and the G_{2254} genotype described by Nugent et al., (2006)[17]. However, there is an increasing body of compelling evidence to indicate that this nucleotide substitution is not the only determinant of enhanced neuropathogenicity[21]. In the survey of Perkins et al., (2009), 24% of isolates from horses with neurologic disease possessed the A_{2254} and not the G_{2254} genotype. This finding is supported by our own investigations comparing results from the real-time allelic discrimination assay with detailed case-histories provided by attending veterinarians[21]. We identified a number of A_{2254} genotype EHV-1 isolates from cases of neurologic disease, as well as the isolates of the G_{2254} genotype from numerous horses with no evidence of neurological involvement. In addition, we have identified viruses with non-synonymous nucleotide substitutions in ORF30 besides A→$G_{2254,}$ from horses without signs of neurologic disease; this presents the possibility that these may have an attenuating effect on the viral phenotype. Therefore, identifying and genotyping EHV-1 field strains using allelic discrimination real-time PCR raise several questions that need to be considered at this point but for which we do not yet have answers (Table 1).

Table 1. Key questions and answers in relation to detecting A→G_{2254} substitution (A/G_{2254} genotype) by allelic discrimination real-time PCR.

Questions	Answer
Does A→G_{2254} substitution always correlate with the development of EHM?	No. A or G_{2254} genotype identified by allelic discrimination real-time PCR may not always correlate with EHM.

Could horses shed both genotypes (A+G_{2254})?	Yes. Both genotypes can be shed in nasal secretions. Similarly, both genotypes can be present in lymph nodes of latently infected horses.
Implementation of control and regulatory (quarantine) measures should based on the A/G_{2254} genotype?	No. Regardless of the A/G_{2254} genotype, appropriate prevention and control measures should be taken during EHV-1 outbreaks associated with neurologic signs, abortions or respiratory disease.
Should we continue to do A/G_{2254} genotype testing?	Yes. Since we have not identified any other virulence determinants of EHV-1 neurovirulence, we should continue to monitor the association between A/G_{2254} and EHM.
What real-time PCR assay should be used in routine diagnosis of EHV-1 infection?	Allelic discrimination rPCR targeting ORF30 can be used as a routine diagnostic assay, as well as genotyping assay compared to other real-time qPCR assays targeting gB, gD genes and LAT (used to identify the latent virus).

As indicated earlier, VI and real-time PCR results will depend on the sample type (e.g. nasal/nasopharyngeal swabs and buffy coat cells) and time of collection of the clinical sample in the course of the infection. Sometimes, the VI and real-time PCR may not agree with each other. In such situations, the respective results should be evaluated carefully (Table 2). In addition to this, there are some caveats when using real-time PCR to confirm a clinical diagnosis of EHV-1 infection during disease outbreaks:

1. All real-time PCR assays need to be carefully developed and validated. Real-time qPCR assays need to include appropriate internal controls to normalize for DNA purification and PCR amplification efficiencies.
2. Results may vary between laboratories due to the use of various nucleic acid extraction methods, target gene and specific commercial PCR reagents. This variation can be reduced by sending samples to the same diagnostic laboratory with proven expertise and experience in testing for EHV-1 infections. This is especially important when paired samples from the same animal (taken at two different time points) or from the same outbreak are submitted for laboratory testing.
3. Sensitivity and specificity of real-time PCR assays can be affected by a variety of factors such as sample type, sample volume, viral nucleic acid extraction method, target gene, primers and probes and their concentrations, commercial PCR reagent kits, number of cycles and cutoff point. Thus, harmonization of real-time PCR protocols between diagnostic laboratories is very important.
4. The cycle threshold (C_T) values can be used to indicate the approximate viral DNA concentration in samples. C_T <25 = high (acute stage of infection), C_T 25-30 = moderate, C_T 30-35 = low, C_T 35-40 = suspect.
5. The real-time qPCR should only be used for better characterization of the stage of the disease, assessment of risk of exposure to other horses, monitoring of response to treatment and in research studies.

Table 2. Suggested actions to be taken based on real-time PCR test results.

