Proceedings of the

AMERICAN ASSOCIATION OF VETERINARY LABORATORY DIAGNOSTICIANS

49TH ANNUAL CONFERENCE

Minneapolis Hilton
Minneapolis, Minnesota
October 12 – 18, 2006

AMERICAN ASSOCIATION OF VETERINARY LABORATORY DIAGNOSTICIANS

Purpose

- Dissemination of information relating to the diagnosis of animal diseases
- Coordination of diagnostic activities of regulatory, research and service laboratories
- Establishment of uniform diagnostic techniques
- Improvement of existing diagnostic techniques
- Development of new diagnostic techniques
- Establishment of accepted guidelines for the improvement of diagnostic laboratory organizations relative to personnel qualifications and facilities
- Consultant to the United States Animal Health Association on uniform diagnostic criteria involved in regulatory animal disease programs

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ACKNOWLEDGEMENTS

The success of a meeting is a function of both presenters and attendees. A special thank you to all who presented their data and findings, all exhibitors and sponsors, and all who attended the meeting. A special thank you also to all our invited speakers for the First Plenary Session and the Joint Plenary Session.

The program committee (listed below) deserves a special acknowledgement for all their hard work, organization, review and editing of the abstracts, and moderation of sessions. Jay Kammerzell was instrumental in organizing the review process and assisting in compilation of the program book. Pat Blanchard, Donna Dare, Allison Reitz, Jackie Cassarly (of the Planning Committee) and Linda Ragland coordinated the meeting room arrangements, exhibitor booth arrangements, sponsor agreements, breaks and all the other details that make a meeting a success.

* *

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First Plenary Session

Saturday, October 14, 2006
Salon AB

Moderator: Barb Powers

8:00 am	**WELCOME AND ANNOUNCEMENTS –** Barb Powers President Elect AAVLD	Page
8:05 am	**Interspecies Transmission of Influenza Viruses** - Ruben Donis, CDC	23
8:45 am	**Pathobiology of Avian Influenza Viruses** – Mary Pantin-Jackwood, SERPL	24
9:15 am	**Rapid Diagnostics for Avian Influenza Advances in testing** – David Suarez, SERPL	25
9:45 am	BREAK	
10:00 am	**Advanced Diagnostics and Expanded Capabilities for Foreign Animal Disease Detection and Surveillance** – Ben Hindson LLNL	26
10:30 am	**Trends in Veterinary Diagnostic Laboratory Medicine – Implications for the Future -** John Andrews, CSU	27
11:15 am	House of Delegates	

Bovine Virus Diarrhea Scientific Session

Saturday, October 14, 2006
Salon A

Moderator: Hong Li and David Steffan

Avian Influenza and Canine Influenza Scientific Session

Saturday, October 14, 2006

Salon B

Moderator: Kristy Pabilonia and Letitia Curley

Pathology Scientific Session

Saturday, October 14, 2006
Salon C

Moderator: Scott Fitzgerald and Peg Miller

Transmissible Spongiform Encephalopathy and Virology Scientific Session

Saturday, October 14, 2006

Duluth

Moderator: Tim Baszler

Epidemiology Scientific Session

Saturday, October 14, 2006

Rochester

Moderator: Francois Elvinger and Lindsay Oaks

Virology Scientific Session

Sunday, October 15, 2006

Salon A

Moderator: Steve Bolin and Kyoung-Jin Yoon

10:30am	**Porcine Circovirus Type 2: ELISA for the detection of Antigen and Antibodies in Feces** - S. Guillossou, E. Deshaies, N. Brajon, P. Lopez, S Leterme	84
10:45am	**Do Elk Catch Foot-and-Mouth Disease?** - G.B. Ward, J. Rhyan, M. Kenney, H. Wang, M.Y. Deng, T. Gidlewski, M. McCollum, M. Salman, T.S. McKenna	85
11:00am	**A Liquid Phase Blocking ELISA for the Detection of Antibodies to 3D Non-structural Protein of Foot-and-Mouth Disease Virus** - H. Wang, M.Y. Deng, J. Barrera , L. Zsak, G. Mayr, B. Cerino, and T.S. McKenna	86
11:15am	**Experimental Aerosol Infection of Cattle (Bos taurus) with Ovine Herpesvirus 2 using Nasal Secretions from Infected Sheep**. - N. Taus, JL Oaks, K Galbreath, D.L. Traul, D. O'Toole, H. Li	87
11:30am	**Ovine Herpesvirus 2 mRNA Expression in Cattle and Bison with Malignant Catarrhal Fever** - J.L. Oaks, S. Pritchard, N.S. Taus, D.L. Traul, D. O'Toole, and H. Li	88
11:45am	**Serologic Survey for Vector-borne Disease Agents,Bovine Viral Diarrhea Virus, and *Mycobacterium avium subsp. paratuberculosis* in Free-Ranging White-Tailed Deer in Michigan** - A. A. Dye, L. Meader, N. L. Grosjean, S. R. Bolin, S. M. Schmitt, D. J. O'Brien, T. M. Cooley, M. K. Cosgtove, and S. Schaefer	89

Pathology Scientific Session

Sunday, October 15, 2006
Salon B

Moderator: Grant Maxie and Bill Layton

10:45am	**Mycoplasma bovis in Wild and Captive White-tailed Deer (*Odocoileus virginianus*) in Ohio: The other M. bovis in Deer** - J.R. Hayes, C.M. Almgren, S.D. Grimes, Y. Zhang	102
11:00am	**Foot-and-Mouth Disease in Pronghorn (Antilocapra americana) and Mule Deer (*Odocoileus hemionus*): Susceptibility, Clinical Signs, and Lesions** - J.C. Rhyan, T. Gidlewski, M. McCollum, G.B. Ward, F.M. Mohamed, M.Y. Deng, T.S. McKenna, M. Shalev, M. Salman	103
11:15am	**An Outbreak of Eastern Equine Encephalomyelitis in Wild White-tailed Deer** - S.D. Fitzgerald, S. Bolin, T.M. Colley, M. Kiupel, S.M. Schmitt, and Daniel J. O'Brien	104
11:30am **GRAD**	**Fatal necrotizing Fasciitis and Pneumonia in a Domestic Shorthair Cat associated with *Streptococcus canis***. - R. Sura, L.S. Hinckley, G.R. Risatti, and J. A. Smyth	105
11:45am **GRAD**	**Mortality-Associated Lesions of Endurance Sled Dog** - M.M. Dennis, R.J. Basaraba, D.A. Mosier, G.H. Cantor	106

Bacteriology Scientific Session

Sunday, October 15, 2006

Salon C

Moderator: Glen Songer and Loraine Hoffman

10:30am	**Experimental Challenge Model for *Actinobacillus suis* in Pigs** - D. M. Jordan, R. Taylor-Vokes, P. G. Halbur, L.J. Hoffman, R.F. Ross	118
10:45am	**Equine Colitis X Associated with Infection by *Clostridium difficile* NAP1/027** - JG Songer, S Dial, JS Brazier, RD Glock, GE Killgore, A. Thompson, B. Limbago	119
11:00am **GRAD**	**Immunohistochemical, Microbiological, and Molecular Detection of *Brucella abortus* in Rocky Mountain Elk** –A.M. Fluegel, W.H. Edwards, K.W. Mills, T.E. Cornish	120
11:15am	**Preliminary results from *Mycobacterium bovis* surveillance of free-ranging white-tailed deer in Minnesota** - A. Wünschmann, J. Schefers, Susan McClanahan, L . Cornicelli, M. Carstensen Powell, B. Hartmann, N.B. Harris, J.T. Nelson, B.V. Thomsen	121
11:30am	**Vaccination of White-tailed Deer with *Mycobacterium bovis* bacillus Calmette Guerín (BCG)** - M.V. Palmer, T.C. Thacker, W.R. Waters	122
11:45am	**Development of an Immunohistochemistry Assay for the Detection of *Erysipelothrix rhusiopthiae* Antigen in Formalin-fixed Tissues** - T. Opriessnig, L.J. Hoffman, and P.G. Halbur	123

Molecular Diagnostics Scientific Session

Sunday, October 15, 2006

Duluth

Moderator: Sharon Hitala and Kathy Kurth

Toxicology Scientific Session

Sunday, October 15, 2006

Rochester

Moderator: Catherine Barr and Patricia Talcott

10:45am	**Presumptive Zinc Toxicosis in a Dog Following Ingestion of a Brassiere** - K. Meyerholtz, C. Wilson, R. Everson, J. Weitzel, , J. Ellis, E. Whitcomb, and S. Hooser	153
11:00am	**Exposure of California Mountain Lions (*Puma concolor*) to Anticoagulant Rodenticides** - R. H. Poppenga, F. Uzal, S. Riley, W. Boyce, P. Swift, and R. Sauvajot	154
11:15am	**Two Cases of Barium Poisoning in Cattle** - T. Richards, D.L.Erickson, P.A. Talcott and T.V. Bazler	155
11:30am **GRAD**	**Toxicity of the Lichen Substance (+)-Usnic Acid in Ruminants** - R.N. Dailey, M.F. Raisbeck, D.L. Montgomery, R.S. Siemion, T.E. Cornish, and M.J. Vasquez	156
11:45am	**Evaluation of the Toxicity of *Adonis aestivalis* in Calves** – L. W. Woods, L. W. George, M. L. Anderson, D.M. Woods, M.S. Filigenzi, B. Puschner	157

USAHA/AAVLD Scientific Session

Monday, October 16, 2006
Salon AB

Co Chairs: Barbara Powers President-Elect AAAVLD; Lee Myers President-Elect USAHA

		Page
7:30 am	**WELCOME AND ANNOUNCEMENTS –** Barb Powers President Elect AAVLD Lee Myers President-Elect USAHA	
7:45 am	**Global Animal Surveillance for Public Health** – Juan Lubroth, United Nations Food and Agriculture Organization	159
8:15 am	**Zoonotic Disease Surveillance for Public Health** - Tracee Treadwell, Centers for Disease Control and Surveillance	160
8:45 am	**Integration of Food Surveillance Systems** – Pat McCaskey, Food Emergency Response Network	161
9:15 am	**Questions**	
9:30 am	**BREAK**	
9:45 am	**Transforming Animal Health Surveillance** – Brian McCluskey, USDA APHIS VS National Surveillance Unit	162
10:15 am	**Monitoring the health of wildlife and implementing the Global Avian Influenza Network for Surveillance of Wild Birds (GAINS) –** Robert Cook, Wildlife Conservation Society	163
10:45 am	**The Future of Bio Surveillance Systems** – Kimothy Smith, US Department of Homeland Security	164
11:15 am	**Questions**	
11:25 am	**Briefing on FAD Rapid Test Technology** – Barbara Powers, Colorado State University	
11:30 am	**Closing Remarks and Adjournment**	

First Plenary Session

Saturday, October 14, 2006
Salon AB

Moderator: Barb Powers

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Interspecies transmission of influenza viruses

Rueben O. Donis

Influenza viruses are important pathogens of humans and livestock, however they rarely cause disease in the primary host species from which they originate; ducks and shorebirds. Disease severity in humans and other mammalian species seems to be highest immediately after the introduction of a new influenza virus from the avian reservoir and decrease progressively over the years. Eventually, the adapted virus is replaced by a newly emerged influenza strain and the cycle repeats itself.

Influenza viruses are capable of genetic exchange and high rates of evolution. It has been suggested that pathogens with higher mutation rates will produce more genetic variants and be more likely to expand their host range. The population sizes of influenza viruses in its multiple hosts may also be a key factor in interspecies transmission leading to a new cycle of re-emergence, i.e.; a pandemic in the case of the human population.

The concept of interspecies transmission includes the ability to infect a new host species as well as the capacity to undergo sustained transmission in it. Transmission of influenza viruses across species barriers is a rare event due to the multiplicity of functional changes required for acquiring efficient infectivity and transmissibility in the new host. Interestingly, there are examples of a modest number of genetic changes being sufficient for transmission to occur but the underlying functions often remain elusive.

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Pathobiology of Avian Influenza viruses

M.J. Pantin-Jackwood[1]

Avian influenza virus causes serious disease in a wide variety of birds and mammals. Its natural hosts are wild aquatic birds, in which most infections are unapparent. Avian Influenza (AI) viruses are classified into 16 hemagglutinin (H1-16) and nine neuraminidase (N1-9) subtypes. Each virus has one H and one N antigen, apparently in any combination. Influenza viruses infecting poultry can be divided into two distinct groups based on their ability to cause disease in the major poultry species, chickens. The very virulent viruses cause highly pathogenic avian influenza (HPAI), in which mortality can be as high as 100%. These viruses have been restricted to subtypes H5 and H7, although not all of these subtypes can cause HPAI. Infections caused by these subtype viruses have been responsible for devastating epidemics in poultry. All other AI viruses can cause a milder, primarily respiratory disease, and is referred to as low pathogenic AI (LPAI).

Experimentally, HPAI viruses typically produce a similar severe, systemic disease with high mortality in chickens and other galliforme birds. However, these same viruses usually produce no infection or only mild disease in domestic ducks. Over the past decade, the emergent HPAI viruses have shifted to increased virulence for chickens as evident by shorter mean death times. Furthermore, the Asian H5N1 HPAI viruses have changed from producing inconsistent respiratory infections in ducks, to some strains being highly lethal with virus found in internal organs including the brain. Natural infection with HPAI virus in wild birds had been rare, until recently, when HPAI H5N1 outbreaks in wild birds have been reported in Asia, Europe, Near East and Africa. Across all bird species, the ability to produce severe disease and death is associated with high virus replication titers in the host, especially in specific tissues such as brain and heart.

Periodically, avian influenza viruses are transmitted to other hosts, including mammals, causing transitory infections and occasionally deaths. A small number of mammalian species, including pigs, seals, whales, minks, cats, tigers, leopards, and humans, are susceptible to natural infection with influenza viruses of purely avian genetic makeup. Transmission of avian influenza to humans has occurred with AI subtypes H5N1, H9N2, H7N7, H7N3, and H7N2, causing a spectrum of clinical disease, from conjunctivitis or mild respiratory infections to severe and fatal disease. The ongoing outbreak of H5N1 AI virus is of great concern because of the high human case fatality rate and the threat of a new influenza pandemic. Mammalian models, including mice, ferrets, pigs, primates and cats, are being used to evaluate the potential of AI viruses to cross the species barriers and infect domestic animal species and humans. AI viruses in these species have shown to be either highly pathogenic, replicating systemically (mice, ferrets, cats), or of low pathogenicity and replicating only in the respiratory tract (mice, pigs, primates), indicating that the outcome of infection with AI viruses is host and virus dependent.

[1]Southeast Poultry Research Laboratory, USDA-ARS, Athens, GA

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Rapid Diagnostics for Avian Influenza-Advances in Testing

David L. Suarez, Southeast Poultry Research Laboratory, ARS, USDA

A variety of tools are available for the diagnosis of avian influenza virus. They can be generally divided into the serologic diagnostic tests and direct virus detection tests. The serologic tests are important primarily for active surveillance to assure our poultry flocks are free of avian influenza for trade purposes. However in an outbreak, serologic detection plays only a limited role for identifying infected flocks because of the delay in antibody production. In an outbreak the rapid diagnosis of direct diagnostic tests are more crucial to identify and quarantine (or euthanize) infected flocks to limit the spread of the virus. In the U.S. three methods of direct diagnosis are commonly used, virus isolation, real-time RT-PCR (RRT-PCR), and antigen capture tests. Virus isolation is still a critical tool for the initial diagnosis and official reporting of an avian influenza outbreak, but because of the time required for a diagnosis, the use of virus isolation plays more of a supportive role for outbreak control. The primary frontline tests for avian influenza in an outbreak are the RRT-PCR and antigen capture tests.

The RRT-PCR was first used to control an avian influenza outbreak in Virginia in 2002. Since it's successful use in Virginia, the test has become the National Animal Health Laboratory Network standard for testing and is currently available in approved labs in 49 of the 50 states. This test has two steps, first screening with the matrix primers to identify any Type A influenza virus, and identification of subtype. Currently only primers to the H5 and H7 subtype are in common use, although primer sets for the other subtypes are in development. The basic test has not changed in the last 5 years, but several enhancements to the procedure are available. The RNA extraction step, critical for success of the test, has been a major area of research emphasis. This has included the validation of magnetic bead RNA extraction procedures that are amenable to robotics. Robotics to increase processing throughput has become a priority goal in the NAHLN. Several robotic systems are commercially available, but considerable work will be needed to validate a system that provides high quality RNA from a variety of samples at a reasonable cost. The equipment costs in particular need to be low enough to allow most or all NAHLN labs to work with the same standard. A second area of research is how to extract RNA from complex and dirty samples, including wild bird cloacal samples and tissue samples that commonly contain PCR inhibitors that can cause false negative results. Another area of improvement is the availability of an internal positive control for both quality control purposes and to help identify false negatives caused by PCR inhibition. The development of lyophilized or bead reagents have also been developed to improve quality control.

The antigen capture tests for the detection of Type A influenza virus has been around for many years. Up until just recently these antigen capture tests were human use tests that were redirected for veterinary use. However, in the last year a veterinary licensed test has become available. The antigen capture tests can provide a rapid diagnosis with a minimum of equipment. The primary drawback is lower sensitivity than virus isolation or RRT-PCR. The antigen capture tests do well from samples from birds that are sick or have died from avian influenza infection, but they may miss birds that are infected but appear healthy.

The U.S. has achieved a high level of preparedness for avian influenza, as shown by the response of outbreaks in Texas and DelMarVa. Additional research to integrate recent advances in testing will improve the sensitivity, repeatability, and through-put for the diagnosis of avian influenza and other veterinary pathogens.

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October 12 – 18, 2006
Minneapolis Hilton – Minneapolis, MN

Advanced Diagnostics and Expanded Capabilities for Foreign Animal Disease Detection and Surveillance

B.J. Hindson*[1], P.M. Hullinger[1], L.T. Tammero[1], R.J. Lenhoff[1], J. Perkins[1], M.T.McBride[1], T.R. Beckham[2] and B.M. Martin[3]

An introduction of foot-and-mouth disease (FMD) into the United States would be devastating to the agricultural community. FMD is a highly communicable viral disease of domestic and wild ruminants and swine. FMD is endemic to many countries but has not been seen in the US since 1929. Lawrence Livermore National Laboratory (LLNL), in collaboration with the Department of Homeland Security (DHS) and the United States Department of Agriculture Animal and Plant Health Inspection Service (USDA APHIS), has developed a candidate multiplexed diagnostic assay that simultaneously tests samples for foot-and-mouth disease virus and six other viruses that cause clinical signs in animals that are indistinguishable from FMD. The assay could enable early detection of FMD, critical for the reduction of spread and economic impact of the disease.

The National Animal Health Laboratory Network (NAHLN) together with the National Veterinary Services Laboratory (NVSL) at the Plum Island Animal Disease Center (PIADC) are the front-line for FMD diagnosis and response and are potential end-users of this new technology. During November and December of 2005, thirteen NAHLN laboratories and the NVSL, PIADC received training, "leave-behind" instrumentation, reagents and consumables to conduct the assay. These labs then participated in a nationwide interlaboratory comparison of the multiplexed assay, during which more than 3,000 blinded samples were analyzed and greater than 52,000 individual assays conducted. The overall assay success rate was greater than 92%.

As a part of this collaborative effort, two pilot demonstrations of a rapid, scaleable, high-throughput laboratory system were conducted at the California Animal Health and Food Safety Laboratory (CAHFS), University of California at Davis, CA and the Veterinary Diagnostic Laboratory, Colorado State University, Fort Collins, CO. This high-throughput system could be used to provide timely, scaleable diagnostic laboratory support during a foreign animal disease outbreak. During each demonstration, one-thousand clinical samples were processed within ten hours using only two technicians. Automation encompassed the transfer of liquid samples from collection vials to a 96-well plate, addition of an internal control, nucleic acid purification, multiplexed reverse transcriptase polymerase chain reaction (RT-PCR) amplification, liquid array hybridization, detection and data analysis. Integration of USDA APHIS's electronic sample identification, tracking, and results reporting technology with each participating laboratory's LIMS system enabled the live demonstration of a functional end-to-end system for surge capacity.

The analytical performance characteristics of the multiplex assay will be evaluated in the months to come. Once the acquisition and analysis of the analytical data is complete, diagnostic performance data will be gathered in collaboration with the NAHLN, and other US and international partners.

This presentation will review the development and characterization of the multiplex assay, the NAHLN interlaboratory comparison, the NAHLN high-throughput pilot demonstrations and future work planned for 2006-2007.

[1]Lawrence Livermore National Laboratory, Livermore, CA
[2] USDA, APHIS, VS, NVSL, Foreign Animal Disease Diagnostic Laboratory, Plum Island Animal Disease Center, Orient Point, NY.
[3] USDA, APHIS, VS, NVSL, Ames, IA

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Trends in Veterinary Diagnostic Laboratory Medicine - Implications for the Future

J. J. Andrews[1]

Since the early development of state, provincial and university operated North American veterinary diagnostic laboratories a number of important and significant changes have altered the way diagnostic services are provided. Most of the changes have made it easier for the diagnostician to provide the accurate, high quality service that is needed by our clientele, while others have hampered our efforts. I would like to take this opportunity to discuss with you my observations on what I consider some of the more significant trends and what they may mean for you and the veterinary laboratory diagnosticians of tomorrow.

One of the more apparent changes has been the loss of equal recognition at many "land grant" universities of the professional practice and diagnostic service provided by faculty diagnosticians with an individual faculty member's research accomplishments. Many universities are struggling with the evaluation of the quality of service provided while they have increasing expectations for scholarly or creative activities as a part of the tenure process. Universities have also created or allowed increased numbers of "non-tenure track" faculty who serve in primarily service positions.

State and university diagnostic laboratories are no longer serving limited geographical areas. Involvement is frequently much wider, well beyond state and often national borders. While disease diagnosis has always been an important activity, involvement in disease surveillance, food safety and public health activities, early recognition of emerging or foreign animal diseases (FAD's) and partnerships with Federal agencies such as USDA, CDC, USFWS, etc. are becoming much more common.

As with nearly every scientific discipline, the increased use of improved technology permeates veterinary diagnostic laboratories, enabling us to utilize cutting edge scientific and information technology to improve not only the scientific accuracy of our laboratory testing but the delivery and archival of the results. For example, futuristic tools such as high resolution histologic slide scanners await only the easy availability of high speed, high bandwidth internet connections and they will dramatically change the manner in which pathologists will be able to share slides on difficult cases with colleagues around the world.

Storage, and retrieval of data in most laboratories is now done utilizing one or more of many diagnostic laboratory database programs but sharing data between labs is still problematic. Necessary nomenclature and messaging standards such as SNOMED, LOINC, and HL7 have been adopted by the AAVLD but have not been fully implemented in many laboratories.

Testing methods have become increasingly sophisticated to meet increased the expectations of clientele including, but not limited to, rapid turnaround times and high level of competence. Diagnostic laboratories are increasingly concerned with detecting the presence of a disease causing agent rather than detecting an actual disease condition produced by the agent. Concepts such as "validation of test methods" and "quality assurance" are increasing topics of discussion and the driving force behind many laboratory activities.

While 30 years ago most state and university veterinary diagnostic laboratories were examining specimens mainly from domestic livestock and poultry, most laboratories now have increasing numbers of pet animal and wildlife submissions and decreasing numbers of traditional poultry and livestock disease outbreak investigations.

Veterinary diagnostic laboratories are increasingly asked to be involved in the investigation of suspected animal abuse and other cases with legal implications.

Tools used by epidemiologists to predict, identify and track outbreaks of new or emerging diseases are proving of value in reducing the impact of major disease outbreaks. Laboratories are adding the discipline of epidemiology and epidemiologists as a part of their scientific investigatory activities and teams.

Carcass and biomedical waste disposal and shipment of diagnostic specimens are becoming increasingly regulated, complicated and expensive.

The monetary support needed for veterinary diagnostic laboratories is increasingly being derived from fee and contract income and less from state public support. Because of this, the traditional "out-of-state surcharges" common 20 years ago, are becoming a disappearing entity

In colleges of veterinary medicine there is an increasing role of the veterinary diagnostic laboratories in the educational process. Some schools are replacing traditional "necropsy rotations" with "diagnostic rotations" emphasizing the importance of understanding and interpreting laboratory data from multidisciplinary sources.

If we stand still, we will quickly become obsolete. Veterinary diagnostic laboratories have undergone dramatic changes over the last several decades. These laboratories have and will continue to provide essential and needed services to the animal industries of their state or province and of the wider national and international communities through their rapid adaptation to the needs and technologies of today and the future.

1. Western Slope Veterinary Diagnostic Laboratory, Colorado State University, Grand Junction, CO

Bovine Virus Diarrhea Scientific Session

Saturday, October 14, 2006
Salon A

Moderator: Hong Li and David Steffan

AAVLD 49th Annual Conference
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Evidence for Bovine Viral Diarrhea Virus Infection in Captive Mountain Goats (*Oreamnos americanus*)

D. Nelson, [1] J. Evermann, [1] M. Dark, [1] D. Bradway, [1] N. Call, [2] J. Ridpath, [3] J. Haruna [1] and F. Rurangirwa [1]

Wildlife have emerged as potential factors in the epidemiology of bovine viral diarrhea virus (BVDV). According to VanCampen *et al* (2001) there has been serologic evidence of BVDV infection in more than 40 species of free-ranging and captive mammals. Reports of disease and isolation of BVDV from wild animals have been rare. In this report we examined the histological and virological results of two fatal cases of disease in young captive mountain goats from the same zoologic park.

The initial case was a 7 month old male mountain goat that died acutely. Selected fresh and fixed tissues, including lung, heart, small intestine, liver, and kidney, were submitted for histology and microbiology. A diagnosis of hepatitis and enteritis was made on the basis of the histologic changes. The liver lesions were suspected to be due to systemic bacterial showering from the intestinal lesions. Sections of the kidney, heart, and lung were histologically unremarkable. Sections of the tissues were stained by BVDV immunohistochemistry (IHC) and viral antigen was detected in the lung, liver, heart, and kidney. Virus isolation was positive for BVDV on lung and tissue pool (liver, kidney and spleen). Although the BVDV lesions were not considered severe enough for a fatal case of BVDV, viral-induced immunosuppression was noted as a potential contributing factor.

The second case was a 6 month old male mountain goat that was in respiratory distress. Upon death, selected tissues were collected for histology and microbiology. Sections of liver, kidney, spleen, heart and lung were examined by histology. Comparable fresh tissues were examined by microbiology. The histologic diagnosis was bronchopneumonia, myocarditis, hepatitis, and interstitial nephritis. The lung tissue was positive for *Corynebacterium pseudotuberculosis.* Lung and mesenteric lymph node were positive for BVDV by virus isolation. Staining for BVD by IHC was positive on sections of the spleen and lungs. As with the initial case, BVDV-induced lesions were minimal, although they were systemic, and viral-induced immunosuppression was considered as a potential contributing factor in the disease process.

Further investigation on the molecular epidemiology of the BVDV isolates from the two cases revealed them to be BVDV type 2.

This report is significant in demonstrating that mountain goats are not only susceptible to BVDV infection, but are also susceptible to systemic dissemination of the virus and possible immunosuppression.

[1]Washington Animal Disease Diagnostic Laboratory, Washington State University, Pullman, WA 99164; [2]Upper Valley Veterinary Clinic, Rexburg, ID 83440; [3]National Animal Disease Center/ARS/USDA, Ames, IA 50010

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Bovine Viral Diarrhea Virus in Alpacas from North American

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Bovine viral diarrhea virus (BVDV) infections of new world camelids have been sporadically documented over the past 30 years. Even in those cases of confirmed infections, the extent of the infection in the herd and the clinical outcomes did not warrant great concern in the camelid industry as evidenced by the sparse use of BVDV vaccines in llamas and alpacas. This lack of concern by alpaca owners regarding BVDV was replaced last year with intense interest with the publication of a report on a BVDV persistently infected cria (Carman et.al., J Vet Diagn Invest. 2005 Nov;17(6):589-93). The investigation into the circumstances of the birth of this animal suggested a much more widespread infection by BVDV than was previously recognized. Since this initial publication, we have had access to 18 BVDV infected animals or fetuses; some were defined as persistently infected, some were clearly acutely infected, and some could not be assessed from a single sampling.

The crias defined as persistently infected had characteristics very similar to BVDV persistently infected calves: poor weight gain, sporadic diarrhea or pneumonia, atypical "hairy" fleece and general "poor doer" status. The oldest animal detected was 30 months of age. As with calves, there were no pathognomonic lesions as defined by histopathology that would have identified these crias as persistently infected. Clinical pathology data were equally unhelpful. Staining of tissues by immunohistochemistry demonstrated variable amounts of BVDV antigen in most tissues sampled. Skin showed positive staining in all samples tested and neurons showed prominent staining in CNS tissue. All of the BVDV isolates that have been typed are of the BVDV 1b genotype.

The birth of a persistently infected cria on a breeding farm had a significant impact on herd health and clearly indicated the ease of spread of BVDV in naïve animals. One cria died shortly after birth and was subsequently shown by IHC to be BVDV positive. Another cria became infected near birth and maintained a virus positive status for over 2 months before clearing the virus. A pregnant female that seroconverted was intentionally aborted and the fetus was virus positive. From the time of seroconversion, it was estimated the fetus was infected for at least 50 days. Other infected pregnant females will be delivering crias over the next several months. The first to do so delivered a BVDV infected cria. Clearly, BVDV can spread in alpaca herds in the absence of contact with cattle.

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Osteopetrosis with Long Bone Fractures in Neonatal Calves Persistently Infected with Noncytopathic Bovine Viral Diarrhea Virus

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Osteopetrosis of long bones with fractures is occasionally reported in association with transplacental bovine viral diarrhea virus infection.[a, b] Here we report BVDV-associated osteopetrosis in a herd of beef cattle, of which three cases were confirmed histologically, and by virus isolation and/or immunohistochemistry. The mechanism whereby BVDV induces osteopetrosis was investigated.

Five affected full-term calves were born in a 200-cow commercial Angus-cross herd in western Nebraska between February 17 and 22 2006. The herd was unvaccinated for BVDV since the owners sought to produce 'natural' calves. The owners were also attempting to breed for small adult frame size in order to produce small cows without loss of weaning weight. Two affected calves were out of heifers. The remaining three were out of cows.

Affected calves were small, abnormally stocky, with short limbs and slightly domed heads. Calf #1 developed a fracture of the diaphysis of the right metatarsus when 3 days old, and a fracture of the left metatarsus when 8 days old. Fractures were treated by fiberglass casts, yet after 3 weeks no healing of the bone occurred. The calf was euthanized by the owner and was unavailable for examination. Calf #2 developed compound diaphyseal fractures of both metatarsi when 16 days old. It was euthanized at 20 days. Tissues, including bone, were submitted for examination. Calves #3 and #4 were collected alive for laboratory characterization. The left tibia of calf #3 fractured one day after delivery to the WSVL. Both calves were euthanized when 41 days old, and examined post-mortem. Calf #3 had fractures of three ribs and the calvarium was abnormally thin; no fractures were present in calf #4. Growth plates were unremarkable. The dimensions of long bones were comparable to those of healthy 2 week old calves.

Histological changes in the calves were consistent with cortical osteopetrosis. Noncytopathic BVDV was isolated from calves #3 and #4. BVDV antigen was detected in multiple tissues, including brain and cartilage, in calves #2, 3 and 4.

Details on testing of the rest of herd for persistently infected cattle, and radiographic and immunohistochemical features of the disease are in hand at the time this abstract was submitted, and will be presented.

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a. Constable PD et al: 1993, Femoral and tibial fractures in a newborn calf after transplacental infection with bovine viral diarrhoea virus. Vet Rec 132:383-385.
b. Scruggs DW et al: 1994, Osteopetrosis, anemia, thrombocytopeneia, and marrow necrosis in beef calves naturally infected with bovine viral diarrhea virus. J Vet Diagn Invest 7: 555-559.

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October 12 – 18, 2006
Minneapolis Hilton – Minneapolis, MN

Exposure to a Persistently Infected Heifer Can Cause Persistent Testicular Infection with Bovine Viral Diarrhea Virus

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Persistent testicular infections with bovine viral diarrhea virus (BVDV) have been reported after intranasal inoculation of post pubertal bulls with a BVDV-1 strain of the virus. However, the potential for development of persistent testicular infections after natural exposure to persistently infected animals has not been examined. Thus, the objective of this research was to evaluate the potential for production of persistent testicular infections after natural exposure of naïve bulls to heifers persistently infected with a BVDV-1 or BVDV-2 strain.

In the first trial, 4 seronegative 10-month-old bulls were exposed for 28 days in a pasture environment to a mature heifer persistently infected (PI) with a BVDV-1 strain. In the second trial, 4 seronegative 10-month-old bulls were exposed for 28 days in a pasture environment to a mature heifer PI with a BVDV-2 strain. In the third trial, 2 seronegative 20-month-old bulls were individually exposed for 2 days in a pen breeding situation to the BVDV-1 PI heifer during estrus. In the fourth trial, 2 seronegative 19-month-old bulls were individually exposed for 2 days in a pen breeding situation to the BVDV-2 PI heifer during estrus. Semen was obtained from the 8 young bulls at 59, 90, 120 and 150 days after initial exposure. Testicular biopsies were obtained at 28, 90 and 178 days. Semen was obtained from the 4 older bulls at 59, 90, 120, 150 and 181 days after initial exposure. Testicular biopsies were obtained after 181 days. Semen samples were assayed for BVDV using virus isolation and reverse transcription-nested PCR (RT-nPCR). Testicular biopsies were assayed for BVDV using virus isolation, RT-nPCR and immunohistochemistry.

The first trial resulted in 2 bulls with BVDV persisting in semen and testicular tissue. One bull produced semen positive for BVDV only by RT-nPCR at 90 days after initial exposure and testicular biopsies positive only by RT-nPCR at 90 and 178 days after initial exposure. One bull produced semen positive for BVDV only by RT-nPCR at 59, 120 and 150 days after initial exposure and a testicular biopsy positive only by RT-nPCR at 90 days after initial exposure. The second trial resulted in 1 bull with BVDV persisting in testicular biopsies as detected only by RT-nPCR at 28 and 90 days after initial exposure. The third trial resulted in 1 bull with BVDV persisting in semen and testicular tissue. Semen was positive for BVDV only by RT-nPCR at 90, 120, 150 and 181 days after initial exposure. The testicular biopsy taken from this bull 183 days after initial exposure was positive for BVDV by virus isolation, RT-nPCR and immunohistochemistry. The fourth trial resulted in no persistence of BVDV in testicular tissue or semen.

This research demonstrates that natural exposure of seronegative bulls to a PI heifer can cause persistent infection of testicular tissue with BVDV. The risk of these persistent testicular infections causing subfertility or venereal transmission of virus remains to be elucidated.

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Bovine Viral Diarrhea Virus Persistent Infections in Beef Breeding Herds: Utilization of Immunohistochemistry and Antigen Captive ELISA on Ear Notches

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Bovine viral diarrhea virus (BVDV) infections represent significant disease manifestations in cattle. Persistently infected (PI) cattle from fetal infections are considered the major reservoir of virus for susceptible cattle. Beef cattle in stocker and feedlot operations are often where diseases are recorded, yet the breeding herd is where the PI cattle begin. Control programs for BVDV PI cattle identification and removal should start in the breeding herd with proper biosecurity and vaccination programs. An education program was available in 2006 utilizing 29 ranches in southern Oklahoma and northern Texas represented by approximately 4500 breeding cows. The vaccination history for each ranch was available. The purpose of the project (which is ongoing in 2006 and will be repeated in 2007) is to perform a survey for PI cattle in these 29 ranches using available and validated assays. The objectives of the study included: (1) testing the 2006 calf crop using validated tests including the antigen capture ELISA (ACE) test on fresh notches and immunohistochemistry (IHC) on formalin notches. If positive animals were found the tests were repeated and serums obtained for viral growth and BVDV subtyping ; (2) testing new additions to the herd including bulls and replacement females; and (3) if PI calves were found in a herd, the dam of each PI calf was also tested.

There have been 4407 cattle tested representing the 29 herds. These 4407 cattle represent 4013 calves, 199 bulls, and 195 breeding females. There have been 26/4407 PI cattle identified (0.59%). There have been 5/29 (17.2%) herds with PI calves. Of the 4013 calves tested 25 were PI (0.62%). There were 0/199 bulls positive by ACE or IHC. There were 5 herds with PI calves: (herd A, 12/349 (3.4%); herd B, 1/134 (0.8%); herd C, 1/68 (1.5%); herd D, 1/338 (0.3%); and herd E, 10/191 (5.2%) . Thus in this study over half (3/5) had only a single PI calf rather than multiples or groups of PI calves. All the dams of the 25 PI calves were tested and 1 PI calf's dam was PI . For the breeding females only 1/195 was PI. Sequencing of the isolates from herd A , including the calves and PI dam indicate the subtype is BVDV1b, and there is 100% homology among all but one isolate, with that one with a 99.8% homology. By using PCR on the serum or fresh notch of calves from four herds, BVDV1 was identified. Subtyping for those isolates is in progress. In addition to the 25 cattle positive by both IHC and ACE we found 5 additional ACE positive calves which were IHC negative.

Results of the study reaffirm the detection of less than 1% BVDV PI prevalence of a survey population of 4013 calves. Reports indicate 0.13% to 2% of the US cattle as PI BVDV, and 4% to 10% of the herds with PI cattle. This study was in the range for PI, 0.59% but there were 5/29 herds (17.2%) with PI cattle. Finding herds with only one PI calf , is somewhat against the prevailing opinion that PI calves come in multiple numbers. However, the finding of a single PI calf in three herds emphasizes the requirement that any testing utilized, must detect a single PI animal.

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October 12 – 18, 2006
Minneapolis Hilton – Minneapolis, MN

Field Application of Pooled Reverse Transcriptase Polymerase Chain Reaction to Detect BVD Positive AC-ELISA Results

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Ear notch samples from 12,528 mixed breed cattle under 12 months of age were submitted for screening to detect the presence of persistent BVDV infection (PI) animals using a pooled testing technique. Conventional Reverse Transcriptase PCR (RT-PCR) was used to detect the presence of BVDV in pooled supernatant from individual tissue samples placed in individual tubes containing one ear notch and 1 ml of phosphate buffered saline (PBS). The 12,528 samples were randomly pooled by laboratory accession into pools no larger than 100 samples. A total of 174 pools were formed from all samples and compared with AC-ELISA for the corresponding samples. The average pool contained 72 samples with the range of pool size from as few as 12 samples to the maximum number of 100. BVDV was detected in twenty-seven of the pools by RT-PCR comparison of pool constituents by individual AC-ELISA revealed at least one AC-ELISA positive in 23 of the pools and four pools that an AC-ELISA could not be demonstrated. The total number of individual AC-ELISA positives detected in the 23 positive pools was 38 ranging from 1 to 8 positive AC-ELISA in a single pool the average number of AC-ELISA positives successfully screened using pooled RT-PCR was 1.4 with a median of 1. Follow-up testing on negative pools did not detect any positive AC-ELISA results on individual pool constituents. This information further supports the use of pooled conventional RT-PCR to screen for BVDV PI animals. However follow-up testing to confirm the suspect animal is truly a PI must be accomplished.

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October 12 – 18, 2006
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A Taqman-based Real-time RT-PCR with Internal Control for the Detection of BVD Virus in Ear Notch Samples

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BVD is one of the most economically important diseases of cattle. Persistently infected (PI) animals, as a result of *in utero* exposure prior to immunocompetence, are the main source of viral spread. Different sampling strategies and assay formats are currently available to detect PI animals. The sample used most commonly at present is the ear notch. Current assay formats to test ear notches include immunohistochemistry, antigen capture-ELISA (ACE) and direct FA. A small number of laboratories have also started to implement PCR assays for BVDV to test pooled ear notch samples. The size of the pools tested and the potential problems with inhibitors have been the predominant issues recognized.

We have developed a Taqman-based real-time RT-PCR (rRT-PCR) assay for BVDV with an internal RNA control to monitor inhibition of the reaction. The *in vitro* transcribed internal control or "mimic" RNA, designed based upon the method of Kleiboeker (2003), contains the same primer binding sites present in the BVDV target template. The BVDV-specific primers and probe were designed from conserved sequences within the 5' UTR. The assay amplifies both BVDV types 1 and 2 based upon testing of a validation panel provided by Dr. J. Ridpath (NADC, Ames, IA). The detection limit of the assay is 0.8 $TCID_{50}$.

Known FA and ACE BVDV-positive ear notch samples (n=42) were tested by rRT-PCR. When RNA isolated from individual PBS extracts was tested, 38 samples were positive and 4 were true negatives (reactions not inhibited). In contrast, when RNA extracted from pools of 10 ear notch tissue pieces (2 mm x 2 mm) was tested, where one in the pool is a known positive sample and the other nine are previously tested ACE negative samples, all 42 tissue pools were found positive. Subsequently, a total of 257 ear notch tissue pools were analyzed by rRT-PCR, in parallel with individual sample testing (2,570 samples) by ACE. Twenty-one pools were positive, 234 were negative and 2 showed an inhibited reaction. Each of the 21 PCR-positive pools contained at least one positive sample by ACE and all 236 PCR-negative pools were comprised of negative individual samples by ACE. Based upon these data, our current preference is to test pooled ear notch tissue pieces. The use of an internal control in the rRT-PCR assay is essential to rule out false negatives.

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October 12 – 18, 2006
Minneapolis Hilton – Minneapolis, MN

An Improved Integrated Workflow for High Throughput Viral RNA Isolation and Detection of Bovine Viral Diarrhea Virus

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Bovine viral diarrhea virus (BVDV) is a small (12.3 kb single-stranded positive RNA) enveloped virus in genus *Pestivirus* of the Flaviviridae family. BVDV infections are responsible for major economic losses in the dairy and beef industries around the world due primarily to poor reproductive functioning of infected cattle. The infection is maintained in a population by the presence of PI (persistently infected) cattle, which become immunotolerant to BVDV type 1 or type 2 early in utero. PI cattle shed large amounts of the virus intermittently throughout their life infecting other members of the herd, so rapid accurate detection of PI cattle is essential for controlling the spread of the virus.

We have developed an integrated "Sample to Signal" workflow process consisting of a rapid high throughput magnetic bead-based viral RNA isolation method, Ambion's MagMAX™ technology, and a highly sensitive one-step quantitative real-time reverse transcription PCR (qRT-PCR) for the purification and identification of BVDV. The magnetic bead nucleic acid isolation method can be used to process diverse samples such as whole blood, serum, plasma, ear notch, swabs, and Whatman® FTA® cards containing BVDV. The purified BVDV RNA is detected using our highly sensitive one-step qRT-PCR BVDV assay designed to identify BVDV types 1 and 2; the analytical sensitivity of this assay is 20 copies of BVDV RNA. In addition, this assay does not detect BVDV vaccines present in blood or ear notch samples allowing continued BVDV screening of a herd after vaccination. An outstanding feature of our integrated process is the use of XenoRNA-01, a proprietary 1000 nucleotide RNA transcript containing unique nucleotide sequences to serve as the internal process control. XenoRNA-01 is spiked into either the nucleic acid isolation process or the qRT-PCR to assess RNA purification or qRT-PCR efficiency, respectively. The XenoRNA-01 also monitors the presence of PCR inhibitors present in the RNA samples to ensure that false negative results are minimized, if not eliminated.

The BVDV RNA isolation process and qRT-PCR assay can be performed in 96-well formats manually or by automation. The entire simple process, from Sample to Signal, can be completed in approximately 3 hours for 96 samples. The rapid turn around time ensures single day BVDV identification for efficient and effective herd screening. We have validated our assay using greater than 400 field samples, and our results demonstrate 100% detection efficiency and 100% concordance with secondary ELISA and RT-PCR results. Our assay detects various subgenotypes of BVDV-1 and -2 from North America and Europe. Our integrated BVDV detection process contains a superior solution for BVDV identification.