Real-time PCR Assay		Virus Isolation		Type of Infection/Action
Nasal Swab	**Blood Sample**	**Nasal Swabs**	**Blood Sample**	
+	+	+	+	Active infection & virus shedding / Quarantine
+	+	+	-	Active infection & virus shedding / Quarantine
+	+	-	-	Active infection & virus shedding / Quarantine
+	-	-	-	Active infection & virus shedding / Quarantine
-	-	-	+	No virus shedding*/Release**
-	+	-	-	No virus shedding*/Release**

*Convalescent infection or establishment of latency - analysis of nasal swabs for 2-4 consecutive days by real-time PCR before lifting quarantine
**Follow AAEP guidelines

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UPDATE ON AFRICAN HORSE SICKNESS IN SOUTH AFRICA

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African horse sickness (AHS) is a non-contagious, infectious, insect-borne disease of equids caused by African horse sickness virus (AHSV). In horses, the course of the disease is usually peracute to acute and in fully susceptible animals more than 90 percent of those affected die. Clinically, the disease is characterized by pyrexia, oedema of the lungs, pleura and subcutaneous tissues and haemorrhages on the serosal surfaces of internal organs. Mules are less susceptible than horses and donkeys and zebras rarely show clinical signs of disease.

An Arabic document reports the first known historical reference to a disease resembling AHS occurring in Yemen in 1327. Father Monclaro's report of the travels of Francisco Baro in East Africa in 1569 also reports AHS affecting horses imported from India. Although neither horses nor donkeys were indigenous to southern Africa, they were introduced shortly after the arrival of the first settlers of the Dutch East India Company in the Cape of Good Hope in 1652. Records of the Dutch East India Company make frequent reference to "perreziekte" or "pardeziekte" in the Cape of Good Hope. In 1719 nearly 1,700 horses died due to AHS in the Cape. During their exploration and expansion into the interior of southern Africa the Voortrekkers reported severe losses amongst their horses. Exploration of southern, central and east Africa by Livingstone was complicated by his inability to use horses on some of his journeys. Although horses die as a result of AHS every year in southern Africa, major epidemics prior to the 1950's occurred at intervals of roughly 20-30 years. Severe losses were reported in 1780, 1801, 1839, 1855, 1862, 1891, 1914, 1918, 1923, 1940, 1946 and 1953. The 1854/55 epizootic was the most severe with almost 70,000 horses dying, representing more than 40% of the horse population of the Cape of Good Hope.

In the early 1900's, researchers succeeded in transmitting the disease with a bacteria-free filtrate of blood from infected horses confirming that the disease was caused by a virus. The pioneering research of Sir Arnold Theiler, who founded the Veterinary Research Institute at Onderstepoort in 1908, revealed multiple "immunologically distinct strains" of AHSV since immunity acquired against one "strain" did not always afford protection against infection by "heterologous strains". Whilst Theiler proposed that AHS may be transmitted by biting insects in 1903, it was not until 1944 that Du Toit confirmed that *Culicoides* species were probably vectors of both AHS and bluetongue viruses.

Mouse brain attenuated polyvalent vaccines against AHSV were first developed in the 1930's. Since the 1970's tissue culture attenuated modified live virus vaccine strains have been developed to provide polyvalent protection against all serotypes of AHS in South Africa. Approximately 300,000 doses of polyvalent AHSV vaccine are sold annually by Onderstepoort Biological Products, Onderstepoort, South Africa. Monovalent modified live virus vaccines against AHSV serotype 9 are manufactured in a number of North African countries. In endemic areas, severe losses due to AHS have ceased since the development of these polyvalent vaccine. Epidemics of AHS have occurred in the Middle East, south-east Asia and Europe. Due to its high potential for trans-boundary spread AHS is a World Organisation of Animal Health (OIE) listed disease and there are specific chapters devoted to AHS in the OIE Code and OIE Manual. AHS is one of the important diseases to consider when moving equids internationally but movement can be accomplished safely following appropriate quarantine and testing procedures as described in the OIE's Terrestrial Animal Health Code.

Nine distinct serotypes of AHSV have been described. All nine serotypes have been documented in eastern and southern Africa while serotype 9 is more widespread and appears to predominate in the northern parts of sub-Saharan Africa. Serotypes 3 and 6 were isolated in Ethiopia for the first time in 2003. Serotype 2 of AHS has recently been isolated for the first time in Nigeria, Ghana, The Gambia and Senegal. Multiple serotypes of AHS, including serotypes 2, 6, 7, 8 and 9 have also been identified in Ethiopia. These more recent findings may be an indication of changes to the traditional distribution ranges of the various strains of AHS which may be associated with climate change.

Research activities in South Africa over the last ten years have focused on the development of new generation vaccines and molecularly based diagnostics for AHS. These studies have shown that a canarypox vectored recombinant vaccine against AHSV serotype 4 is highly effective in protecting horses against challenge with virulent AHSV serotype 4. Further studies on canarypox vectored recombinant vaccines against other serotypes of AHSV are currently being developed. Real-time polymerase chain reaction assays (RT-qPCR) using Taqman probes have been developed which have a high diagnostic accuracy and are currently being validated. These assays are suitable for high throughput application and can be applied during outbreaks. More recently serotype specific RT-qPCR's for all nine serotypes of AHSV have been developed which allows the serotype of the AHSV to be identified directly from clinical samples (whole blood or organ samples) within four hours of receipt of samples in the laboratory.

Whilst AHS is a disease which has been recognised for many years, the changes observed in the epidemiology of many arboviruses observed in many parts of the world including Europe and more recently in the USA, there is increased International awareness of AHS and many countries have become actively involved in developing contingency plans for AHS. It is anticipated that recent developments on new generation vaccines and diagnostics will be incorporated into control strategies for AHS.

Evaluating the Immunogenicity of a Novel Inactivated Vaccine Against Leptospirosis for Horses: Project Overview and Draft Budget

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A. Rationale for an Equine Leptospirosis Vaccine

1. Leptospirosis is an important disease of horses – Infection with the bacterium *Leptospira* occurs worldwide, and infects humans, domestic animals, and wildlife. In horses, infection with *Leptospira* is most commonly associated with abortion and recurrent uveitis; however, disease of the kidneys, liver, and systemic illness may also occur. The disease is likely under-recognized. In areas such as central Kentucky where horse-breeding is intensive, the importance of equine leptospirosis is frequently identified. Over the last twenty years, 541 cases of leptospiral abortion from the Bluegrass region have been diagnosed by the University of Kentucky Veterinary (UKY) Diagnostic Laboratory. One unpublished study in Kentucky estimates the value of foals lost in the twenty year period at over $100M. A recent national serological survey indicates that horses throughout the continental United States and Ontario are commonly exposed to the bacterium. Finally, a recent graduate student in the UKY College of Public Health demonstrated that equine veterinarians and horse farm workers develop leptospiral antibodies related to their exposure to horses and newborn foals. These data are to be presented at the meeting of the American Public Health Association (APHA) in October, 2012.

2. A vaccine is needed – The fact that wildlife may serve as a maintenance reservoir for infection means that eradication of equine leptospirosis will be difficult because eliminating infections of wildlife, including amphibians and reptiles, will not be possible. Treatment of infection will not help prevent abortions and fetal losses. Thus, there is need for an effective vaccine. Vaccines against leptospirosis exist for other domestic animals including dogs, cattle and pigs. A challenge for vaccine development is the need to include multiple serovars of Leptospira. The proposed vaccine strategy will allow us to include serovars of particular importance to the horse, viz., Pomona var kennewicki and Grippotyphosa.

3. Mucosal vaccination as a new approach – Existing vaccines in other species use the parenteral route (primarily intramuscular or subcutaneous routes). Infection with *Leptospira* is thought to occur primarily through direct contact with infected fluids. Thus, infection is likely to occur primarily by the mucosal route. For mucosal infections, vaccination at mucosal surfaces is likely critical. Thus, we propose using a mucosal route of vaccination against *Leptospira* for horses. Evidence exists that inactivated vaccines administered by the mucosal route can provide protection in the respiratory tract and intestinal tract against respiratory and intestinal pathogens, respectively.[33-39] Moreover, sublingual (oral) administration of inactivated influenza virus protected mice against respiratory infection with live influenza virus.[41] Thus, it is feasible that a sublingual, intranasal, or oral vaccination against *Leptospira* might protect horses against developing disease.

4. Inactivated candidate vaccine – Investigators at Texas A&M University have developed a proprietary method for inactivating bacteria. Preliminary data show that inactivated *Rhodococcus equi* given orally are capable of inducing both cell-mediated and humoral mucosal immune responses against this bacterium. A critical advantage of our approach is that the bacteria are whole and recognized by the immune system similar to live bacteria; however, they are incapable of replicating and causing disease. We propose to administer *Leptospira* that have been inactivated by this proprietary method via oral and intranasal routes to evaluate whether immune serological and nasal mucosal immune responses can be induced. If immune responses can be generated, further studies of the vaccine can be investigated for effectiveness. The inactivated bacteria will be administered with a mucosal adjuvant to enhance immune responses.