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Avian Influenza and Canine Influenza Scientific Session

Saturday, October 14, 2006

Salon B

Moderator: Kristy Pabilonia and Letitia Curley

AAVLD 49th Annual Conference
October 12 – 18, 2006
Minneapolis Hilton – Minneapolis, MN

Comparison of RRT-PCR and Virus Isolation Findings from Live Bird Market Samples, New York

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Currently several laboratories are utilizing rapid diagnostic tests for the detection of avian influenza (AI) viruses. The traditionally recognized gold standard has been virus isolation which can take several days or weeks. Recently the real time reverse transcriptase-polymerase chain reaction (RRT-PCR) has been used to detect the presence of AI in tracheal samples. Application of the RRT-PCR can decrease the time to reporting to a day or less for appropriate samples. Surveillance sampling of tracheal, environmental and cloacal samples are collected from the live bird market system, though the latter two samples types do not lend themselves to the rapid diagnostics of the RRT-PCR at this time.

Since 2004, the RRT-PCR test has been used to detect AI in tracheal samples collected from the live bird market system in New York. Samples are tested against the RRT-PCR and if found positive (or suspect) via the matrix sequence, the sample is also tested with PCR primers and probes specific for H_5 and H_7 hemagglutinin genes of the AI virus. Samples identified as positive or suspect for the matrix sequence are forwarded to a second reference laboratory (Laboratory B) to confirm the initial testing and conduct virus isolation.

Laboratory A sent 468 matrix positive and 14 suspect samples to Laboratory B. Of the 468 positive samples, Laboratory B confirmed 373 of the samples as positive via RRT-PCR AI matrix. Of the 14 suspect samples forwarded to Laboratory B, nine were reported as positive by Laboratory B. Samples were then tested specifically for evidence of H_5 and H_7 antigen. Results from H_7 specific testing found concordance among 340 of the 447 samples classified as either positive or negative. In 262 instances both laboratories agreed that the samples were positive via the H_7 specific testing and for 78 samples both laboratories agreed that the results of the same test were negative for the sample. Of the 107 discordant results, 106 samples were classified as H_7 positive by Laboratory A and negative by Laboratory B. The Kappa statistic for this comparison is 0.460 indicating good reproducibility (Landis and Koch). Of the 106 samples positive at Laboratory A and negative via RRT-PCR at Laboratory B, 69 were virus isolation positive.

Using virus isolation as the 'gold standard' and comparing the H_7 RRT-PCR results for these same samples, Laboratory A had a sensitivity of 95.9% (331/345) and a specificity of 58.6% (68/116). Laboratory B had a sensitivity of 74.8% (255/341) and a specificity of 92.6% (125/135).

Regulatory action (orders to depopulate, clean and disinfect the facility) is dependant upon laboratory findings. In this instance, the reporting of RRT-PCR matrix and H_7 specific positive findings from Laboratory A initiate these actions. Ultimately ALL samples (cloacal, environmental and tracheal) are reported as either positive or negative for the AI virus. Considering all sample results, 103 markets were virus isolation positive for one or more samples. Twenty-five markets were classified as 'negative' based upon virus isolation and RRT-PCR findings. In 10 instances markets that were AI virus negative, had been instructed to depopulate, clean and disinfect based upon initial RRT-PCR results (positive matrix and positive H_7 specific RRT-PCR).

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Minneapolis Hilton – Minneapolis, MN

Increased pathogenicity of H5N1 Vietnam viruses in ducks

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Ducks and other wild aquatic birds are the natural reservoir of influenza type A viruses, which usually are nonpathogenic in these birds. The Asian H5N1 HPAI viruses have changed from producing a mild respiratory infection in ducks to some strains causing systemic disease and death. In order to further understand the change in pathogenicity of these new viruses in ducks, we studied the clinical disease, gross and microscopic lesions, and the tissue distribution of viral antigen in 2-week-old white Pekin ducks inoculated intranasally with two different strains of Asian origin H5N1 HPAI viruses isolated from ducks in Vietnam during 2006: A/duck/Vietnam-Ninh Binh/203/2005 and A/duck/Vietnam-Nam Dinh/218/2005. Ducks inoculated with these viruses were severely depressed the day after inoculation and presented neurological signs including tremors, uncontrollable shaking, marked loss of balance, tilted head, seizures, and paralysis. All ducks died, with a mean death time (MDT) of 3.3 days for ducks inoculated with A/duck/VN-Ninh Binh/203/2005 and 2.7 days for ducks inoculated with A/duck/Vietnam-Nam Dinh/218/2005. These ducks died more than a day earlier than ducks inoculated with an H5N1 Vietnam strain from 2004 (A/Vietnam/1203/04) or with any other previously studied Asian H5N1 virus. Grossly, dehydration, lung congestion, empty intestines, thymus atrophy and splenomegaly was observed in most ducks. In some ducks, congested and malacic brain, impacted proventriculus and gizzard full with intense bile staining of the mucosa, and pale pancreas, was also observed. Microscopically, the brain, heart, pancreas, skeletal muscle, and adrenal glands were the organs most consistently affected and viral antigen was most often detected in the parenchyma of these organs. These lesions were similar to the lesions produced by previously reported AI viruses pathogenic for ducks, however, and different from these, lesions and viral staining were also present in other organs including the lung, liver, spleen, thymus, proventriculus, gizzard, kidney and intestine, indicating an expanded tissue tropism. Both viruses studied were isolated in high titers from oropharyngeal and cloacal swabs and also from brain, heart, spleen, lung and muscle tissues collected at 2 days post-inoculation. These viruses are more pathogenic to ducks than previously studied AI strains, and this pathogenicity is related to increased viral replication in tissues.

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AAVLD 49th Annual Conference
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Detection of H5N1 in Meat Samples from Experimentally Infected Chickens

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The Asian H5N1 highly pathogenic Avian Influenza (HPAI) virus is a major threat to the poultry industry and is potentially zoonotic. In this study we monitored the infection of H5N1 in different meat samples (breast, thigh and heart) and tracheal swabs of infected chickens at different time points after inoculation with the Asian H5N1 HPAI virus A/WhooperSwan/Mongolia/244/05. Birds were sampled every 6 hours for 48 hours, with at least 10 birds being sampled at each time point. At the earlier time points and prior to the development of any clinical sign, birds were euthanized, and at later time points, sick or dead birds were examined preferentially. The tracheal swabs were evaluated for the detection of the virus by real-time RT-PCR (RRT-PCR), and with a commercially available AIV antigen capture test (FluDetect, Synbiotics). The meat samples were analyzed by an RRT-PCR test with a modified RNA extraction protocol and by virus isolation in embryonated chicken eggs. The virus was sporadically detected in meat and the tracheas of infected birds without any clinical signs of disease as early as 6 hrs post inoculation (PI), and was found in 100% of birds tested at each time point 24 hours PI and later. Similar results were seen among all meat sample types, but the heart had a higher concentration of virus in the early stages of infection (between 24 and 30 hrs PI), while breast and thigh meat had a higher concentration of virus in the later stages of infection (between 36 to 48 hrs PI). This study describes a rapid and sensitive method for the detection of HPAI virus in meat samples, which in conjunction with tracheal swabbing can provide an increased assurance for early detection of AIV in infected poultry.

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AAVLD 49th Annual Conference
October 12 – 18, 2006
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Demonstration of H5N1 Highly Pathogenic Avian Influenza Viral Antigen in Formalin-fixed Avian Tissue Specimens by an Avidin-biotin Immunohistochemistry Procedure

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The avidin-biotin immunohistochemistry (IHC) procedure has been used successfully to identify a variety of bacterial, viral and cellular antigens in formalin-fixed tissues. The procedure is rapid, reproducible, and specific making it an extremely useful method for screening diagnostic specimens. The objective of this work was to adapt this procedure to detect H5N1 highly pathogenic avian influenza (HPAI) viral antigen in avian specimens.

The Mongolian strain of H5N1 HPAI was inoculated into 8 15-week-old chickens. All 8 chickens exhibited clinical signs of avian influenza. The chickens died and were necropsied 16 hrs post exposure. Gross lesions observed in all 8 chickens included: cyanosis of comb and wattles, multifocal subcutaneous hemorrhage in the musculoskeletal system, severe peritracheal hemorrhage, multifocal pulmonary hemorrhage with edema, marked splenomegaly and multifocal mucosal congestion and hemorrhage in the digestive tract extending from the proventriculus, ventriculus, small and large intestine.

An avidin-biotin IHC procedure adapted from a method described by L.Vincent et. al. (*JVDI*, Vol 9:191-195, 1997) was performed on the tissue specimens collected at necropsy. The HPAI viral antigen was successfully demonstrated in formalin-fixed specimens of brain, comb, wattle, lung, spleen, pancreas, heart, proventriculus, ventriculus, large intestine, and small intestine.

The results of this limited study indicate the avidin-biotin IHC procedure may be a useful tool to screen avian tissue specimens for HPAI but it should be noted that there is little if any research data to indicate that this specific IHC procedure can distinguish between highly pathogenic and low pathogenic strains of Avian Influenza.

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AAVLD 49th Annual Conference
October 12 – 18, 2006
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Comparison of the WHO RT-PCR and WVDL rRT-PCR Assays for the Identification of N1 Avian Influenza Neuraminidase in Isolates and Clinical Specimens

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Avian Influenza (AI) is an important disease of poultry that is caused by an RNA virus of the orthomyxoviridae family. Waterfowl and shorebirds, the natural reservoirs of influenza A viruses, are the primary targets for the planned surveillance of the Asian strain of highly pathogenic (HP) H5N1 AI virus in 2006. Surveillance and early detection are keys to the control, management and eradication of the disease. An estimated 80,000 swab specimens will be collected in 2006 by the USDA Wildlife Services (WS) and the Department of Interior for the surveillance of H5N1 virus. The specimens will be screened for AI by the real-time RT-PCR (rRT-PCR) matrix (M) assay; those positive by the M assay will be further tested by the H5 and H7 rRT-PCR assays. Specimens positive for H5 will also be tested for N1 subtype RNA by the WHO RT-PCR and the WVDL rRT-PCR assays to establish a presumptive positive for H5N1.

The WHO conventional RT-PCR and the WVDL rRT-PCR assays (both designed to detect N1 neuraminidase gene) were compared with the gold standard, the neuraminidase-inhibition test. Dilution series of H5N1, H1N1, and H3N1 viruses were tested with the two procedures to compare endpoint detection. Specificity studies were conducted with a panel of reference and wild bird viruses with H5 and N1 antigens. Additionally, specificity studies were conducted with H5 positive clinical swab specimens.

The WHO RT-PCR and WVDL rRT-PCR assays were comparable in the detection of N1 RNA of the Asian H5N1 virus as well as reference viruses possessing the N1 neuraminidase. The WHO assay detected 17 of 18 N1 reference viruses while the WVDL assay detected all 18 viruses tested. Both assays were specific for AI N1 RNA as shown by negative test results when a panel of non AI avian respiratory viruses and H5-positive, N1-negative clinical specimens were tested. As for sensitivity, the WVDL rRT-PCR detected one more log of virus when tested with serial dilutions of SW/Mongolia/395549/05 H5N1 and SW/IA/31 H1N1 viruses compared to the WHO RT-PCR assay. Both the WHO RT-PCR and the WVDL rRT-PCR will be used to screen H5-positive surveillance specimens for N1 RNA. The assays will reduce the time needed to make a presumptive H5N1 diagnosis in clinical specimens.

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WVDL Real-time PCR Neuraminidase Typing Assay

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Waterfowl are the natural reservoir of influenza A, however many other species can be infected including humans, swine, seals, whales, ferrets, mink, canines and numerous orders of domesticated birds. Surveillance of influenza viruses in multiple species is critical for assessing risk and determining what measures are necessary to halt a pandemic. In the surveillance of influenza, the main criteria investigated are the subtype of the viruses and their antigenic properties. The subtype of influenza A is determined by the two major surface glycoproteins of the virus, hemagglutinin and neuraminidase. There are 16 H types and 9 N types. Typing of influenza neuraminidase has conventionally been done by the neuraminidase inhibition (NI) assay. The NI assay is complex, expensive, and time consuming. In the NI assay, the enzymatic activity of the neuraminidase must first be measured by titration of the virus; typing could take twenty-four hours. Those issues have been resolved in part by the application of conventional and real-time RT-PCR methods to subtype neuraminidases N1 and N2 from human isolates, but the assays are not developed to work with isolates from animal lineages. To further develop a molecular diagnostic panel applicable to isolates of animal lineages, primers and probes for real-time RT-PCR were designed to subtype the neuraminidases N1, N2, N3, N6, N7, N8, N9 of influenza A viruses, regardless of the species from which the virus originated.

Multiple alignments were performed with sequences taken from a national influenza database using NTI Align X software (Invitrogen). Primers and probes were designed in the most conserved areas of the neuraminidases that would not interact with other neuraminidase subtypes. The neuraminidase sub-typing sets were analyzed with human and veterinary samples (equine, swine and avian). Results from these real-time PCR assays were compared to typing done serologically by a neuraminidase inhibition assay or WHO conventional PCR. To date, the N1 and N2 assays have been tested and found to be specific for their intended neuraminidase subtype and there is no cross reaction with other respiratory pathogens. Preliminary validation data shows: 73/73 strains of human, swine, equine and avian origin tested correctly as N1; 69/70 strains of human and swine origin tested correctly as N2; 12/12 strains of avian origin tested correctly as N3; 19/21 strains of avian origin tested correctly as N6; 20/20 equine and avian strains tested correctly as N7; 17/17 strains of equine and avian origin tested correctly as N8; 10/10 strains of avian origin tested correctly as N9. The N2 and N6 assays are undergoing further development including sequencing to resolve the neuraminidase type of discrepant samples. Further cross reactivity tests will be performed for the remaining assays. The N4 and N5 assays are in development.

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AAVLD 49th Annual Conference
October 12 – 18, 2006
Minneapolis Hilton – Minneapolis, MN

Canine Influenza Virus Agglutination of Avian and Mammalian Red Blood Cells

T.C. Anderson[*1], J.M. Katz[2], E.P.J. Gibbs[1], and P.C. Crawford[3]

Canine influenza A subtype H3N8 virus (CIV) is a newly emerging, highly contagious respiratory pathogen of dogs. Molecular analyses of all eight genes indicate that the entire genome of an equine influenza H3N8 virus (EIV) has been transferred to dogs with subsequent dog-to-dog transmission. EIV H3 recognizes α2,3-galactose (Gal) sialic acid residues in host cell membranes as the receptor for virus attachment. Compared to EIV H3, there are several conserved amino acid changes in the CIV H3, including sites around the receptor binding pocket. However, little is known about the sialic acid receptor specificity of CIV H3. Receptor specificity correlates with agglutination of red blood cells (RBC) from different animal species that express α2,3-Gal, α2,6-Gal, or a mixture of both types of sialic acid linkages. Therefore, the purpose of this preliminary study was determination of CIV H3 receptor preference by agglutination of RBC from various avian and mammalian species.

For the hemagglutination assays, serial dilutions of the A/canine/FL/04 (H3N8) isolate were incubated with 0.5%, 0.75%, and 1% suspensions of chicken, turkey, goose, horse, dog, sheep, cow, pig, and human type O RBC. The RBC suspensions were prepared in phosphate-buffered saline (PBS) and PBS containing 0.5% bovine serum albumin (PBS/BSA). Incubations were performed in "V" and "U" bottom microtiter plates for 30 minutes and 1 hour at room temperature and 4°C. Hemagglutination units (HAU) were defined as the reciprocal of the last virus dilution that agglutinated the RBC. The HAU were compared for the different RBC species and incubation conditions.

The highest HAU occurred with 0.5% chicken and turkey RBC suspensions prepared in PBS and incubated with CIV in "V" bottom plates for 30 minutes at room temperature. A similar HAU was obtained with 1.0% dog RBC suspensions in PBS/BSA incubated in "V" bottom plates at room temperature for 1 hour. The HAU for 1.0% human RBC in PBS/BSA was similar to that for dog when incubated in "U" bottom plates for 1 hour at 4°C. Agglutination of chicken, turkey, dog, and human RBC was inhibited by preincubation of the virus with serum from dogs infected with CIV. The HAU was lower for goose RBC than turkey and chicken RBC. The HAU were much lower for horse, cow, and sheep RBC regardless of the incubation conditions. There was nonspecific agglutination of pig RBC under all incubation conditions.

Chicken and turkey RBC express a mixture of α2,3-Gal and α2,6-Gal sialic acids, while goose, horse, cow, and sheep RBC express α2,3-Gal sialic acids. Human RBC primarily express α2,6-Gal sialic acids. Previous reports suggest that dog cells contain a mixture of both types of sialic acids similar to the chicken and turkey. The results of this study suggest that CIV H3 preferentially agglutinates RBC containing both α2,3-Gal and α2,6-Gal sialic acid receptors (chicken, turkey, dog).

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October 12 – 18, 2006
Minneapolis Hilton – Minneapolis, MN

Development of a Hemagglutination Inhibition Assay for Diagnosis of Canine Influenza Virus Infection

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Canine influenza A subtype H3N8 virus (CIV) is a newly emerging, highly contagious respiratory pathogen for dogs. Molecular and phylogenetic analyses indicate that CIV is genetically related to 2002 and 2003 isolates of equine influenza H3N8 virus (EIV). Hemagglutination inhibition (HAI) assays are routinely used for serological diagnosis of influenza virus infections. Specific attachment of antibodies to the virus hemagglutinin protein interferes with virus binding to sialic acid receptors on red blood cells (RBC), thereby inhibiting hemagglutination. The optimal reagents and incubation conditions for CIV HAI are currently not well-defined. The purpose of this study was to determine the most appropriate RBC species to use, if canine serum should be pretreated to remove nonspecific inhibitors, and whether EIV can be used instead of CIV.

To determine the best RBC species and optimal incubation conditions, hemagglutination assays were performed by incubating serial dilutions of A/canine/FL/04 virus stock with 0.5%, 0.75%, and 1% suspensions of chicken, turkey, goose, horse, dog, sheep, cow, pig, and human type O RBC. Incubations were performed in "V" and "U" bottom microtiter plates for 30 minutes and 1 hour at room temperature and 4^{o}C. Hemagglutination units (HAU), defined as the reciprocal of the last virus dilution that agglutinated RBC, were compared for the different RBC species and incubation conditions. The highest HAU occurred with 0.5% chicken and turkey RBC suspensions incubated in "V" bottom plates for 30 minutes at room temperature. Therefore, these RBC species and incubation conditions were selected for the HAI assay.

For the HAI assay, A/canine/FL/04 virus was incubated for 30 minutes at room temperature with serial dilutions of serum from uninfected and CIV-infected dogs. The sera were incubated with virus directly or after pretreatment with RDE or periodate. A 0.5% turkey RBC suspension was added and incubated for 30 minutes at room temperature. Antibody titers, defined as the reciprocal of the last serum dilution that inhibited hemagglutination, were compared for the untreated and treated sera. Untreated canine sera contained nonspecific inhibitors of hemagglutination that were effectively removed by pretreatment with RDE or periodate. The assay had a sensitivity and specificity of 100% with a cut-off antibody titer of 32.

Sera from 41 CIV-infected dogs were tested in the HAI assay with canine/FL/04, equine/KY/91, equine/KY/93, equine/NY/99, and equine/OH/03 influenza virus strains. Antibody titers to canine/FL/04 ranged from 32 to 2048. Titers to equine/OH/03 were 2-to 4-fold lower than corresponding titers to canine/FL/04 and misdiagnosed one dog as uninfected. Titers to equine/NY/99 were 8- to 32-fold lower than corresponding titers to canine/FL/04 and misdiagnosed 12 (29%) of the dogs as uninfected. Titers were negative in all samples when equine/KY/93 and equine/KY/91 were used resulting in misdiagnosis of all dogs.

In conclusion, the optimum HAI assay for accurate diagnosis of CIV included the use of 0.5% chicken or turkey RBC suspensions, RDE- or periodate-treated sera, a CIV virus isolate, and incubation in "V" bottom microtiter plates for 30 minutes at room temperature.

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Pathology Scientific Session

Saturday, October 14, 2006

Salon C

Moderator: Scott Fitzgerald and Peg Miller

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October 12 – 18, 2006
Minneapolis Hilton – Minneapolis, MN

DIAGNOSTIC AGREEMENT AMONG SURGICAL PATHOLOGISTS EVALUATING DIGITS AMPUTATED FROM CATS AND DOGS

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Agreement among pathologists interpreting histologic specimens is an area of interest within human pathology, but little work has been done in veterinary pathology. Diagnostic agreement among pathologists of histopathologic sections of amputated digits from dogs and cats submitted to multiple diagnostic laboratories was examined. Histologic sections previously used to make a diagnosis were examined using blinded review to determine the repeatability of diagnosis. Five hundred-thirteen total cases were reviewed and complete agreement was reached in 409 (79.7%). Of the 104 instances of disagreement, 77 (74.0%) had important implications for clinical management of the animal. Blinded third party review of these cases of disagreement produced a third diagnosis that was different from both the original and second diagnosis 24 times (23.1%). Agreement rates among cases submitted by various institutions or species were not statistically significantly different from each other. The diagnosis of keratoacanthoma was disagreed with in 19 of 21 diagnoses (90.4%). No other individual diagnosis was similarly disputed. The overall level of disagreement is large and was similar to that reported in human pathology and suggests that further study would be useful in veterinary pathology.

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October 12 – 18, 2006
Minneapolis Hilton – Minneapolis, MN

Expression of MUM1 Protein in Canine Lymphomas and Other Round cell Tumors

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MUM1 protein (multiple myeloma oncogene-1) is encoded by the gene originally identified in a rare translocation associated with multiple myeloma. This protein is involved in the normal development of germinal centers and plasma cells. We reported (proceedings 48th Annual Conference AAVLD, Hershey, PA) that most canine plasmacytomas express MUM1 protein as detected by immunohistochemistry. This protein is also expressed in some human lymphomas (lymphoplasmacytic, diffuse large B-cell) and activated T-cells. MUM1 protein is detected mainly in medullary cords of canine lymph nodes but rare lymphocytes are also labeled in the light zone of the germinal center and in the paracortex. Our hypothesis is that MUM1 is expressed in some canine lymphomas.

As part of the validation of the mouse monoclonal antibody MUM1p for the diagnosis of canine plasmacytomas we examined its expression in 90 formalin-fixed, paraffin-embedded canine round cell tumors. These tumors included 66 lymphomas (33 B-cell type and 33 T-cell type), 16 cutaneous histiocytomas, 3 histiocytic proliferations, 3 mast cell tumors, 1 thymoma, and 1 hyperplastic lymph node. Antigen retrieval consisted of incubating slides in EDTA buffer, pH 9.0, in an autoclave at 90-95 C for 20 min. A mouse monoclonal antibody (Dako laboratories, code M7259) against recombinant GST-MUM 1 protein, was diluted 1/500. Sections were incubated with the primary antibody for 60 min. at room temperature. The detection system was a nonavidin-biotin, polymer-based, peroxidase system (ImmPRESS™). Immunophenotyping of lymphomas was done using antibodies to CD3, CD79a, and CD20 antigens.

Eight of 90 tumors were positive for MUM1; all 8 were B-cell lymphomas (6 nodal, 1 cutaneous, 1 intestinal). The staining was nuclear. MUM1 was expressed strongly in 4 cases although it was detected in fewer neoplastic cells than with antibodies to CD20 and CD79a. Rare positive cells (less than 1%) were observed in many cases and were considered to be reactive lymphocytes.

Our results indicate that MUM1 expression in canine lymphomas is uncommon but limited to those of B-cell type. Approximately 12% of lymphomas in our series were positive for MUM1. Two of the MUM1-positive lymphomas (1 cutaneous, 1 intestinal) had distinct plasmacytoid differentiation and might be considered plasmacytomas. Based on these and previous results MUM1 protein is most useful to support a diagnosis of plasmacytoma although some B-cell lymphomas may also be positive.

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AAVLD 49th Annual Conference
October 12 – 18, 2006
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Subpial necrotising myelopathy following parenteral copper administration in sheep

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The cause of neurological disease following parenteral copper administration was investigated in 11 sheep flocks. Between 1 and 4% of ewes developed ataxia, paresis and recumbency 2 – 3 days following parenteral administration into the neck of copper-containing compounds (including copper heptonate) intended for intramuscular injection. All died or were killed humanely for welfare reasons 3 days - 12 weeks later. Nineteen of these sheep were examined post mortem. Histological examination of the neuraxis revealed circumferential subpial white matter necrosis with abundant Marchi-positive degenerate myelin and Gitter cell accumulation in cervical spinal cord with similar lesions sometimes also involving subpial white matter in thoracic spinal cord and ventral aspect of caudal medulla oblongata. Immunohistochemistry for glial fibrillary acidic protein and neurofilament revealed marked intralesional white matter astrocyte depletion and relative axonal preservation. In one affected ewe, fixed spinal cord (C2) copper concentration was 310 umol/kg DM compared with 56 umol/kg DM in an unaffected adult ewe submitted for PME on same day to the same laboratory. Parenteral copper had been administered 2-3 weeks earlier to this unaffected ewe and sample handling including duration of fixation, and tissue selection for copper analysis was identical to the affected ewe.

This subpial lesion does not appear to have been recorded previously in sheep. However, marked degeneration of subpial white matter of spinal cord has been reported to follow infusion of various copper compounds into the CSF (e.g. via lateral ventricle) in cats and rabbits, whereas administering iron containing compounds by the same route caused no histological change (Vogel and Evans, 1961; Wisniewski et al 1965). The striking similarity between these lesions in cats and rabbits and those observed in the sheep strongly suggest that these cases of ovine myelopathy arose as a consequence of high local copper concentrations, either by diffusion of copper compounds for example via intervertebral foramina or following inadvertent epidural or subdural introduction of copper-containing preparation. Lipid peroxidation mediated by copper ions may have resulted in the observed injury to myelin and white matter astrocytes.

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Minneapolis Hilton – Minneapolis, MN

Association Between Aneurysm and Rupture of Abdominal Arteries and Low-Normal Liver Iron Concentrations in Cattle

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Aneurysm and rupture of abdominal arteries in diary cattle is a sporadic disease characterized by exsanguination and sudden death. Studies in swine and turkeys have demonstrated a correlation between heavy metals and arterial rupture. The purpose of this study was to determine if the levels of Cu, Fe, Mn, S, and Zn in fresh/frozen, fixed, and paraffin-embedded liver samples are associated with the development of abdominal artery rupture in cattle. Twenty affected cattle and nine age-matched control cattle were included in the study. Heavy metal concentrations within the liver were determined for each sample type available for each animal using Inductive Couple Plasma Analysis. The Wilcoxon rank-sum test was used to compare the liver values of heavy metals from affected and control animals for each sample type. Iron levels were significantly lower in the affected cattle group when compared to the control group for all sample types (fresh/frozen: p=0.0121, fixed: p=0.0006, and paraffin: p=0.0067). There was no significant difference in the liver concentrations of Cu, Mn, S, and Zn between groups for all sample types. These results indicate that low normal iron concentrations in the liver are strongly correlated with the development of abdominal artery rupture in cattle.

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October 12 – 18, 2006
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Role of Iron in Inflammation in liver in dogs and rats

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An increased concentration of iron in the liver was found in 54 of 95 dogs that were suspected to have liver disease based on clinical signs or increased serum enzymes that indicate liver damage. The iron was present in Kuppfer cells and macrophages in the portal areas, as visualized with Prussian blue stain. Many dogs with increased liver iron had inflammatory lesions and in general those with the highest iron concentrations had the most severe inflammation. However, any causal relationship between iron and the inflammation in these dogs remains unknown. It is theorized that iron can serve as a catalyst for damaging oxidative reactions and could exacerbate inflammatory lesions.

A rat model was used to study the relationship of high iron concentration and hepatocellular injury. A high concentration of iron in the liver was achieved by feeding rats an iron enriched diet for 6 weeks. The average liver iron concentration was 2900 ppm on a dry weight basis. The iron was present in hepatocytes and Kuppfer cells in portal areas. These rats also had a very slight increase in serum enzymes that indicate hepatocellular injury. Hepatocellular injury was induced by intraperitoneal administration of carbon tetrachloride which caused necrosis of central lobular hepatocytes. Livers of rats fed the high iron diet and given carbon tetrachloride and rats fed a standard diet and given carbon tetrachloride had the same histologic appearance and similar levels of serum enzymes that indicate liver damage.

It may be that iron does not exacerbate lesions or that iron only exacerbates lesions that are induced in the zones of the liver in which the iron is present. The relationship between increased iron concentration and inflammation in liver is unclear.

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AAVLD 49th Annual Conference
October 12 – 18, 2006
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An Outbreak of Porcine Dermatitis and Nephropathy Syndrome: Pathology and Unanswered Questions

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Porcine dermatitis and nephropathy syndrome (PDNS) and postweaning multisystemic wasting syndrome (PMWS) are often categorized as porcine circovirus associated diseases (PCVAD). Historically, PDNS has a low incidence (usually < 1%) but a high fatality rate, and is characterized by necrotizing, exudative glomerulonephritis, interstitial nephritis, and necrotizing vasculitis in the kidneys, dermis, and, in many cases, other tissues. PMWS is more common and characterized by failure to thrive and microscopic lesions of lymphoid depletion and granulomatous inflammation in lymphoid and other tissues, especially the liver and lungs. Large quantities of porcine cirovirus 2 (PCV2) can be demonstrated in PMWS lesions, but PCV2 is typically present in low quantities in tissues from pigs with PDNS. Although categorized as a PDVAD, the cause of PDNS is unknown. We report a series of cases with a dramatically high incidence of PDNS.

Beginning in December 2005, numerous swine producers in central and eastern Kansas noted a dramatic increase in mortality beginning 4 to 5 weeks after placing pigs in the finisher buildings when the pigs were 14 to 15 weeks-old. In many cases mortality reached 10% to 20% within the month following onset. Many pigs had clinical signs and pathologic lesions of PMWS, but of 81 pigs or sets of tissues submitted to the diagnostic laboratory from 13 herds, 23 had gross and microscopic lesions of PDNS and most producers reported having multiple other pigs with skin lesions of PDNS. Pigs with PDNS were in good body condition and the microscopic lesions consisted of necrosis in glomerular tufts with fibrin filling Bowman's spaces, protein in most tubules, fibrinoid necrosis of renal arteries, interstitial nephritis, and necrotizing vasculitis of the dermis with neutrophilic inflammation and epidermal necrosis. The proportion of affected glomeruli varied from 100% to less than 50% and the chronicity of renal lesions varied considerably. Many pigs also had fibrinoid necrosis of arteries in a wide variety of tissues that included the spleen, lymph nodes, epicardium, adrenal glands, stomach, intestines, and meninges, with the spleen most often affected. Arteritis was not found in the lungs. Lymphoid follicles in lymphoid tissues from most pigs contained groups of epitheloid macrophages and a few multinucleated giant cells. Immunohistochemical staining demonstrated either very small quantities or no PCV2 antigen, which was most commonly located in lymphoid follicles. In addition, many pigs had infiltrates of lymphocytes and plasma cells in many tissues, including the brain, spinal cord, heart, and adrenal glands. PCV2 antigen was not found in association with this mononuclear inflammation. There were an additional 3 pigs with marked interstitial nephritis and vascular necrosis in the kidney that did not have the exudative glomerulonephritis or epidermal necrosis characteristic of PDNS. They also had little PCV2 antigen in their tissues. In addition, 6 pigs from 3 herds had severe pulomonary edema and, in 3 cases, pleural and pericardial effusions. These pigs did not have vascular lesions in the kidneys or other tissues except pigs with severe pulmonary edema had necrotizing, fibrinoid vasculitis of pulmonary arteries. Many veins and/or lymphatics contained fibrin thrombi and there was fibroplasia within and surrounding many arteries. Between the luminal epithelium and smooth muscle of many bronchi there was a thick layer of immature spindle cells, which has been described as a PCV2 associated change. The lungs with pulmonary vasculitis contained considerable PCV2 antigen, as did the lymphoid tissue of pigs from which it was available.

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AAVLD 49th Annual Conference
October 12 – 18, 2006
Minneapolis Hilton – Minneapolis, MN

Rickets-like Osteodystrophy in Transgenic Pigs Generated by Nuclear Transfer

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Somatic cell nuclear transfer (SCNT) has been used to create transgenic pigs. This technique is relatively inefficient and may produce animals exhibiting cardiovascular and other abnormalities. During the creation of pigs transgenic for n-3 fatty acid desaturase from *Caenorhabditis elegans* we observed a rickets-like syndrome with widening of the costochrondral junctions and of the physes of all long bones resulting in lameness in 8/8 pigs alive at weaning. Pigs exhibiting these lesions had elevated serum parathyroid hormone and less than normal ionized Ca^{++}. These data were suggestive of renal secondary hyperparathyroidism; however there were minimal to mild renal DZ on histopathology with a minimal elevation of BUN and no alteration of creatinine. There was also elevation of serum creatine kinase, an enzyme marker of skeletal muscle disease/ rhabdomyolysis. Histologically there were abnormal retention of cartilage, deficient mineralization of bone and mild increase of calcitonin-positive cells in the thyroid glands.

Dietary n-3 fatty acids decrease osteoclastogenesis and loss of bone mass in ovariectomized mice and have been speculated to protect post-menopausal women against osteoporosis. However, this rickets-like syndrome was observed in both transgenic and non-transgenic control animals suggesting that it is the result of the nuclear transfer technique and not the n-3 fatty acid desaturase transgene.

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Soft bone and eggshells in commercial egg layer flocks

A. Singh Dhillon and Curt Nelson

Nine houses containing 800,000 egg layers were affected by severe soft shell egg problems with an increase in mortality. Signs of a respiratory disease were not reported from any of the flocks. Chickens submitted for necropsy from two houses had lesions of soft fragile and crooked breastbone. The ribs were soft and caved in. Rupture of ovarian follicles and associated egg yolk peritonitis was present in all dead birds necropsied. Choanal swab samples tested from two affected flocks were negative for AI and NDV by RRT-PCR. Results of virus isolations were negative. Analysis of vitamin and mineral supplement received was found to contain no Vitamin D3. The egg production loss and mortality continued for three weeks. Excess mortality of 7, 144 hens and a loss of 4,412 cases of eggs were present as result of Vitamin D3 deficiency.

Avian Health and Food Safety Laboratory

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Transmissible Spongiform Encephalopathy and Virology Scientific Session

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Identification and Characterization of U.S. BSE Cases

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Bovine spongiform encephalopathy (BSE) surveillance has been ongoing in the United States since the early 1990's and initial testing was done at the U. S. Department of Agriculture (USDA), Animal and Plant Health Inspection Service (APHIS), Veterinary Services (VS), National Veterinary Services Laboratories (NVSL), utilizing routine histopathology exclusively. In 1995, the immunohistochemistry (IHC) test was incorporated into surveillance testing in addition to routine histopathology. By 1999, virtually all BSE screening was performed by IHC, and by 2001 the NVSL had switched to an automated IHC procedure. In 2002 and 2003, the NVSL tested about 20,000 high risk animals each year by IHC. In December 2003, an animal was identified by IHC as positive for BSE (Case 1); this animal was determined to be imported from Canada. In June 2004, the USDA began its enhanced surveillance program as a shared effort between selected state veterinary diagnostic laboratories and NVSL, as part of the National Animal Health Laboratory Network. The plan called for testing as many targeted high risk animals as possible in a 12-18 month period. From June 1, 2004, through June 12, 2006, over 730,000 animals have been tested (Bio-Rad ELISA). Of those tested, two animals (Cases 2 and 3) have been identified as positive for BSE.

While all three cases were strongly positive by Bio-Rad ELISA, Cases 2 and 3 have common features which are distinct from Case 1. Definitive spongiform changes in the obex, strong immunohistochemical reactions, and Western Blot patterns similar to European BSE cases were observed in Case 1. In contrast, Cases 2 and 3 did not contain definitive histological lesions of BSE and the IHC staining was less intense than Case 1. In addition, Cases 2 (approximately 12 years) and Case 3 (approximately ten years) were older animals while Case 1 was 6.5 years old.

Using Western Blot analysis, PrPSc from Case 1 showed molecular features similar to typical BSE isolates, whereas PrPSc from Cases 2 and 3 revealed an unusual molecular PrPSc pattern; molecular mass of the unglycosylated and monoglycosylated isoform was higher than that of typical BSE isolates. Case 1 contained more PrPSc per brain tissue mg equivalent compared with Cases 2 and 3 using antibody 6H4. In Western Blot analysis, Case 2 and Case 3 were strongly positive with antibody P4, while Case 1 was negative or weakly positive with P4.

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Lymphoid involvement in pre-clinical chronic wasting disease in adult mule deer (*Odocileue hemionus hemionus*)

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The exact dissemination mechanism of the chronic wasting disease (CWD) associated prion, PrP^{CWD}, within natural cervid hosts is not completely understood. Strong evidence suggests that the peripheral nervous system is important in this process. In addition, the tropism of PrP^{CWD} for lymphoid tissue has been extensively described. PrP^{CWD} has been demonstrated in various lymphoid tissues of experimentally and naturally infected mule deer (*Odocoileus hemionus*). However, the extent to which PrP^{CWD} is distributed in lymphoid tissues of pre-clinical free-ranging mule deer has not been reported. We describe six, apparently healthy, male and female mule deer, sampled as part of an ongoing test and cull CWD management program at Rocky Mountain National Park, Colorado, USA. Each deer was found to have immunohistochemical staining (IHC) consistent with CWD in a palatine tonsil biopsy sample. Deer were euthanized and full necropsies performed. Numerous tissues were collected to investigate the distribution of PrP^{CWD} during the pre-clinical stages of disease. Five of the animals were considered to be in the early stages of CWD and one was in a mid disease stage. Lymphoid tissues of the head, neck, thorax, and abdomen including peripheral lymph nodes were IHC positive. Additionally, hemal nodes of the neck, thorax and abdomen were positive. The thymus glands were IHC negative. This report documents the wide distribution of lymphoid tissues where PrP^{CWD} may be found during the early stages of CWD in mule deer. Furthermore, PrP^{CWD} accumulation in hemal nodes suggests the potential for hematogenous dissemination of PrP^{CWD}.

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October 12 – 18, 2006
Minneapolis Hilton – Minneapolis, MN

Utility of rectal mucosal biopsies for detecting chronic wasting disease in Rocky Mountain elk (*Cervus elaphus nelsoni*)

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Preclinical diagnostic tests for transmissible spongiform encephalopathies have been described for mule deer (*Odocoileus hemionus*), using biopsy tissues of palatine tonsil; for sheep, using lymphoid tissues from palatine tonsil, third eyelid and rectal mucosa. The utility of rectal mucosal biopsies was investigated in Rocky Mountain elk (*Cervus elaphus nelsoni*), a species for which there is not a live-animal diagnostic test. Postmortem rectal mucosal sections (PRMS) were examined from 308 elk from two herds that were depopulated. The results of the PRMS were compared to immunohistochemical (IHC) staining of the brain stem (vagus nucleus), retropharyngeal lymph nodes and palatine tonsil. Seven elk were found positive in the brain stem and cranial lymphoid tissues, however, only six of these elk were positive in the PRMS. The remaining 301 elk in which PrP^{CWD} was not detected in the brain stem and cranial lymphoid tissues also were free of PrP^{CWD} in the PRMS. Rectal mucosal biopsies (RMB) were taken from 421 live elk from three ranches in which at least two previous cases of CWD had occurred. Three positive non-clinical elk were found in one farm. These elk were killed and CWD was confirmed. PrP^{CWD} also was found around neurons and in non-myelinated nerves within the submucosa. PrP^{CWD} also was found in the connective tissue of the lamina propria of the infected elk. The number of follicles counted in PRMS and biopsies decreased with age of animal. Examination of postmortem rectal mucosal sections is easy and rapid to perform and may be suitable as a diagnostic test as part of an integrated management strategy to detect CWD in elk.

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AAVLD 49th Annual Conference
October 12 – 18, 2006
Minneapolis Hilton – Minneapolis, MN

Preclinical Detection of Ovine Scrapie in Rectal Biopsy Tissue using an ELISA

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Earlier studies of rectoanal mucosa-associated lymphoid tissue (RAMALT), obtained from animals at various stages of disease, were sourced and subjected to immunohistochemistry (IHC), and Western blot (WB) analysis. These tests have shown that PrP^{sc} could be detected with a consistency comparable to central nervous system (CNS), and other lymphoreticular system (LRS) tissues. The aim of the present work was to investigate the suitability of using an IDEXX post mortem PrP^{sc} ELISA for the detection of preclinical scrapie in rectal biopsy tissue as a diagnostic tool for live animal testing.

An initial small scale evaluation was conducted with the IDEXX Herdchek Chronic Wasting Disease Antigen Test Kit (CWD-EIA). The test was conducted according to the package insert with ovine rectal mucosal tissue replacing the cervid retropharyngeal tissue normally evaluated in the test. This preliminary evaluation resulted in a specificity of 100% (n=38) and diagnostic sensitivity of 75% (56/75 positive). Based on this promising data, the decision was made to further optimize the IDEXX CWD-EIA for rectal mucosa. A small panel of IHC positive and negative tissue was made available for optimization purposes. The key steps evaluated were tissue disruption, PrP^{sc} sample capture and PrP detection conditions. Release of PrP^{sc} from the tissue matrix was optimised by the addition of a large ceramic bead to the disruption step and increasing the number of cycles. The addition of digestive enzymes was investigated but was found to have no significant effect on signal strength. An increase in capture time, during the sample incubation step along with the addition of a gentle motion was used to optimize sensitivity. Experiments also demonstrated that there was no significant loss of signal when biopsy sized tissue (approximately 120mg) was used, compared to the standard kit weight of 300mg. Using the optimized protocol, a population of 256 scrapie negative RAMALT samples and 213 scrapie positive RAMALT samples (IHC positive) were evaluated on the IDEXX CWD-EIA Test Kit using the modified protocol. The specificity of the optimized protocol was 100% and sensitivity was 94%.

In conclusion, we were able to modify the IDEXX CWD Antigen test Kit protocol for optimal detection of PrP^{sc} in ovine rectal mucosal tissue.

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AAVLD 49th Annual Conference
October 12 – 18, 2006
Minneapolis Hilton – Minneapolis, MN

Survival and Causes of Mortality of Free-ranging White-tailed Deer (*Odocoileus virginianus*) Within a High Chronic Wasting Disease Prevalence Area

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Chronic wasting disease (CWD) is classified as a transmissible spongiform encephalopathy, a group of prion diseases that includes scrapie and bovine spongiform encephalopathy. Chronic wasting disease has been diagnosed in mule deer (*Odocoileus hemionus*), white-tailed deer (*Odocoileus virginianus*), Rocky Mountain elk (*Cervus elaphus nelsoni*), and moose (*Alces alces*) within the historic endemic area, which includes northern Colorado, southeastern Wyoming, and Nebraska panhandle. Transmission and geographic spread of CWD are poorly understood. Our study, which began in 2003, will determine the effects of CWD on the behavior and survival of free-ranging white-tailed deer (WTD) and provide information on the spread of CWD. One of the specific objectives of this study is to monitor CWD-positive and CWD-negative WTD throughout their lifespan via radio-telemetry and global positioning system (GPS) collars to determine differences in survival rates and causes of mortalities. To meet this objective deer were captured as fawns, tested for CWD by tonsil biopsy immunohistochemistry (IHC), marked with ear tag radio-transmitters, and recaptured on a yearly basis to re-test for CWD and to replace radio-transmitters with GPS collars. All mortalities are subjected to complete necropsies with thorough CWD testing, including immunohistochemistry examination of tonsil, retropharyngeal lymph node, and obex region of the medulla oblongata as well as enzyme-linked immunosorbent assay (ELISA) testing of retropharyngeal lymph node. In four years, we have captured, marked and CWD tested 152 WTD. The CWD prevalence for adult deer captured in 2006 is 29% (13/45), while the CWD prevalence for all deer including fawns is 23% (14/62). The yearly survival rates are lowest for male deer [50% (13/26)] and CWD-positive male deer [33% (2/6)]. For deer where cause of death was determined, hunter harvest (19), capture-related mortality (13), emaciation associated with clinical CWD (9), predation (5), vehicle collision (4), epizootic hemorrhagic disease (3), and entanglement in fence (2) are the major causes of mortality. We were unable to determine cause of death for 10 mortalities. The proportions of deer killed by hunter harvest (47%) and motor vehicle collisions (50%) that were CWD-positive was higher than expected, based on the CWD prevalence in the study population. These findings suggest that wildlife professionals and diagnosticians need to rule out CWD in otherwise apparently healthy deer killed by hunters, motor vehicle collisions, and other anthropogenic causes of mortality in areas where CWD is known to occur. These findings also support previously published reports that inclusion of hunter-killed and/or road-killed deer should be considered where applicable for CWD surveillance programs.