B. Preliminary Data

Proprietary restrictions preclude us from providing full representation of our technology and preliminary data. We have demonstrated using dose-titration experiments demonstrating that inactivation renders *R. equi*, *Salmonella*, and *Streptococcus equi* subsp *equi* unable to grow on enriched media; the surface structure of these bacteria remain intact (Figure 1) and, when refrigerated, remain intact for at least 14 days. For *R. equi*, we have documented that the surface-expressed protein vapA necessary for virulence remains detectable by flow cytometry on inactivated *R. equi*. A critical aspect of our approach is that the bacteria are whole and recognized by the immune system similar to live bacteria; however, they are incapable of replicating within the host and causing disease.

Moreover, we have demonstrated that inactivated *R. equi* administered orally can elicit cell-mediated immune responses (↑ interferon-gamma expression by CD4+ T cells), and anti-*R. equi* IgA in the upper respiratory tract of foals that is not detectable in control foals.

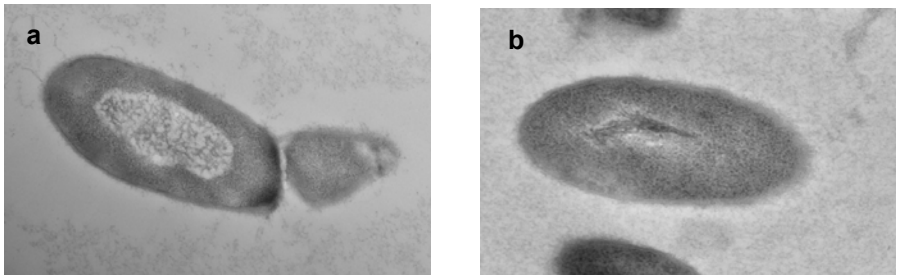

Figure 1. Scanning transmission electron microscopic images of *R. equi* that were live and at exponential growth at the time of processing (a) or inactivated using our proprietary method (b); Note that the cell surface is not disrupted following treatment, but that the nucleoid (center of cell) of the treated bacterium has changed, presumably reflecting damage to DNA that prevents replication. Similar results were seen with other bacteria

C. Approach

We will use the following approach:

1. **Optimize the protocol for inactivating Leptospira**: We will need to optimize the dose for inactivating the bacteria and ensure that the bacteria are inactivated, using standard approaches such as growth curves. We also will need to verify membrane integrity using electron microscopy and membrane permeability assays.
2. **Vaccine route and dose** – We will evaluate 2 doses of inactivated *Leptospira* administered either orally or intranasally in horses to evaluate whether the vaccine is immunogenic by either route.
3. **Immunogenicity** – We will evaluate serologic responses to *Leptospira*, using both standard MAT titer testing as well as IgG-subisotype-specific testing for antibodies against a whole bacterial lysate. We will evaluate mucosal immunity by testing for Leptospira-specific IgA from nasal swabs and possibly vaginal/uterine swabs as well.

D. Budget

1. Horse Use: 40 horse x $150/horse = **$6,000**
(four groups: high dose, low dose, negative control, bovine vaccine)
2. Vaccine Preparation: **$12,000**
Includes dose titration, membrane integrity testing, etc.
3. Immunological Testing* (need to adjust if we do fourth group [40 horses])
   a. Serological testing: MAT –
      three times per horse x 40 horses=**$1,800**
   b. Nasal washes and swabs collection (supplies, sedation, etc.):
      three times per horse x 30 horses x $25/horse = **$2,250**
   c. Uterine washes and swabs collection
      two times per horse x 30 horses x $25/horse = **$2,250**
   d. ELISA development and assays (may not be needed if MAT testing suffices)
      i. Development: **$5,000**
      ii. Assays of nasal swabs and washes for IgA:
         30 horses x 3 time-points x $30/horse = **$2,700**
      iii. Assays of uterine washes and swabs for IgA **$2,700**
      iv. Serum IgGa and IgGb:
         30 horses x 3 time-points x $30/horse = **$2,700**
   e. Cell-mediated immune responses (Included for Plan "A"; Excluded for Plan "B"):
      i. Intracellular interferon gamma staining of peripheral blood CD4+ T cells stimulated with *Leptospira* lysate
      30 horses x 3 time-points x $100/horse = **$9,000**
   f. Post-doctoral support: Dr. Angela Bordin will design assays and perform analyses
      Salary is $60,000/yr + $18,000 benefits = $78,000@50% = **$39,000**
   g. Student workers to help with lab work, horse work, etc.:
      2 students @ $10/hr x 10 hr/wk x 40 weeks = **$4,000**
   h. CO2 incubator **$12,500**

Total Plan "A" (includes cell mediated response metrics) $92,900
Total Plan "B" (excludes cell mediated response metrics) $101,900