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October 12 – 18, 2006
Minneapolis Hilton – Minneapolis, MN

Pathogenicity of exotic BTV-1 in sheep and deer

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Bluetongue virus (BTV) was isolated from a moribund white-tailed deer found in Louisiana during the fall of 2004. The initial isolate was made at the Southeastern Cooperative Wildlife Disease Study Laboratory and identified as BTV-1 at the National Veterinary Services Laboratories (NVSL). This was the first documented case of BTV-1 in the United States (DJ Johnson *et al.*, JVDI, in press). Subsequently, white-tailed deer and sheep were challenged with the US BTV-1 virus to assess its pathogenicity in these species.

Ruminants naturally infected with BTV from the bite of *Culicoides* midges can develop an array of clinical signs which may include pyrexia, oral erosions accompanied by excessive salivation, hyperemia, and coronary band lesions. Female midges ingest bluetongue virus as they feed on viremic ruminants. The virus is amplified in both insect and ruminant hosts. BTV is not contagious; there is no direct transmission between infected ruminants. The severity of disease is influenced by the strain and virulence of the virus and the susceptibility of the ruminant host. Sheep and deer may demonstrate clinical disease which is rarely seen in cattle.

This study commenced during the winter of 2006. Five deer and seven sheep were housed in an insect proof facility at the NVSL. Animals were segregated by species with one control animal per species. Temperature probes were inserted subcutaneously into the neck of each animal to facilitate daily monitoring of body temperature. To simulate the natural route of infection, animals were inoculated at multiple subcutaneous and intradermal sites. Animals were challenged with 4 ml of cell culture fluids containing $10^{4.75}$ $TCID_{50}$ BTV-1 per ml. The control sheep and deer were inoculated with 4 ml of virus-free cell culture suspension.

Animals were observed daily for clinical signs and blood samples were collected. Virus-infected sheep displayed transient pyrexia, but no other clinical signs. One infected deer presented with oral lesions and hypersalivation and a second deer with hyperemia and coronary band lesions. No BTV-associated clinical signs were observed in two of the four challenged deer. Blood was screened by nested RT-PCR and BTV positive samples were confirmed by virus isolation in BHK-21 cell cultures. All challenged animals demonstrated viremia and antibody production; the two control animals remained negative. Virus was identified in all challenged sheep by 5 days post inoculation (dpi) and by 2 dpi in all challenged deer. Antibody production was measured by competitive ELISA and was present in all challenged animals by 9 dpi. Selected animals were euthanized and necropsied at 10, 14, 15, and 20 dpi. Tissues were collected at necropsies for virus isolation, typing and histologic exams (see abstract by C. Loiacono *et. al.* in these proceedings). Evidence from this study demonstrates that the BTV-1 isolate is capable of producing bluetongue disease in experimentally inoculated deer.

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AAVLD 49th Annual Conference
October 12 – 18, 2006
Minneapolis Hilton – Minneapolis, MN

Lesions associated with experimentally inoculated exotic BTV-1 in sheep and deer

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Four adult white-tailed deer and six adult domestic sheep were inoculated with Bluetongue virus type 1 (BTV-1) isolated from a white-tailed deer fawn found near Belle Isle, Louisiana in 2004. This is the first isolate of BTV-1 identified in the United States. One deer and one sheep were sham inoculated and housed with the inoculated groups. Whole blood (for virus isolation and PCR) and serum (for serology) were collected daily for the first 10 days post-inoculation and then three times weekly after that for the duration of the experiment. One deer and one sheep were euthanized at 10 days post inoculation (dpi) and at 14 dpi, one deer was euthanized at 15 dpi, and the remainder of the animals (two deer and five sheep) including both control animals were euthanized at 20 dpi. Full necropsies were performed on all animals and a complete selection of tissues was evaluated for microscopic changes. Spleen was collected for virus isolation.

None of the sheep or deer had detectable antibody to bluetongue prior to the challenge. All virus inoculated animals became infected and developed antibodies to BTV-1. Neither of the sham-inoculated animals became infected or developed BTV-1-specific antibodies. Significant clinical signs were observed in two of the BTV-1 inoculated deer including depression and excessive salivation in one and lameness and muscle tremors in the second. The remaining two BTV-1 inoculated deer as well as the sham inoculated deer showed no clinical signs. Gross lesions were present in the two clinical deer. Excessive salivation was associated with severe glossal and buccal necrosis. This deer also had mucosal necrosis in the reticulum and omasum. The lame deer had several areas of necrosis along the coronary band of each hind limb as well as multiple areas of hemorrhage along fascial planes among muscles of the neck and hind limbs. A third BTV-1 inoculated deer with no clinical signs had an area of hemorrhage (3X4 cm) in the right ventricular epicardium that extended into the myocardium.

The six BTV-1 inoculated and one sham inoculated sheep did not exhibit any overt clinical signs. One of these BTV-1 inoculated sheep did, however, have hemorrhage within the tunica media of the pulmonary artery. Another BTV-1 inoculated sheep had myocardial vasculitis and multifocal myocardial necrosis with mineralization.

According to results from this study, BTV-1, an exotic bluetongue virus strain for the US, appears to be pathogenic for white-tailed deer; however, lesions produced were mild and less hemorrhagic when compared to reported lesions associated with infection in deer by domestic strains of BTV. Although the sheep in this study did not show clinical signs, two of the animals experimentally inoculated with BTV-1 had gross and/or histologic lesions that have been associated with domestic BTV strains. These most likely represent the normal inter-species and intra-species variation in lesions known to occur with bluetongue virus. Monitoring white-tailed deer populations may represent a good indicator for the presence of BTV-1 in this country.

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Minneapolis Hilton – Minneapolis, MN

Comparison of two commercial radial immunodiffusion assays for detection of bovine IgG in newborn calves

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The transfer of maternal antibodies from the dam to the neonatal calf, referred to as passive transfer of immunity, is important in reducing mortality in calves. In ruminants, the syndesmochorial placenta prevents transmission of immunoglobulins to the fetus in utero. Newborn calves need to absorb colostrum containing maternal antibody by ingestion soon after birth. Failure of passive transfer (FPT) of adequate amounts of maternal antibody (≥ 1,000 mg/dL) is an important condition to identify soon after birth because newborn calves are predisposed to infections which can lead to high morbidity and mortality.

Various methods have been used to measure the passive transfer status in neonatal calves. The radial immunodiffusion test (RID), the enzyme-linked immunoassay, and the turbidimetric immunoassay (TMI) using species specific antibody are the methods that directly measure serum bovine IgG concentration, whereas all other available tests estimate serum IgG concentration based on concentration of total globulin or other proteins whose passive transfer is statistically associated with transfer of IgG. Currently, RID is considered the gold standard method to determine FPT in calves. Two commercial RID assays routinely used for serodiagnosis of FPT in calves include the VET-RID kit manufactured by Bethyl Laboratories (Montgomery, TX) and the SRID kit manufactured by VMRD (Pullman, WA). We have noted discrepancies in the results of these two RID assays in our laboratory. Our hypothesis was that the variation in the results was due to inaccuracies in the internal standards. Therefore, the objective of this study was to compare the bovine IgG concentration determined by the VET-RID and SRID kits using i) pre- and post-colostrum serum samples collected from 30 neonatal Holstein calves prior to and 24 hours after ingestion of colostrum, ii) the internal standards in each commercial RID kit, and iii) two known purified bovine IgG products obtained from Sigma (St. Louis, MO) and NVSL (USDA, Ames, IA) spiked in a precolostral serum pool using a recovery technique.

Both commercial kits showed no detectable IgG in duplicate samples obtained from 30 calves prior to colostrum ingestion. Eight of the 30 postcolostral serum samples had IgG concentrations higher than the detection limit of the VET-RID kit (> 5,000 mg/dL; 2 samples) and of the SRID kit (> 3,200 mg/dL; 6 samples). All remaining 22 postcolostral serum samples had different IgG concentrations using both RID kits (range of differences 50-1,200 mg/dL). At a cutoff value of 1,000 mg/dL, one sample showed failure of passive transfer using the VET-RID kit, whereas all samples had IgG concentrations higher than 1,000 mg/dL using the SRID kit. Using a Deming regression to compare methods, there was a proportional and constant bias towards the SRID kit. Cross comparison of the internal standards in each commercial RID kit demonstrated that the VMRD standards in the VET-RID kit measured lower than expected, whereas the Bethyl standards in the SRID kit measured higher than expected. The spiking and recovery assay demonstrated that for the VET-RID kit purified bovine IgG (Sigma) and the standard bovine IgG (NVSL) had an average recovery of 101% and 99% whereas the average recovery for the SRID kit was 144% and 133%, respectively. Collectively, these results indicate a bias in the SRID kit compared to the VET-RID kit and that the VET-RID kit more closely approximates the expected concentrations of the Sigma and NVSL standards.

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Epidemiology Scientific Session

Saturday, October 14, 2006

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Moderator: Francois Elvinger and Lindsay Oaks

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Development of a graphic tool to assess the influence of historical data in Bayesian estimation of diagnostic test parameters

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Introduction: Decision making relies often partially on diagnostic test interpretation. Unfortunately, it is well admitted that there is no perfect reference test. Bayesian approach for latent class analysis has been one of several statistical methods proposed to estimate tests parameters such as sensitivity and specificity. One critical step of this approach is determination of informative priors. These have to be identified and built from historical data in order to avoid bias in estimation and inference. The weight of these priors cannot be easily appreciated.

Materials & Methods : A prior distribution may be seen as the product of a non-informative prior distribution by the likelihood of historical data. The power-prior approach consists in raising to a power varying from zero to one the likelihood of historical data in order to vary the weight of historical data on the global prior distribution. Existing data from a comparative study of two diagnostic tests are used to study the influence of the power prior on the mean estimations and credibility intervals of the population prevalence as well as the sensitivities and specificities of the two tests. These two tests measure the titer of neutralizing antibody against rabies virus in vaccinated dogs and cats in order to identify which are protected. Simulations creating hypothetic data sets with different prevalences were also used.

Results: Posterior parameter distributions, described by their mean**s** and credibility interval**s**, are estimated in function of different power priors. The resulting curves are used to determine the cutoff where power priors affect posterior estimations by more than ±5% compared to the values obtained with a power prior of zero. Cutoffs for different parameters are compared. The available data set, confirmed by simulations, shows that an unbalanced data set could lead to one test-parameter estimation that is very sensitive to prior information whereas others are not.

Conclusion: This original approach shows to be a useful and easy-to-handle tool to assess the weight of historical data on Bayesian estimation of diagnostic test performances. This tool is sensitive to highlight the parameters that would be moderately to strongly influenced by historical data. Critical informative prior distributions could then be easily identified and carefully chosen using literature review and experts' knowledge.

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Value of Diagnostic Test Performance Indicators in Surveillance Program Design

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Diagnostic test validation often is regarded as an academic exercise with limited practical relevance for decision making. In many instances, diagnostic characteristics of tests have never been appropriately determined or the information may be nowhere available for the diagnostician, regulator, veterinarian or client to peruse. Often test results have only ancillary relevance when used to reinforce other findings in a diagnostic work-up and diagnostician falsely ignore the potential lack of sensitivity or specificity of tests.

With deployment of new tests in major surveillance efforts for high impact diseases (e.g. foot and mouth disease, classical swine fever, avian influenza, exotic Newcastle disease, and others), which require fast turn-around and high volume testing and for which there are severe penalties for a missed infected animal (continued unchecked spread of catastrophic disease), or for a false positive test result (movement restrictions, trade implications), it is essential to have properly generated documentation of diagnostic characteristics (e.g. point estimates, number of samples included in validation process for generation of probability intervals). This information is required to design sampling schemes and allocate resources to laboratories by regulators at federal and State levels. Ideally, any deployed test would have very high sensitivity and specificity, say, better than 95% sensitivity and 99.9% specificity. A test with 80% sensitivity, or 99.0% specificity, would however not be disqualified from deployment. Rather, sampling schemes will accommodate the lack of sensitivity at the sample level by compensating by increased sample size at the premises level (e.g. for a test with 80% sensitivity, testing three 'infected' samples from an infected premises results in 99.2% premises sensitivity); and resources for follow-up investigation or response would need to be allocated by State Veterinarians and other stakeholders according to the specificity of the test: lower specificity will increase the frequency of regulatory responses (movement restrictions and others) to positive test results that may be false positive (e.g. reduced specificity from 99.9% to 99.5% results in 40 more 'response' decisions to be made for every 10,000 samples tested).

In conclusion, knowledge of diagnostic performance of deployed tests is essential for all diagnosticians, veterinarians, regulators and animal owners who are stakeholders in the health and economic well-being of our livestock and poultry populations. Incomplete information on diagnostic characteristics of deployed tests will lead either to decreased effectiveness of our surveillance efforts (lower sensitivity of surveillance systems), or to waste of resources (unnecessary testing, unnecessary regulatory action), as it clearly hampers the ability for compensatory adjustments of sampling or response strategies when designing and implementing highly efficient surveillance system.

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Rift Valley fever, a potential emerging threat to livestock, wildlife, and humans in the U.S. - a review and a GIS early warning system for RVF vectors

Seth C. Britch and Kenneth J. Linthicum

Rift Valley fever (RVF) virus is a mosquito-borne zoonotic hemorrhagic disease that causes 100% abortions in ungulates such as cattle, sheep, and goats, and is often fatal to young animals. Though currently confined mainly to Africa this disease could be introduced into the U.S. and spread via mosquitoes at least as rapidly as WNV. Unlike WNV, Rift Valley fever is also transmitted by contact with infected tissues or aerosolized material, and there is no approved vaccine for humans or animals. We discuss work being done on RVF by collaborators in agencies within and outside of the USDA, such as pathways analysis, development of vaccines and test kits, and GIS modeling of vectors and vector habitat. Of particular concern is the relationship between the geography of human settlement and the livestock industry, the biogeography of wild ungulates, and the biogeography of potential RVF vectors. Our contribution is developing a GIS and remote sensing platform for early warning of elevated vector populations in the U.S. using satellite climate data and long-term mosquito surveillance data from mosquito control and public health agencies. Currently, the best strategy against Rift is preparation. By monitoring climate in Africa and the U.S., reports of RVF activity around the world, and vector populations in the U.S., we can target and implement control and containment resources to minimize effects of Rift should it appear here. Importantly, many of the systems we develop in preparation for RVF can be laterally transferred to inform strategies against any mosquito-borne disease threat.

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Interrelationships of *Salmonella* status of broiler flocks at sequential time points through the production continuum

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The ultimate goal of food safety interventions in poultry production and processing continuum is to reduce load of foodborne pathogens on the final product. The objective of this research was to determine interrelationships among broiler flock *Salmonella* status at sequential time points through the continuum. This information could then be used to determine what sample types are the best predictors of subsequent *Salmonella* status of the flock, and where in the continuum interventions should be directed.

Sixty two commercial broiler flocks reared at 31 grow-out farms by two production companies in the Southeast US were tested. *Salmonella* status of a flock was determined by sampling the flock upon arrival at a grow-out farm (30 transport tray pads, and gastrointestinal tract samples from 30 chicks from the corresponding trays); approximately a week before harvest (whole carcass rinse, ceca and crop samples from each of 30 birds); upon arrival at the processing plant (whole carcass rinse, ceca and crop samples from each of 30 birds); prior to the chill tank (rinses from 30 carcasses); and post-chill (rinses from 30 carcasses). *Salmonella* contamination of the grow-out house was evaluated by drag swabs (4) and litter samples (4) prior to the flock placement and at the end of grow-out. Logistic regression was used to model the relationships between broiler flock *Salmonella* status at the sequential time points through the production and processing continuum, accounting for random effects of the farm, complex and company.

Flock *Salmonella* status post-chill was positively associated ($p \leq 0.05$) with the degree of broiler litter *Salmonella* contamination prior to the flock placement (OR=1.64 for each 1 positive out of 4 samples increase); with the degree of broiler litter *Salmonella* contamination at the end of grow-out (OR=1.42 for each 1 positive out of 4 increase); with the flock status upon arrival to the processing plant as determined by crop samples (OR=1.18 for each 10 % increase), and with the flock status prior to the chill tank (OR=1.24 for each 10 % increase in the proportion of *Salmonella* positive samples prior to chilling). Flock *Salmonella* status post-chill was negatively associated with the degree of grow-out house *Salmonella* contamination prior to the flock placement as determined by drag swabs (OR=0.75 for each 1 positive out of 4 increase).

The results suggest that exposure of a newly placed broiler flock to *Salmonellas* present in the broiler litter at the time of placement has a long-lasting effect and affects broiler flock *Salmonella* status post-chill. We currently can not explain the negative association observed between grow-out house *Salmonella* contamination prior to flock placement as determined by drag swabs and the flock *Salmonella* status post-chill. However, insight into this relationship may be provided by investigation of the distribution of *Salmonella* serotypes in the litter, drag swab and post-chill samples.

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Tracking the spread of skunk rabies in Wyoming (20+years)

K. Mills, A. Boerger-Fields, and K. Sato

Wyoming, being a headwaters state with varied and physically separated environments, offers an interesting setting for tracking the spread of rabies in terrestrial species. The WSVL is charged with doing all rabies testing within the state which includes more than just human or animal exposure submissions. All specimens are examined using the fluorescent antibody staining. During the course of gathering this data the reagents have changed from fluorescent labeled polyclonal antibody to two sources of labeled monoclonal antibody. Skunk rabies entered the state in 1984 along the Powder River, a tributary to the Missouri River drainage into Montana. We tend to refer to river drainages when talking about skunk rabies because skunks prefer habitat found along rivers, streams or water sources. Rabies spread through skunks in the northeast part of the state and included animals along the Powder, Tongue and the Belle Fourche rivers. The number of skunk rabies case has been somewhat cyclic with as many as 221 cases in 1986, 1 in 2003 and 15 in 2005. In the early 1990s we had cases appear in the Big Horn River basin which also drains into the Missouri but separated from the original outbreak by the Big Horn Mountains. Rabies spread south but did not extend past the Wind River canyon, which has marginal skunk habitat. In 1999 we had cases south of that canyon which spread through the Riverton area and on south to Lander. In late 2000 we had cases appear further south in the Farson area which lies on a tributary to the Green River. This is important because the Green River drains into the Colorado River which has never had rabies established in its skunk population. After a very short burst of cases limited to a very small area there have been no additional cases of rabies around Farson. As mentioned, we currently have very few positive skunks in Wyoming and diagnose less than 6 rabid bats each year. Bat rabies can be transmitted to terrestrial species but generally does not establish a perpetuating cycle. Over the space of 20+ years we have had a few cases of rabies in skunks in isolated areas far away from any other cases but in each of these situations the virus was found to be of bat origin and did not establish itself in skunks. It has been generally believed that once rabies has infected a population of skunks it will be maintained at some cyclic level. This does not seem to be the situation in Wyoming in that skunk rabies seems to have completely disappeared from areas of the state.

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AAVLD 49th Annual Conference
October 12 – 18, 2006
Minneapolis Hilton – Minneapolis, MN

West Nile Virus Surveillance in Mississippi

M. Zhang[1], L. Pace[1], J. Watson[2], S. Zhang[*, 1]

West Nile virus (WNV) and Eastern equine encephalitis virus (EEEV) are two major arboviral pathogens in Mississippi. Since its introduction from neighboring states in 2001, WNV has spread to all 82 counties in the state. In general, WNV season starts at the beginning of June and ends in mid-November with a peak time in August. Although cases are concentrated in late summer and early fall, WNV has been detected in winter and spring. While horses are very susceptible to WNV infection, other animals, such as alligators, cattle, deer, dogs, and wild turkey can also be infected.

The surveillance programs, including vector surveillance, dead/ill bird surveillance, equine case surveillance, and human case surveillance, were initiated in 2002. The first three programs were conducted by Mississippi Veterinary Research and Diagnostic Laboratory and human case surveillance was performed by Mississippi State Department of Health. In the vector surveillance program, mosquitoes were collected in the field, sorted by species, and pooled (50 or fewer mosquitoes) in the laboratory. A total of 2482 mosquito pools were tested for the presence of WNV-specific RNA using real-time reverse transcription PCR (RRT-PCR). Forty-four pools were found to be WNV-positive. Among the fifty plus mosquito species in Mississippi, only *Aedes albopictus*, *Aedes vexans*, *Culex qinquefasciatus*, and *Culex salinarius* were involved in WNV transmission. The prevalence of infection varied among mosquito species. *Culex qinquefasciatus*, with the highest prevalence of infection at 3.33%, was identified as the major mosquito vector for WNV transmission in Mississippi.

In the dead bird testing program, cloacal swabs or tissue samples were collected for VecTest WNV antigen assay or RRT-PCR, respectively. WNV antigen or WNV-specific RNA was detected in the following avian species: blue jay (*Cyanocitta cristata*), American crow (*Corvus brachyrhynchos*), sparrow (*Passer domesticus*), cardinal (*Cardinalis cardinalis*), grackle (*Quiscalus quiscula*), lorikeet (*Trichoglossus haematodus*), mallard (*Anas platyrhynchos*), blackbird (*Turdus merula*), mockingbird (*Mimus polyglottos*), thrasher (*Toxostoma rufum*), hummingbird (*Amazilia yucatanensis*), finch (*Carpodacus purpureus*), chickadee (*Poecile carolinensis*), swan (*Cygnus buccinator*), and wild turkey (*Meleagris gallopavo*). Of 2654 birds tested in four years, 586 were WNV-positive. Blue jay and American crow constituted 72.9% and 14.7% of WNV-positive birds, respectively. Other species accounted for 12.4% of positive birds.

To detect WNV infection in horses, serum samples from horses exhibiting clinical signs of encephalitis were tested using IgM capture ELISA. Postmortem brain tissue samples were tested using RRT-PCR. Of 1613 samples tested in a four-year period, 539 were WNV positive. Analysis of seasonal and geographical distribution of WNV cases indicates that dead birds are an indicator of WNV activity, most WNV cases occur around urban areas and along the coastal region, and the number of WNV cases has declined.

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October 12 – 18, 2006
Minneapolis Hilton – Minneapolis, MN

Prevalence of Q-fever agent *Coxiella burnetii* in raw milk from Wisconsin dairy herds

S.N. Gibbons-Burgener[1,2], F.K. Cigel[1], O. Okwumabua[1,2], K.L Toohey-Kurth[1,2]

Coxiella burnetii, an obligate intracellular bacteria included on the U.S. Department of Homeland Security Select Agents list, is the causative agent of Q-fever in humans and ruminants. It is a zoonotic disease most often spread by exposure to aerosolized organisms shed in urine, milk, feces or birthing fluids and contaminated airborne barnyard dust. Consumption of contaminated milk and tick bites are additional, less common transmission routes in the United States. Pasteurization of milk kills most bacterial contaminants, but the consumption of raw milk persists and poses a public health risk, especially to vulnerable populations including pregnant women and immunocompromised individuals. Therefore, to further assess potential public health and occupational risks for Q-fever, a study to estimate the prevalence of *C. burnetii* in Wisconsin's raw milk supply was undertaken in 2005. A stratified random sample of bulk-tank milk from 900 geographically representative dairy herds in Wisconsin was tested for *C. burnetii* by real-time PCR. Approximately 75% of the milk samples had detectable *C. burnetii* DNA. Ranging between 52% and 96%, the prevalence varied between geographic regions, Grade A and B licensed farms and herd size. Farms in the southern regions, Grade A farms, and large herds appear to have a larger risk of having detectable *C. burnetii* in their milk. In lieu of the minimum prevalence of any stratum in this study being >50%, and the very low infectious dose required, the consumption of unpasteurized milk or dairy products by consumers and environmental exposure on dairy farms may pose a significantly greater risk of zoonotic *C. burnetii* transmission than previously recognized.

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October 12 – 18, 2006
Minneapolis Hilton – Minneapolis, MN

The emergence of *Cryptococcus gattii* in North America

C.Duncan[1,2*], C.Stephen[3], K.Bartlett[4], S.Kidd[4], , E.Galanis[5], S.Mak[5], L.MacDougall[5]

Cryptococcosis is a sporadic disease of humans and animals with a global distribution. Since 1999 there has been a marked increase in human and animal cryptococcosis on Vancouver Island in southwestern British Columbia, Canada. *Cryptococcus gattii* was isolated from humans, animals and environmental samples within the region. Identification of this organism in a temperate climate challenges previously accepted ecology and dictated the need for investigation into the emergence of *C. gattii* in this region.

A series of presumed or confirmed *Cryptococcus gattii* cases was compiled through record reviews of veterinary laboratories and human diagnostic services. Results showed a continual increase in the annual number of cases diagnosed; no seasonality was observed. Animal cases exceeded human cases even though it was hypothesized that animal cases are more likely to go undiagnosed or unreported when compared to humans. Animal cryptococcosis was identified on Vancouver Island prior to 1999 suggesting the organism may have emerged in the region prior to its identification as a causative agent for human disease; therefore animals may serve as a good sentinel for human cryptococcosis infection.

The clinical presentation of companion animals was consistent with that previously reported. Multivariate survival analysis identified only the presence of neurological symptoms as a statistically significant predictor of mortality. A case-control study identified host and environmental risk factors for clinical *C. gattii* infection in dogs and cats suggesting that where an infectious agent is not uniformly distributed, individual risk increases when the organism is re-distributed through large scale environmental disturbance, or when the animal has increased opportunities for exposure through travel or activity level.

Serum samples and material for fungal culture were collected from dogs, cats, horses and terrestrial mammal species residing within the region where clinical cases had been diagnosed. Nasal colonization and asymptomatic infection, defined as the presence of cryptococcal antigen in the bloodstream in the absence of clinical symptoms, was identified in a small percentage of animals. All positive animals resided in areas of heavy environmental colonization supporting the role of animals as environmental sentinels.

Since 2003 both human and animal cases have been diagnosed on the British Columbia mainland and Washington, USA without a history of travel to the island. The organism has been isolated from rare environmental samples near the homes of mainland cases suggesting colonization of the organism throughout a much broader range. The organism has been shown to spread via air, water and on fomites such as shoes and car tires. Sporadic, presumably travel related, *C. gattii* cases have been diagnosed by veterinary and human health laboratories outside of the endemic region. Diagnosticians should be aware of the recent emergence of this organism with North America.

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Virology Scientific Session

Sunday, October 15, 2006

Salon A

Moderator: Steve Bolin and Kyoung-Jin Yoon

10:30am	**Porcine Circovirus Type 2: ELISA for the detection of Antigen and Antibodies in Feces** - S. Guillossou, E. Deshaies, N. Brajon, P. Lopez, S Leterme	84
10:45am	**Do Elk Catch Foot-and-Mouth Disease?** - G.B. Ward, J. Rhyan, M. Kenney, H. Wang, M.Y. Deng, T. Gidlewski, M. McCollum, M. Salman, T.S. McKenna	85
11:00am	**A Liquid Phase Blocking ELISA for the Detection of Antibodies to 3D Non-structural Protein of Foot-and-Mouth Disease Virus** - H. Wang, M.Y. Deng, J. Barrera , L. Zsak, G. Mayr, B. Cerino, and T.S. McKenna	86
11:15am	**Experimental Aerosol Infection of Cattle (Bos taurus) with Ovine Herpesvirus 2 using Nasal Secretions from Infected Sheep**. - N. Taus, JL Oaks, K Galbreath, D.L. Traul, D. O'Toole, H. Li	87
11:30am	**Ovine Herpesvirus 2 mRNA Expression in Cattle and Bison with Malignant Catarrhal Fever** - J.L. Oaks, S. Pritchard, N.S. Taus, D.L. Traul, D. O'Toole, and H. Li	88
11:45am	**Serologic Survey for Vector-borne Disease Agents,Bovine Viral Diarrhea Virus, and *Mycobacterium avium subsp. paratuberculosis* in Free-Ranging White-Tailed Deer in Michigan** - A. A. Dye, L. Meader, N. L. Grosjean, S. R. Bolin, S. M. Schmitt, D. J. O'Brien, T. M. Cooley, M. K. Cosgtove, and S. Schaefer	89

AAVLD 49th Annual Conference
October 12 – 18, 2006
Minneapolis Hilton – Minneapolis, MN

Stability of Infectious Porcine Reproductive and Respiratory Syndrome Virus in Aerosols.

J.R. Hermann[1], K-J Yoon[2], S.J. Hoff[3], J. Zimmerman[2]

Transmission via aerosols was once considered the primary route of PRRSV transmission, but it has been difficult to consistently reproduce airborne transmission of PRRSV from infected to susceptible pigs under experimental conditions. Inconsistent replication of airborne transmission of PRRSV under experimental conditions suggests that we do not understand the conditions required for its occurrence. Determining the probability of airborne transmission requires estimates on viral shedding, stability of airborne virus, and infectious dose of susceptible pigs by the aerosol route. The objective of this experiment was to derive estimates of stability of aerosolized infectious PRRSV as a function of relative humidity and temperature to better understand the process of airborne transmission.

A suspension of PRRSV was aerosolized into a dynamic aerosol toroid (DAT) rotating at 5 revolutions per minute and maintained at a pre-determined temperature and relative humidity. The cloud of PRRSV contained within the DAT was sampled repeatedly over time, the concentration of infectious PRRSV ($TCID_{50}$) in the samples was determined, and the T1/2 for the specific combination of relative humidity and temperature was estimated based on the inactivation of infectious virus observed over time. T1/2 of PRRSV for each of the experimental runs was calculated using linear regression analysis. A total of 18 T1/2 estimates were used in a regression analysis to derive an equation estimating the T1/2 of aerosolized PRRSV for any combination of temperature and relative humidity.

Results:

1. The experimental results showed that aerosolized PRRSV was least stable (T1/2 = 3.6 min) at 41.0°C and 73.0% relative humidity and most stable (T1/2 = 192.7 min) at 5.0°C and 17.1% relative humidity
2. A non-linear regression model revealed that the effects of temperature ($p < 0.001$) and relative humidity ($p = 0.003$) on the T1/2 of airborne PRRSV were statistically significant, but the interaction between temperature and relative humidity was not ($p = 0.21$).

Based on the results of this experiment an equation was derived to predict PRRSV T1/2 for any combination of temperature and relative humidity.

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October 12 – 18, 2006
Minneapolis Hilton – Minneapolis, MN

2b peptide based ELISA as a potential DIVA test for PRRS virus

Wai-Hong Wu[1], Sang-Ho Cha[1], Won-Il Kim[1], Kyoung-Jin Yoon[1]

Porcine reproductive and respiratory syndrome (PRRS) is considered to be the most economically significant viral disease in U.S. swine industry. Such an economic significance of PRRS has recently re-directed the industry's focus to looking into eradication of the disease. However, unlike the situation with the pseudorabies eradication program, no marker vaccines and accompanying differential diagnostic tests are available. The following study was conducted to find a serological way to differentiate pigs exposed a wild type PRRS virus from animals vaccinated with Ingelvac® PRRS MLV.

Peptide sequences of 2b protein from MLV, which did not serologically cross-react with VR-2332 (the parental strain of Ingelvac® PRRS MLV), were identified when attempting to detect 2b protein from different PRRS viruses by using antisera raised against the VR-2332 strain. The MLV version of 2b was also found in other MLV-like PRRS viruses (i.e., vaccine-like viruses) and maintained its stability during serial passages in pigs. Synthetic peptides and recombinant proteins of the identified sequences were then prepared as differential markers and used as antigens for an indirect ELISA to detect the vaccine virus- or wild type-specific antibodies, thus differentiating vaccinated pigs and infected animals. Various panels of sera collected from vaccinated pigs that were or were not challenged with a field isolate or pigs experimentally infected with various field isolates were used to evaluate the performance of the ELISA.

Pigs vaccinated with Ingelvac® PRRS MLV could be serologically differentiated from pigs exposed to a wild-type PRRSV using these differential markers. While no serological response to 2b antigen of VR-2332 was detected in the vaccinated pigs, specific antibody response to the VR-2332 2b antigen was detected in pigs infected with VR-2332 or other wild type PRRS viruses by 35 days post inoculation. Although the sensitivity of 2b peptide based ELISA may need to be improved as the assay detected antibody response at a later stage of infection as compared to IDEXX PRRS ELISA, the 2b ELISA can be a DIVA test for Ingelvac® PRRS MLV vaccine. It remains to be further studied whether differential markers employed in this ELISA can successfully differentiate antibody responses induced by vaccine revertants.

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Probability of porcine reproductive and respiratory syndrome (PRRS) virus infection via the aerosol route of exposure

J.R. Hermann[1], K-J Yoon[2], S.J. Hoff[3], J. Zimmerman[2]

Transmission via aerosols was once considered the primary route of PRRSV transmission. Experimentally airborne transmission of PRRSV from infected to susceptible pigs has been demonstrated but difficult to consistently reproduce. Inconsistent replication of airborne transmission of PRRSV under experimental conditions suggests that we do not understand the conditions required for its occurrence. Determining the probability of airborne transmission requires estimates on: 1.) viral shedding, 2.) stability of airborne virus, and 3.) infectious dose of susceptible pigs by the aerosol route. The objective of this experiment was to derive a dose-response curve to estimate the infectious dose 50 (ID_{50}) of PRRSV for aerosol route of exposure in young pigs.

In each of 6 replicates, 10 pigs were administered a specific dose of PRRSV isolate VR-2332 by direct aerosol exposure. Pigs were exposed to aerosolized infectious PRRSV contained in a dynamic aerosol torrid (DAT). Each pig respired 10 liters of air from the DAT. SKC BioSampler® impingers were used to collect air samples from the DAT. Microinfectivity assays ($TCID_{50}$) were performed on impinger samples to determine the titer of infectious PRRSV per liter of air. Following exposure, pigs were individually housed in hepa-filtered units. Serum samples were collected on 0, 5, and 10 days post exposure to establish whether exposure resulted in infection. Infection status post exposure was based on the detection of virus in serum by virus isolation (VI) and reverse transcription-polymerase chain reaction (RT-PCR). The number of pigs infected among the total number exposed by dose was used to derive the dose-response curve for aerosol exposure.

Results:

3. All pigs were VI negative and ELISA negative on DPE -7 and 0. All samples from negative control animals were VI negative and ELISA negative.
4. All infected animals were VI and RT-PCR on 5 and 10 DPE.
5. Infectivity data indicated pigs were susceptible to infection via aerosol exposure.
6. Aerosol exposure dose of $10^{3.6}$ $TCID_{50}$ was sufficient cause infection in young swine.
7. Data from this experiment was used to establish a dose response curve and an ID_{50} for aerosol exposure.

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An Alternate Method for PRRSV Surveillance: Experimental Data

John Prickett[1*], Robert Simer[2], En-Min Zhou[1], Kyoung-Jin Yoon[1], Jeff Zimmerman[1]

Annual economic losses in U.S. pork production due to PRRSV (porcine respiratory and reproductive syndrome virus) have been estimated at $560.32 million (Neumann et al., 2005). For this reason, PRRSV has been targeted as a candidate for eradication. The most fundamental component of any successful eradication program is surveillance. Current diagnostic methods for identifying PRRSV in the production setting are laborious and expensive.

Preliminary studies indicated that PRRSV was shed in oral fluids (Wills et al., 1997). We recently completed a study (1) to determine whether PRRSV was in oral fluids at diagnostically useful levels and, if so, (2) to determine whether the duration and/or level of PRRSV in oral fluids differed by pig age at the time of infection.

Three age groups, i.e., 4-, 8-, and 12-week-old pigs, were selected for evaluation. Each age group consisted of 16 pigs (12 PRRSV-inoculated, 4 negative controls) housed in pens of 4 pigs except that the 3-week old PRRSV-inoculated pigs were housed in 2 pens of 6 pigs each. Oral fluid samples were collected by hanging a section of braided cotton rope in each pen; the pigs chewed on the rope, depositing oral fluids in the process. The ropes were retrieved after 20-30 minutes and oral fluid was then mechanically extracted from the rope. Oral fluids were collected at regular intervals throughout the 63 day study. Blood was also drawn from each pig at regular intervals, and serum harvested. Quantitative RT-PCR (North-American strain) was used to generate $TCID_{50}$ estimates of all samples. Individual pig serum samples were averaged by pen and week and compared to pen-based oral fluid samples.

1. Results to date show a positive correlation between PRRSV titers in serum and oral fluid samples.

2. RT-PCR on oral fluids was positive in over 75% of the known positive pens through day 28.

These results indicated that oral fluid samples may offer a promising alternative to current methods in regards to the development of an efficient, cost effective, and practical method of PRRSV surveillance in the production setting.

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An Alternate Method for PRRSV Surveillance: Field Study

John Prickett[1*], Robert Simer[2], En-Min Zhou[1], Kyoung-Jin Yoon[1], Jeff Zimmerman[1]

Alternate methods for the detection of PRRSV (porcine respiratory and reproductive syndrome virus) are currently under investigation. Pen-based sampling of oral fluids has proven to be a simple, reliable, and accurate diagnostic approach under experimental conditions. The method is non-invasive, and reduces the time and labor involved in sample collection. Validation of this new technique requires field testing to truly measure the potential, practicality, and validity of the approach.

On three finishing sites, pens of approximately 25 pigs were monitored over time for PRRSV infection using oral fluid and serum samples. Samples were collected when pigs entered the facilities at 3 weeks of age, then at 5, 8, 12, and 16 weeks of age. Oral fluids were collected by suspending cotton rope in each pen for approximately 20 minutes. The oral fluids were then extracted and stored frozen until assayed. Serum samples were collected from a convenience sample of 5 pigs per pen, processed, and stored frozen until assayed. Prior to analysis, samples were completely randomized and then analyzed for PRRSV by quantitative RT-PCR at the Iowa State University Veterinary Diagnostic Laboratory.

The results from this field study paralleled those from the experimental study reported at this meeting.

1. Estimated virus titers in serum and oral fluids were positively correlated.
2. On a pen basis, qualitative results of serum and oral fluid samples resulted in a high level of agreement in regards to the infection status of pens.

On the basis of these results, we hypothesize that oral fluid samples may provide a promising alternative to current procedures for infectious disease surveillance in production settings.

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AAVLD 49th Annual Conference
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Minneapolis Hilton – Minneapolis, MN

Genetic determinants for different phenotypes of wild-type and attenuated PRRS virus

W.-I. Kim[*1], K.-J. Yoon[1,2]

Porcine reproductive and respiratory syndrome (PRRS) virus has caused enormous economical loss in swine industry since it was identified in the early 1990's. Modified live vaccines (MLVs) attenuated by serial passages in MARC-145 have been made available to veterinarians and producers and proven to reduce the severity of the disease. Because MLVs were adapted well to MARC-145, they tend to replicate faster and grow to a higher titer in this particular cell line than their parental strains or wild-type viruses. As a result, we found that MLVs produced much bigger sized plaques than wild-type virus. In contrast, the replication of MLVs in porcine alveolar macrophages (PAM), the major target cells of PRRSV in pigs, was greatly diminished by the cell-attenuation. In addition, MLVs produced a much lower level of viremia than does wild-type virus. Furthermore, a close relation between the level of viremia and degree of virulence was observed. Therefore, the following study was conducted to identify the genetic determinants for the phenotype of MLVs in MARC-145 and PAM.

PRRS virus designated CC-01, which was originated from VR-2332, was used for the study. The CC-01 strain had the MLV phenotype and closer genetic identity to MLV due to the continuous passages in MARC-145 and grew much better in MARC-145 than in PAM and produced as big plaque as MLVs do. The virus also produced a poor level of viremia and showed low virulence in challenged pigs. The CC-01 strain was sequentially passed 13 times in three different lines of pigs. During pig-to-pig passages, the stability of phenotypic characteristics of CC-01 was assessed. Sequence analysis, mutagenesis and plaque assays were employed to identify sequence element(s) accounting for such characteristics (i.e., virus growth in MARC-145 cell line and PAM; size of plaques formed in MARC-145).

The MLV phenotype of CC-01 (i.e. big sized plaque in MARC-145 and/or poor growth in PAM) was completely reverted to the wild phenotype after 3 or 5 passages in the pigs. Comparisons of full-length genomic sequences of CC-01 and two pig-passed viruses (3A1 and 3B1) that had the wild phenotype revealed the genetic factors responsible for these different phenotypes. Five, single amino acid point mutations in ORF1a, 1b, 2, 5 and 6 were recognized from the sequence comparison. Subsequently, mutants containing each or a combination of the identified sequences were generated using infectious clone constructed based on the sequence of VR-2332. The single mutations in ORF2 and 6 were related to the change of the virus replication preference between MARC-145 and PAM. In contrast, multiple structural genes other than identified five sequences were involved in the determination of plaque size.

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October 12 – 18, 2006
Minneapolis Hilton – Minneapolis, MN

Experimental reproduction of porcine reproductive and neurologic syndrome by a novel pestivirus-like virus

Roman Pogranichniy[1,2], Kent Schwartz[2], Kyoung-Jin Yoon[2]

Disease outbreaks characterized by reproductive failure and/or neurologic disorders, which is commonly referred as "Porcine Reproductive and Neurologic Syndrome (PRNS)", were observed in many swine farms in Iowa and other states. Acute death loss of both adult and growing swine has been substantial to many of affected farms. Extensive laboratory diagnostic investigation on suspect cases repeatedly resulted in the isolation of a cytopathic enveloped RNA virus of 50-60 nm in size from nervous and secondary lymphoid tissues and/or sera that were negative for common viral pathogens to swine. Isolates were not readily recognized by antibodies raised against any known viral pathogens to swine in the United States but by antisera collected from animals that survived a clinical episode. To reflect its unknown identity, the isolates were initially named "Virus X" but later determined to be a novel virus of the genus *Pestivirus*. The following study was conducted to determine the role of the new virus in PRNS.

Pregnant sows experimentally inoculated with Virus X (ISUYP604671) or homogenate (filtrate) of tissues from a clinically infected animal developed clinical signs and pathological changes similar to field observations including the loss of pregnancy. Furthermore, caesarian-derived, colostrum-deprived young pigs developed mild encephalomyelitis lesions in the brain after experimental inoculation with the virus or the tissue homogenate although clinical neurologic signs were not observed. More importantly, Virus X was re-isolated from all inoculated animals while control pigs remained negative for the virus during the study and amplified by PCR based on NS3 region (polymerase) sequence information for border disease.

Collectively, the novel pestivirus-like virus is responsible for PRNS that is present in the field and should be taken into consideration when diagnostic investigations are conducted on reproductive and/or neurologic cases along with relatively high mortality.

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AAVLD 49th Annual Conference
October 12 – 18, 2006
Minneapolis Hilton – Minneapolis, MN

Isolation of a novel viral agent associated with reproductive and/or neurological disorder in pigs

Roman Pogranichniy[1,2], Kyoung-Jin Yoon[2]

Disease outbreaks characterized by reproductive failure and/or neurologic disorders, which is commonly referred as "Porcine Reproductive and Neurologic Syndrome (PRNS)", were observed in many swine farms in Iowa and other states. Although an infectious cause was suspected to account for the disease, no conclusive diagnosis had been reached with respect to conventional infectious agents.

Extensive laboratory diagnostic investigation on suspect cases repeatedly resulted in the isolation of a cytopathic enveloped virus of 50-60 nm in size from nervous and secondary lymphoid tissues and sera that were negative for PRRS virus, pseudorabies virus, porcine teschovirus, PCV2, CSF virus, Japanese encephalitis virus, and hemagglutinating encephalomyelitis virus. To reflect its unknown identity, the isolates were named "Virus X". The presence of a virus particle with morphological characteristics similar to Virus X in tissues from affected animals was also observed on thin-section positive-staining electron microscopy.

Virus X was determined to contain RNA genome. Isolates of Virus X were not readily recognized by antibodies raised against any known viruses pathogenic to swine but by antisera collected from animals that survived a clinical episode, indicating that Virus X is likely a previously unrecognized agent in US swine population. Further laboratory studies demonstrated weak two-way cross reactivity between Virus X and ruminant pestiviruses (i.e., BVDV and BDV) when tested by monospecific polyclonal rabbit antisera raised against individual viruses. Virus X infection to a permissive cell line was partially blocked by anti-BDV polyclonal antibody. Partial sequences of NS3 region (polymerase) of Virus X showed some degree of sequence homology with that of the existing pestiviruses but were phylogenetically very distinct from the known pestiviruses.

Collectively, Virus X is a novel pestivirus whose role in the clinical disease remains to be determined.

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AAVLD 49th Annual Conference
October 12 – 18, 2006
Minneapolis Hilton – Minneapolis, MN

Comparison of ELISA assays and PCR for the detection of anti-PCV2-antibodies and PCV2-antigen or DNA in porcine serum and fecal samples

T. Opriessnig, J. Johnson, K.-J. Yoon, and P.G. Halbur

Porcine circovirus type 2 (PCV2) associated diseases are considered economically important to the US swine industry. With the introduction of several PCV2 vaccines on the US market in spring 2006 there are increasing requests to determine the anti-PCV2-antibody status of swine herds in order to determine the best time for vaccination. However, there is no commercial PCV2 ELISA assay kit available in the US. Some laboratories offer immunofluorescence assays (IFA) and Iowa State University Veterinary Diagnostic Laboratory (ISUVDL) offers an in-house PCV2 ELISA assay which is based on recombinant ORF2 protein of PCV2. We used serum and fecal samples to compare the ISUVDL antibody ELISA assay and a commercially available European competitive antibody ELISA assay (Synbiotics, France). In addition, we compared a commercial European PCV2-antigen capture ELISA assay (Synbiotics, France) and a PCV2 real-time PCR assay on fecal samples for their ability to detect PCV2 shedding. Samples were obtained from 40 pigs: 10 pigs were inoculated with PCV2 14 days prior to sample collection, 10 pigs were vaccinated with a PCV2 killed vaccine 42 days prior to sample collection and were inoculated with PCV2 14 days prior to sample collection, and 20 pigs were naïve to PCV2 at the time of sample collection at 4 weeks of age (10 weeks of age) and at 15 weeks of age (n=10). There was a good correlation between the two antibody assays on serum samples: 26/40 results were identical and 6/40 samples were in one of the assays in the suspect positive range and in the other assay either low positive or negative. Three of forty pigs were found to be suspect positive for PCV2-antibodies on fecal samples and 1/40 was positive. Finally, it was possible to detect PCV2-DNA in fecal samples of 4/40 pigs (all non-vaccinated and PCV2-infected) whereas the PCV2-antigen capture ELISA was negative on all fecal samples tested.

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Porcine Circovirus Type 2: ELISA for the detection of Antigen and Antibodies in Feces.

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Introduction: Postweaning Multisystemic Wasting Syndrome (PMWS), a swine disease first identified in 1991, in Canada, has been since observed in the United States, Europe, and other Asian countries. Ultimately, the definitive diagnosis of a PCV-related disease will be based on the presence of the PCV2 associated with lesions and clinical symptoms.

Objectives: Due to high prevalence, detection of serum antibodies is usually of poor value. This study report on the development of an antibody-detection blocking ELISA and an antigen-detection capture ELISA, both optimized for an original use on fecal samples. Concordance with the disease was investigated.

Materials and Methods: Fecal samples (n=49) were collected from pigs with suspicious clinical signs (diarrhea, progressive weight loss, dermatitis, nephritis) in 22 herds in Britania, France, with history of PMWS demonstrated by immunohistochemistry. For the specificity study, fecal samples (n=139) were collected from pigs from one herd with no history of PMWS. Samples were collected on dry swabs. Following an extraction step of the swabs, samples were incubated in the two ELISAs and compare to disease status established by PCR, histology investigation and clinical symptoms. Both tests use an HRP-labeled specific anti-PCV2 monoclonal antibody for improved specificity.

Results: Amongst 27 PCR positive fecal samples from infected herds, 21 were found positive for PCV2 antigen and 14 for antibodies. However, a sensitivity of 100% was achieved by adding results from both tests (antigen and/or antibody positive results). When run on samples from herds with no history of PMWS, the antigen detection and antibody detection tests showed a specificity of 96% and 95%, respectively. The specificity of the antibody detection test was significantly higher for young pigs (<8 wk), 98%, than for older ones, 90%. A very broad distribution of the responses obtained with the two tests when sampling infected herds (STD=0.972 for antigen detection and 0.609 for antibody detection), compared to the narrow one for healthy herds (STD=0.080 for antigen detection and 0,288 for antibody detection) was also observed.

Discussion: Some samples from pigs with severe clinical signs had no ELISA detectable antigen in their feces but presented a very strong fecal antibody response, involving mainly IgA antibodies. This could be explained by a blocking action of the epitopes of the antigen by an excess of antibody, as can be observed for canine parvovirus.
Conclusions: The original combined use of antigen and antibody ELISAs on fecal samples has demonstrated high concordance with PMWS status at the herd level and may provide ante-mortem tools. More valuable information was obtained than with conventional serology approach and this may assist in herd management decision.

Keywords: Diagnostic, PCV2, PMWS, Feces, ELISA

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Do Elk Catch Foot-and-Mouth Disease?

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Foot-and-mouth disease (FMD) is a serious threat to livestock. In the western United States cattle and sheep co-range with a number of wild ruminants including elk (*Cervus elaphus*). An active domesticated elk breeding industry produces both meat and antlers for sale. Susceptibility of elk, viral shedding and infectiousness, and cattle to elk, elk to cattle and elk to elk transmission of FMD virus (FMDV) have not been characterized. We conducted experiments to determine if elk could be infected with FMDV by inoculation, if they could be infected by contact with cattle carrying the virus, if they could infect cattle, and if there was elk-to-elk transmission.

One bovine and one elk were each inoculated into the tongue intradermally with 6×10^3 bovine tongue infectious doses of O_1 Manissa FMDV. The inoculated animals were co-housed with a naïve bovine and a naïve elk. All the animals were monitored for clinical signs and blood and nasal and oral swab samples were taken daily from each animal for virus isolation and real-time RT-PCR. When typical clinical signs of FMD were observed in the contact bovine, the bovine was moved into a room housing two naïve elk. Although no clinical signs were observed in the contact elk, it was moved into a room housing three naïve elk at the same time as the contact bovine was moved. Because we observed no evidence of disease transmission to or by elk, an additional experiment was conducted by inoculating FMDV into two elk as described above and housing them with naïve cattle and naïve elk immediately after virus inoculation. One inoculated elk was co-housed with two naïve bovines and the other with three naïve elk. All animals were monitored for clinical signs and sampled as described above. Sera were sampled and assayed for neutralizing antibodies by a virus neutralization test and for antibodies to FMDV non-structural proteins by ELISAs.

It was demonstrated that elk could be infected by direct inoculation of FMDV, O1 Manissa but the susceptibility of elk was at least 1,000-fold lower than that of cattle. Sera collected from inoculated elk were positive for antibodies to non-structural proteins 3ABC and 3D of FMDV in ELISAs. Virus neutralization titers of these sera, however, were lower than those of sera collected from FMDV-infected cattle. Although FMDV was isolated from the blood of virus-inoculated elk during the early stage of infection, the virus was not detected from nasal or oral swab samples collected from these animals. There was no transmission of FMDV from cattle to elk or from elk to cattle. None of the three elk housed with the virus-inoculated elk showed typical clinical signs of FMD and only one of them was positive for antibodies to FMDV. There was no evidence of viremia in this elk.

These results all indicate that transmission of FMDV O_1 Manissa either to or from elk is very difficult and unlikely.

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A Liquid Phase Blocking ELISA for the Detection of Antibodies to 3D Non-structural Protein of Foot-and-Mouth Disease Virus

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The RNA polymerase of foot-and-mouth disease virus (FMDV), 3D, is highly conserved in amino acid sequence and antigenicity among all seven serotypes of the virus. Consequently, a single immunoassay for antibody to this protein has the potential for detecting FMDV infection regardless of viral serotypes. In this work, a liquid phase blocking ELISA (LPBE) for the detection of antibodies to 3D protein was developed. In this assay, polyclonal capture antibodies to 3D, a purified antigen 3D protein produced in insect cells infected with recombinant baculovirus, and a biotinylated detector antibody are used. Cut-off values of this assay were established by testing over one thousand negative serum samples. Applying these cut-off values, 99% of known negative bovine and swine samples and all of known negative ovine samples were negative in the assay. In testing serum samples of bovine, swine and ovine, the specificity of the assay was shown to be equivalent to that of the agarose gel immunodiffusion (AGID), a traditional method for detecting antibodies to 3D. For early detection of FMDV infections in bovine and swine using over three hundred sera collected sequentially following viral infection, the 3D LPBE was comparable to the AGID, the virus neutralization (VN) test, and the commercially available Ceditest FMDV-NS (Cedi-Diagnostics B.V., the Netherlands). For early detection of FMDV infections in ovine using close to one hundred sera collected sequentially following viral infection, the 3D LPBE was more sensitive than the AGID, the VN test and the Ceditest FMDV-NS. Similarly, in testing convalescent sera from animals infected with six different FMDV, the sensitivity of 3D LPBE was comparable to that of AGID and Ceditest FMDV-NS for bovine and swine samples and it was higher than that of the other two methods for ovine samples. In addition to being comparable to the AGID, the VN test and the Ceditest FMDV-NS in sensitivity, the 3D LPBE takes only four hours to run, providing results sooner than the other assays. The suitability of the 3D LPBE for samples from a wide range of animal species including goat, bison and pronghorn antelope was demonstrated. This assay is being further evaluated.

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EXPERIMENTAL AEROSOL INFECTION OF CATTLE (*BOS TAURUS*) WITH OVINE HERPESVIRUS 2 USING NASAL SECRETIONS FROM INFECTED SHEEP

N. S. Taus[a], J. L. Oaks[b], K. Gailbreath[a], D. L. Traul[a], D. O'Toole[c] and H. Li [a]

Infection of clinically susceptible ruminants, including domesticated cattle (*Bos taurus*) and American bison (*Bison bison*), with ovine herpesvirus 2 (OvHV-2) can result in the fatal lymphoproliferative and vasculitis syndrome known as malignant catarrhal fever (MCF). Studies of MCF pathogenesis have been constrained by the lack of an *in vitro* OvHV-2 propagation system, which precludes the generation of conventional virus stocks for use in such studies. Furthermore, experimental transmission of MCF due to OvHV-2 has generally been difficult to achieve and has relied on inoculation of whole blood, tissues, and/or cells obtained from naturally occurring cases of MCF, most often in red deer (*Cervus elaphus*) and domesticated cattle, into species such as red deer, cattle and laboratory rabbits (*Oryctolagus cuniculus*). A reliable experimental infection model is needed to study the pathogenesis of MCF and to develop effective vaccination strategies to control the disease.

We recently characterized the use of nasal secretions collected from sheep experiencing virus shedding episodes as a source of infectious OvHV-2, and established an experimental aerosol infection model in sheep (1). In this study we sought to extend the results of that work by using the same pool of nasal secretions to establish an experimental infection model that, for the first time, produced infection and MCF in domesticated cattle using the natural route of exposure. Using the protocol and OvHV-2 inoculum established in the previous study, eight calves were nebulized with four different doses of OvHV-2 in nasal secretions from infected sheep. Two control calves were nebulized with nasal secretions from uninfected sheep. Infection status of all calves was monitored using competitive inhibition ELISA, PCR and clinical parameters. Six of eight nebulized calves became infected with OvHV-2. One calf receiving the highest dose of virus developed typical clinical, gross and histological changes of MCF. This study showed that nasal secretions collected from sheep experiencing OvHV-2 shedding episodes were infectious for cattle and capable of inducing MCF. The data also indicate that cattle are relatively resistant to disease following infection. More susceptible ruminant species, such as bison and deer, might be considered for future studies of virus pathogenesis and vaccine development.

1. Taus, N. S., Traul, D. L., Oaks, J. L., Crawford, T. B., Lewis, G. S. & Li, H., 2005. Experimental infection of sheep with ovine herpesvirus 2 via aerosolization of nasal secretions. J Gen Virol. 86, 575-579.

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Ovine herpesvirus 2 mRNA expression in cattle and bison with malignant catarrhal fever

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Malignant catarrhal fever (MCF) is a fulminant disease of certain susceptible ruminants caused by ovine herpesvirus 2 (OvHV-2). Sheep are subclinical reservoirs of the virus that intermittently shed virus and can transmit OvHV-2 to other ruminants. Bison and deer are highly susceptible to disease, and exposure of these ruminants to sheep can lead to significant morbidity and mortality. Cattle can also be infected with OvHV-2, but are more resistant to disease and are affected more sporadically. In the past, OvHV-2 infections were regarded as uniformly fatal in susceptible species such as cattle, bison, and deer. More recently, however, chronic forms of disease have been described, and subclinical infection rates of up to about 20% have been reported in cattle and bison.

The characteristic lesions of MCF include lymphoproliferation, vasculitis and mucosal ulceration. The pathogenesis of these lesions is very poorly understood, but is most likely due to virally-induced dysregulation of cellular immune responses to host and/or viral antigens resulting in immune-mediated damage to vascular and epithelial tissues. However, the inability to culture OvHV-2 in vitro, and the lack of genetic information about OvHV-2, has precluded studies of viral gene expression in vivo. Recently two complete and annotated genome sequences of OvHV-2 have become available. Based on this information, we used PCR to detect viral mRNA for genes associated with both latent (ORF 73) and lytic infections (ORF 25 – capsid protein), as well as key immunomodulatory genes (Ov 2.5 - homolog of IL-10; Ov 4.5 - homolog of Bcl-2; Ov 5 - homolog of G-protein coupled receptor), in tissues of bison and cattle experimentally infected with OvHV-2. In four bison with primarily necrotizing lesions, we detected mRNA for ORF 25, Ov 4.5, and Ov 5. The presence of ORF 25 suggests that the lesions may be due to direct viral lysis and antiviral immune responses. In an infected steer in which the lesions were primarily lymphoproliferative, only mRNA for Ov 4.5 was consistently detected. These findings suggest that lymphoproliferative lesions are not associated with productive viral infection, and that the anti-apoptotic viral Bcl-2 mediates lymphoproliferation. In addition to providing new insights into the pathogenesis of MCF, these studies may also identify mRNA targets that distinguish animals with active infections from those with subclinical latent infections.

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Serologic survey for vector-borne disease agents, bovine viral diarrhea virus, and *Mycobacterium avium subsp. paratuberculosis* in free-ranging white-tailed deer in Michigan

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An outbreak of eastern equine encephalitis virus (EEEV) in free-ranging white-tailed deer (*Odocoileus virginianus*) occurred in Michigan in September of 2005. As a result of that outbreak, a serologic survey was conducted by the Michigan Department Natural Resources (MDNR) and the Diagnostic Center for Population and Animal Health to assess the prevalence of EEEV and other disease causing agents in deer that might infect humans, companion animals, or livestock. Blood was obtained from deer harvested by licensed hunters in 3 regions of the lower peninsula of Michigan during November of 2005. The age of the deer, gender, and harvest location was recorded by MDNR field staff at deer check stations. A plaque reduction assay was performed by the National Veterinary Services Laboratory, USDA/APHIS, for detection of antibody against EEEV. Antibody against West Nile virus and type 1 and type 2 bovine viral diarrhea viruses was detected using virus neutralization assays. Antibody against *Mycobacterium avium subsp. paratuberculosis* was detected using a commercially available ELISA. Antibody against *Borrelia burgdorferi*, *Rickettsia rickettsii*, *Babesia bovis*, and *Anaplasma phagocytophilum* was detected by indirect fluorescent antibody staining using fluorescein conjugated rabbit anti-goat IgG and commercially available antigen slides. A total of 133 samples of serum were tested were from the lower peninsula's southwestern region (Cass, Kalamazoo, Van Buren, and St. Joseph counties [n=58]), west-central region (Kent and Montcalm counties [n=21]), and northeastern region (Alpena, Montmorency, Alcona, and Oscoda counties [n=54]. Antibody against *Rickettsia rickettsii* was not detected. The prevalence of antibody against EEEV, and BVDV Type 1 and 2 varied among the 3 regions. The prevalence of antibodies against the other infectious diseases showed some regional variation.

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Pathology Scientific Session

Sunday, October 15, 2006
Salon B

Moderator: Grant Maxie and Bill Layton

10:45am	**Mycoplasma bovis in Wild and Captive White-tailed Deer (*Odocoileus virginianus*) in Ohio: The other M. bovis in Deer** - J.R. Hayes, C.M. Almgren, S.D. Grimes, Y. Zhang	102
11:00am	**Foot-and-Mouth Disease in Pronghorn (Antilocapra americana) and Mule Deer (*Odocoileus hemionus*): Susceptibility, Clinical Signs, and Lesions** - J.C. Rhyan, T. Gidlewski, M. McCollum, G.B. Ward, F.M. Mohamed, M.Y. Deng, T.S. McKenna, M. Shalev, M. Salman	103
11:15am	**An Outbreak of Eastern Equine Encephalomyelitis in Wild White-tailed Deer** - S.D. Fitzgerald, S. Bolin, T.M. Colley, M. Kiupel, S.M. Schmitt, and Daniel J. O'Brien	104
11:30am **GRAD**	**Fatal necrotizing Fasciitis and Pneumonia in a Domestic Shorthair Cat associated with *Streptococcus canis***. - R. Sura, L.S. Hinckley, G.R. Risatti, and J. A. Smyth	105
11:45am **GRAD**	**Mortality-Associated Lesions of Endurance Sled Dog** - M.M. Dennis, R.J. Basaraba, D.A. Mosier, G.H. Cantor	106

AAVLD 49th Annual Conference
October 12 – 18, 2006
Minneapolis Hilton – Minneapolis, MN

Abortions due to *Arcobacter skirrowii* in a flock of sheep

A.L. Allen[1] and M. Ngeleka[2]

A Saskatchewan farmer had a flock of sheep that included 75 ewes that he owned previously and 125 ewes that were pregnant when purchased. Lambing was expected from January to May of 2006, but most of the ewes were due to lamb in April. In early March, four ewes aborted within a 10-day period. In mid-March, twin fetuses were aborted and submitted to Prairie Diagnostic Services in Saskatoon, SK, for postmortem diagnostic investigation. No lesions were detected grossly. Microscopically, both fetuses had a diffuse, moderate to severe, neutrophil rich meningitis and mild, multifocal, neutrophil rich pneumonia. Large numbers (4+) of *Campylobacter*-like bacteria were detected during microscopic examination of Gram-stained abomasal fluid. These bacteria were identified as *Arcobacter* sp. based on culture and biochemical test results.

The attending veterinary practitioner and owner were asked to submit additional aborted material if problems continued. Triplet fetuses were aborted on March 27 and transported, along with two placentas, to Prairie Diagnostic Services in Saskatoon. Grossly, the liver of one fetus contained numerous, relatively well demarcated, 0.5- to 1.5-cm diameter, white foci, interpreted to be areas of necrosis, inflammation, or both. Neither placenta had visible lesions, although one of the placentas had two umbilical cords. Microscopically, the lesions in the liver were composed of areas of necrosis, often surrounding large blood vessels, outlined by bands of increased cellularity. The cells involved were degenerated and necrotic, but a few were reminiscent of neutrophils. The fetus with the necrotic hepatitis, and one of the others, had mild meningitis. There appeared to be mild, multifocal placentitis.

Large numbers (4+) of *Campylobacter*-like bacteria were again detected during the microscopic examination of Gram-stained abomasal fluid. Bacteria, identified as *Arcobacter skirrowii*, were isolated from samples of abomasal fluid (3+) and liver (4+).

The farmer chose not to treat the pregnant ewes prophylactically. In all, 20 to 25 ewes aborted and abortions appeared to stop in April, when the majority of the ewes were due to lamb.

The genus *Arcobacter* was included in the family Campylobacteraceae in 1991 and presently consists of six species. *A. butzleri*, *A. cibarius*, *A. cryaerophilus* and *A. skirrowii* are often associated with animals and humans. Much remains to be learned about the species of *Arcobacter* but those that have been associated with mammals are considered potential zoonotic agents transmitted in water and foods of animal origin. In humans, *Arcobacter* have been associated with gastrointestinal disease and bacteremia. The role of *Arcobacter* spp. as an animal pathogen is unclear, but multiple species of *Arcobacter* have been isolated from aborted fetuses and placentas of bovine, porcine, and ovine origin.

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An Investigation of Enzootic Caprine Coxiellosis (Q Fever) in Colorado

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A meat-goat production facility with previously diagnosed enzootic coxiellosis was prospectively investigated over the course of six months with the goals of describing the pathology of naturally occurring *Coxiella burnetii* infection in goats and identifying potential routes of zoonotic transmission from caprines to humans. Investigation was initiated after the first kidding season in which substantial reproductive failure attributed to *C. burnetii* infection was documented. Diagnostic modalities utilized included conventional gross and histopathology, immunofluorescence serology (IFA), real-time were six post-abortion does, their sixteen stillborn fetuses and two clinically unaffected does and their three kids. Additional animals examined were eight adult does of uncertain reproductive status and one adult buck all found dead with no prior remarkable medical history. A three week course of oral tetracycline in drinking water had been administered to the entire herd subsequent to the original diagnosis of coxiellosis.

Placentas from aborting does had marked necrotizing cotyledonary and intercotyledonary placentitis with variable necrotizing vasculitis and inflammatory infiltrates which varied from neutrophilic to lymphohistiocytic. Myriad intracellular bacilli identified within chorioallantoic epithelial cells and stromal histiocytes by hematoxylin/eosin stain were confirmed as *C. burnetii* by IHC. One post-stillbirth doe had severe, fulminant, transmural metritis and diffuse peritonitis comprised nearly exclusively of mixed mononuclear leukocytes. Seven stillborn fetuses had mild-moderate interstitial pneumonia. *C. burnetii* bacilli and fragmented antigen were identified within histiocytic cells in several maternal and fetal tissues by IHC. The eight adult does found dead had diffuse necrotizing metritis with abundant stout bacilli from which several *Clostridium spp.* were cultured.

C. burnetii DNA was detected by real-time PCR in numerous tissues and body fluids from nearly all animals examined. Aborted placentas and uteri of aborting does consistently had the lowest cycle thresholds (highest genome copy numbers). Vaginal, uterine, and milk/mammary specimens were uniformly positive for all does regardless of reproductive success status. Other specimens which were variably, though similarly, PCR positive across does and the buck included liver, spleen, lung, kidney, feces, bone marrow, and various lymph nodes. In aborted fetuses, the most frequently PCR positive specimens were lung, meconium, and thymus. Multiple specimens from thriving fourteen day old kids were PCR negative with the exception of one kidney and one spleen from the same kid which both were positive at high cycle thresholds. Placentas from healthy doe-kid pairs were PCR positive at substantially higher cycle thresholds relative to aborted placentas. All does and the buck had similar IFA titers against *C. burnetii* phase I and phase II antigens.

The following conclusions may be drawn from this data: *C. burnetii* may cause substantial reproductive losses in goat herds for at least the first two kidding seasons subsequent to initial herd exposure. Oral tetracycline is inefficacious for eradication or mitigation. In an infected herd, all does should be considered as *C. burnetii* shedders regardless of their reproductive success status. Pooled fetal tissues (particularly lung, kidney, and meconium) are similarly useful for diagnosis as placenta by PCR. *C. burnetii* may predispose does to fatal post-partum clostridial metritis. Anti- *C. burnetii* antibody titers, while efficacious for determining herd-level exposure, are not predictive of predilection for an individual animal's reproductive failure. Undetermined titer-independent immune status-related factors are likely responsible for determining the clinical course of *C. burnetii* infection in an individual doe.

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The Predictive Value of Gross and Microscopic Lesions Associated with *Clostridium difficile* Infection in Neonatal Pigs.

MJ Yaeger[1], JG Songer[2], J Kinyon[1]

Clostridium difficile is an anaerobic, gram-positive, spore-forming bacillus that has been associated with enteric disease in man and several animal species, including pigs, horses, cats, dogs, rabbits, calves, hamsters, and ostriches. Disease in swine has most often been described in suckling pigs. This abstract outlines the results of a prospective study designed to assess the correlation between the presence of *C. difficile* toxins (TCD) in the colon contents of neonatal pigs and a number of parameters, including gross evidence for diarrhea, mesocoloninc edema, and colitis. The study population consisted of 100 live piglets, 1-7 days of age, submitted to the Iowa State University Veterinary Diagnostic Laboratory with the clinical complaint of diarrhea, and 29 apparently healthy control pigs purchased from local farms.

Clostridium difficile was isolated from 51% (66/129) of large intestines and TCD were detected in the colon contents of 50% (65/129) of the piglets examined. When the relationship between TCD and gross evidence of diarrhea was evaluated, 58% (38/65) of TCD-positive piglets had normal to pelleted contents in their colon and rectum, whereas 75% (48/64) of TCD-negative pigs had gross evidence of diarrhea. TCD-positive piglets were significantly more likely to have normal to pelleted feces ($p \leq 0.001$). At high toxin levels this difference was more pronounced as 74% (18/25) of +4 TCD-positive pigs had pelleted to normal feces.

The primary gross lesion ascribed to *C. difficile* infection is mesocolonic edema. Edema of the mesocolon was observed in 38/65 (59%) of TCD-positive piglets. Because of the high number of TCD-positive piglets (41%) without edema of the mesocolon and the high number of TCD-negative pigs exhibiting mesocolonic edema (51%), a statistically significant association between mesocolonic edema and CDT was not identified and the positive predictive value (PPV) of mesocolonic edema as an indicator of the presence of TCD was only 54%. The exception was when edema of the mesocolon was severe. 89% (8/9) of the piglets with severe mesocolonic edema had high (+3, +4) TCD.

49/65 (75%) of TCD-positive piglets had colitis and 47/65 (72%) had typhlitis. There was a statically significant association between TCD and both colitis and typhlitis ($p \leq 0.001$). The PPV for colitis and typhlitis as an indicator of TCD was 62% and 60% respectively. As the severity of the lesion increased, so did the association with TCD. 22/28 (79%) of the colitis cases graded moderate to severe were TCD-associated, 32/36 (89%) of the cases with neutrophils in the lamina propria were TCD associated, and 16/17 (94%) of the cases with erosions were TCD-associated. Each of these differences was statistically significant.

TCD was detected in the colon contents of 23/29 (79%) apparently healthy controls pigs obtained from 6 separate sites. Compared to diagnostic cases submitted with a history of diarrhea, a significantly ($p \leq 0.001$) greater proportion of control piglets had low levels of toxin. These generally lower toxin levels may help to explain the lack of clinical disease. 70% of TCD-positive control pigs did have colitis, suggesting that *C. difficile* may represent an important subclinical issue in neonatal swine.

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Navel infections in foals by *Clostridium sordellii*

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Navel infections followed by septicemia are responsible for perinatal mortality in several animal species. In foals, the most important causes of navel infection are *Escherichia coli* and *Streptococcus zooepidemicus.* Several clostridia species have been implicated in omphalophlebitis of animals and humans, but to date no information has been published about the role of *Clostridium sordellii* in these infections of foals. *C. sordellii* is a pathogen of humans and animals that has been recently implicated in a series of severe human infections which were followed by death in most cases. In this paper, we describe 8 cases of perinatal mortality in foals associated with navel infection by *C. sordellii*. The foals studied were between 12 and 21 days old, either male or female and of differing breeds. The diagnosis was established based on the detection of *C. sordellii* by at least one of the following methods: culture; fluorescent antibody test; immunohistochemistry; and gross and histopathological findings. All foals had acute peritonitis, the navel was thickened by edema, hemorrhage and fibrosis, and a moderate amount of serosanguinous fluid with fibrin strands was present in the pericardial sac and pleural cavity. Histopathologically, the urachus and umbilical arterial wall were thickened by edema and exhibited hemorrhage, fibrin and leukocytic infiltration. Gram-positive bacterial rods were observed in subepithelial areas of the urachus, the adventicia of umbilical arteries and interstitium of the navel. *C. sordellii* should be considered as a differential diagnosis for navel infection in foals.

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Leptospira Myocarditis in a two-day old Foal due to Leptospira interrogans serovar pomona.

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Equine abortions, stillbirths, and premature birth of weak foals attributable to leptospirosis in mares have been well documented. Infection is promoted in pregnant mares by wet environmental conditions with contamination by feral or domestic carrier animals through urine shedding. Abortion results from fetal infection, with *L. interrogans* serovar *pomona* being the most commonly implicated serovar. Fetal lesions associated with *Leptospira* abortion include nephritis, pulmonary hemorrhage, pneumonia, and myocarditis. A placentitis is generally present, however gross lesions in the fetus or dead foal may be minimal. A presumptive diagnosis can be made by demonstrating the presence of spirochetes in the kidney or placenta by Warthin-Starry stain.

We report here on an unusual presentation of Leptospirosis in a two-day old foal that presented to CAHFS, UC Davis for postmortem examination. The clinical history was that of a weak, full term foal with undefined neurological symptoms. The maternal history was that of a mare that had over-wintered with a herd of cattle on pasture in Sonoma County, California that had flooded during the rainy season. Gross findings of the foal were unremarkable, with the exception of obvious dehydration. Histological findings were limited to a mild interstitial pneumonia, centrilobular hepatocellular dissociation with piecemeal necrosis, and severe suppurative myocarditis. The myocarditis was comprised of infiltrates of neutrophils with fewer macrophages, many with phagocytosed material, that dissected between cardiac muscle cells with minimal degeneration or necrosis. Myriads of spirochetes were identified in the heart lesion by silver stain, and further confirmed as *Leptospira* spp. by immunohistochemistry (IHC) and electron microscopy. Myocarditis was attributed to the serovar *L. pomona*, based on a microaggluutination titer of 1:204,800 in the mare's sera. A titer of 1:25,600 to *Leptospira interrogans* serovar *brataslava* was also detected and presumed to be serologic cross-reaction. The infection in this case was limited to the heart, as other tissues including the kidney and liver, were negative for *Leptospira* spp by both fluorescent antibody assay and IHC. Similar presentation due to *Leptospira interrogans* serovar *pomona* have been reported in humans.

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Minneapolis Hilton – Minneapolis, MN

Prevention of Malignant Catarrhal Fever (MCF) in a Mixed Species Wild Animal Game Park by Production of Virus-Free Mouflon Sheep

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A mixed species game park in North Carolina experienced an acute outbreak of deaths in Pere David's deer, axis deer, blackbuck antelope, whitetail deer and elk. Clinical signs varied from fulminant disease progressing from depression to bloody scours to death in fewer than four days in Pere David's deer to a more protracted form of disease, ranging from two weeks to three months in axis deer. Laboratory findings revealed high levels of anti-MCF virus antibody by cELISA in axis deer presented in a moribund state to the Rollins North Carolina Diagnostic Laboratory. Ovine herpesvirus-2 (OvHV-2) DNA was also detected in peripheral blood leukocytes of the affected axis deer. No alcelaphine herpesvirus (AlHV-1) or other MCF viruses were detected, although wildebeest, goats and other MCF virus-carriers were present in the park. Retrospective examination of frozen tissue samples from Pere David's deer and blackbuck antelope also confirmed the presence of OvHV-2 DNA.

The possibilities for an OvHV-2 reservoir in the park were several since domestic and non-domestic sheep and many species of deer were commingled. The most prevalent ovine species was the mouflon sheep. Both the mouflon and axis deer, a species in which deaths were numerous, had been on the premise for 9 years. The initial recommendation was removal of the mouflon sheep, Jacob four horn sheep, domestic sheep and goats, and pygmy goats. It was further proposed that mouflon sheep derived from MCF positive ewes by early weaning and PCR verification of negativity at 2 months of age could be reintroduced to the park and, subsequently, commingled safely with clinically susceptible species.

The owner agreed to testing of all sheep and goat species and elimination of positive MCF virus carrier sheep from the collection, segregation of the MCF virus-positive mouflon sheep flock to a barn 3 miles from the park, and removal of mouflon lambs from the positive flock at about 60 days of age. Mouflon lambs were tested by PCR at the time of separation and positive lambs were promptly removed. Mouflon were retested at 7-8 months by cELISA and positive animals were removed. The twice-negative mouflon were then deemed suitable for reintroduction to the park as MCF virus-free. Two MCF virus-negative crops of lambs were harvested from the original positive flock. Prevention of direct contact of animals, workers or equipment between isolated MCF virus-negative animals and MCF virus-positive animals was stressed and achieved by a separate group of caretakers.

With respect to animals in the park, all remaining hoofstock species regarded as clinically susceptible hosts were tested by cELISA and PCR as deemed appropriate, and all animals were ear-tagged. Testing of the entire herd of Bovidae and Cervidae was accomplished during the first quarter of 2004 (426 animals tested) and, subsequently, retested during the first quarter of 2005 (391 animals tested). The result showed that about 4% of the clinically susceptible species in the park were seropositive for MCF virus by cELISA. However, no further cases of MCF have occurred since the removal of OvHV-2 positive mouflon sheep and reintroduction of the virus-free lambs about 2.5 years ago. This is the first time that MCF virus-free mouflon sheep derived by early weaning from positive ewes have been reintroduced to a densely populated animal park with multiple susceptible species. Management control of MCF in high population animal parks is possible and practical with such techniques.

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AAVLD 49th Annual Conference
October 12 – 18, 2006
Minneapolis Hilton – Minneapolis, MN

Pre-clinical and early clinical lesions of malignant catarrhal fever in bison experimentally inoculated with nasal mucus containing ovine herpesvirus-2 (OvHV-2)

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Malignant catarrhal fever (MCF)is a common, fatal disease of American bison. Recently our group demonstrated that MCF can be induced experimentally in American bison by aerosol exposure to nasal secretions derived from sheep experiencing intensive shedding events (O'Toole et al: 2004, Successful experimental induction of acute malignant catarrhal fever in bison using aerosols of ovine nasal mucus containing ovine herpesvirus-2
(OvHV-2). Proceedings p. 55, 47th annual conference, AAVLD, 2004). An infectious dose of >1 x 105 OvHV-2 DNA copies induces MCF in susceptible bison (O'Toole et al: 2005, Defining the minimum intranasal infectious dose of OvHV-2 that is required to induce infection and MCF in bison. Proceedings, P. 114, 48th annual conference, AAVLD, 2005).

In the current study (May 31 - July 9 2006), we sought to define morphological changes during the preclinical and early clinical stages of MCF following experimental challenge. This is part of a promixate goal to define the pathogenesis of MCF, and develop practical immunological and/or genetic strategies for control of the disease.

Eighteen bison negative for OvHV-2 based on PCR testing of peripheral blood leukocytes (PBL) were obtained from a commercial bison operation. Following acclimation, 16 were challenged with OvHV-2 by nebulizing each with 2 ml of an inoculum containing 1 x 107 OvHV-2 DNA copies; the remaining two served as uninoculated controls. Bison had evidence of infected PBLs by 15 days post-aerosolization (DPA). All 16 inoculated bison were positive for OvHV-2 by 22 DPA while both control bison were negative. Pairs of bison were selected for post-mortem examination beginning at 23 DPA. Gross lesions of hemorrhagic cystitis, ulcerative typhlocolitis and laryngitis-pahryngitis were present in two asymptomatic bison at 26 DPA. In inoculated bison multifocal consolidation throughout all lobes of the lung, involving <1-5 % of pulmonary parenchyma were a consistent feature. Clinical signs developed at and after 28 DPA. Bison are scheduled to be examined post-mortem until 40 DPA.

We conclude that aerosol exposure of bison using 1 x 107 OvHV-2 DNA copies reliably induces MCF, with onset of clinical signs of 28 DPI and detectable gross lesions at 26 DPI This is the first report of pre-clinical lesions of MCF in any species.

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AAVLD 49th Annual Conference
October 12 – 18, 2006
Minneapolis Hilton – Minneapolis, MN

Malignant Catarrhal Fever Associated with Ovine Herpesvirus-2 in Free-ranging Mule Deer (*Odocoileus hemionus*) in Colorado

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Malignant catarrhal fever (MCF) was diagnosed in 4 free-ranging mule deer (Odocoileus hemionus) in the winter of 2003. Diagnosis was based on typical histologic lesions of lymphocytic vasculitis in multiple organs and PCR identification of ovine herpesvirus-2 (OHV-2) viral genetic sequences in formalin fixed tissues. The animals were from the Umcomphagre Plateau of southwestern Colorado. Deer were radio-collared in 2 separate winter range study areas as part of an ongoing mule deer study evaluating habitat effects on survival. Deer from these herds occasionally resided in close proximity to domestic sheep *(Ovis aries)*, the reservoir host of OHV-2, in agricultural valleys adjacent to their winter range. MCF has been reported in many domestic and wildlife ruminant species but not confirmed in free ranging mule deer. These cases indicate that fatal OHV-2 associated MCF can occur in free-ranging mule deer exposed to domestic sheep that overlap their range.

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AAVLD 49th Annual Conference
October 12 – 18, 2006
Minneapolis Hilton – Minneapolis, MN

Necrobacillosis in Elk (*Cervus elaphus nelsoni*) on a Winter Feedground in Wyoming

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Necrobacillosis is a clinical syndrome of ungulates caused by infection with the gram-negative obligate anaerobe bacterium *Fusobacterium necrophorum*. Outbreaks of this disease have been reported in free-ranging elk (*Cervus elaphus nelsoni*) on feedgrounds from Wyoming since 1944, and significant mortality events associated with this disease are not rare. In the winter of 2006 increased morbidity and mortality were observed in elk on a large feedground in Wyoming, with common clinical signs including lameness, lethargy, recumbency, variable loss of condition, and terminal loss of response to environmental stimuli. Complete necropsies were performed on 20 elk that died or were euthanized. Eight of these elk demonstrated gross and microscopic lesions of classic necrobacillosis (necrotizing stomatitis, necrotizing reticulorumenitis, necrotizing hepatitis, and/or necrotizing interdigital dermatitis or foot-rot with intralesional gram-negative filamentous bacilli), however we were unable to isolate *F. necrophorum* from tissues with lesions, in part due to overgrowth of contaminants, and sometimes due to decomposition of samples. The diagnosis of necrobacillosis was confirmed by PCR performed on formalin-fixed tissues containing appropriate gross and microscopic lesions using primers specific for the hemaglutinin-related protein gene of *F. necrophorum* subsp. *necrophorum* (*F. n. necrophorum*). We also are in the process of validating an immunohistochemical technique for the diagnosis of *F. necrophorum* infection in formalin-fixed tissue sections. Both subspecies of *Fusobacterium necrophorum* (*F. n. necrophorum* and *F. n. funduliforme*) have been associated with lesions of necrobacillosis in ungulates, but F. *n. necrophorum* is considered to be more pathogenic. Necrobacillosis remains a significant (if sporadic) cause of morbidity and mortality for wild elk on winter feedgrounds in Wyoming. Potential risk factors, including artificial concentration of elk and presumptive heavy environmental contamination with *F. necrophorum*, undoubtedly contribute to outbreaks of this disease.

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AAVLD 49th Annual Conference
October 12 – 18, 2006
Minneapolis Hilton – Minneapolis, MN

Hypertrophic Osteopathy Associated with Mycotic Pneumonia in Two Elk

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Two yearling elk bulls raised on the same farm were presented to Purdue University Large Animal Hospital with a primary complaint of weight loss, decreased appetite, and lameness. On physical examination, hard swellings of the distal aspect of all 4 limbs were noted. Radiographs of the limbs of both elk revealed bony periosteal thickening consistent with hypertrophic osteopathy. Several pulmonary nodules were noted on chest radiographs. Blood work was consistent with chronic disease (mild anemia, hyperglobulinemia). The elk were euthanized due to the poor prognosis.

At necropsy, in both animals the carpal, metacarpal, tarsal, metatarsal bones, and the radii, ulnae, and tibiae had smooth thickening of cortical bone, particularly on their dorsal and proximal portions. Areas of bone lysis, sometimes filled with caseous material, were present in the left metatarsus and left rear P3 of one elk. Pulmonary lesions in both elk consisted of encapsulated nodules that occupied most of the parenchyma in one animal. The capsule was fibrous and the nodules contained abundant caseous material. Tracheobronchial (both elk), pre-scapular and retropharyngeal (one elk) lymph nodes 5-10 times enlarged and contained caseous material. The myocardium, kidney, and spleen of the elk with more severe pulmonary lesions had 1-4 mm in diameter caseous nodules.

Microscopically, pulmonary lesions were granulomas, containing myriads of hyphae. Fungal hyphae were 5-8 micrometer thick, with parallel walls, septate and had dichotomous branching. They formed palisades at the periphery of granulomas and were more disorganized and fragmented in the center. Caseous foci were surrounded by a band of degenerated neutrophils and eosinophils with fewer epithelioid macrophages and rare multinucleated giant cells. A fibrous capsule encircled this lesion. Nodal lesions consisted of ill-defined caseous granulomas with fewer and morphologically different fungal organisms than those in the pulmonary nodules. Lymph node fungi were pleomorphic, from 10-30 micrometer in diameter spherules with thick wall to chains of robust and irregular hyphae or pseudohyphae. The heart and kidney nodules were similar to those in the lung and contained either slender hyphae like those in the lung or spherules like those in the lymph node. Vasculitis with thrombosis was observed in vessels adjacent to some of these nodules. The osseous lesions consisted of marked and homogeneous thickening of periosteal bone characterized by parallel and perpendicularly oriented trabeculae of immature bone with abundant osteoblastic activity and much less active osteoclastic activity. Granulomatous osteomyelitis was observed in the caseous nodules observed grossly. Immunohistochemistry for Aspergillus sp. was strongly positive in both the pulmonary and nodal fungi.

Microscopic findings in both elk were consistent with disseminated fungal granulomas and periosteal hyperostosis (hypertrophic osteopathy). Aspergillus antigens were detected by immunohistochemistry and Aspergillus fumigatus was isolated. Pulmonary aspergillosis is the most common fungal infection observed in elk in our laboratory. The source of infection was undetermined, but inhalation of spores from contaminated feed or bedding was suspected. This case represents the first description of hypertrophic osteopathy (HO) in elk. HO has been described in humans, numerous domestic species and in one deer. The pathogenesis of HO is poorly understood. This condition is usually associated with space-occupying lesions in the thorax, neoplastic or inflammatory processes in the lung, and neoplasms of abdominal organs, particularly in the urinary bladder. HO in our case was most likely the result of severe pulmonary lesions.

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October 12 – 18, 2006
Minneapolis Hilton – Minneapolis, MN

Mycoplasma bovis in wild and captive white-tailed deer (*Odocoileus virginianus*) in Ohio: The other *M. bovis* in deer

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From 2001-2006, *Mycoplasma bovis* was diagnosed in eight deer. One wild and four captive white-tailed deer exhibited bronchopneumonia and multifocal caseous bronchiolitis. In these deer, diagnosis was made by culture of the organism from lung tissue and by positive identification of *Mycoplasma bovis* utilizing polymerase chain reaction (PCR). Review of computerized records from the Ohio ADDL Pathology Section revealed isolation of mycoplasma organisms from lung tissue of three additional deer (one wild; two captive) that had clinical signs, gross and histopathologic lesions similar to those of the first four deer.

Signalment and clinical history were available for seven deer. The age of affected animals ranged from 6 months to five years (mean 1.9 years); another deer was an adult doe carrying twin fetuses and the age of one deer was not reported. Five were female and two were male; the gender of one animal was not provided. Submissions occurred during the months October (2), November (2), December (1), February (2) and April (1) in various years. The five deer from which *Mycoplasma bovis* was confirmed by PCR were from four different counties in Ohio (Gallia, Holmes, Knox, Wayne); the remaining three deer were from three additional counties (Allen Licking, Stark). The whole body of each wild deer was submitted for necropsy; for the other 6 deer, private practitioners submitted chilled and formalin-fixed tissues including lung for examination. Interestingly, both wild deer (one one-year-old male and one pregnant female) were submitted for Chronic Wasting Disease evaluation because they were found emaciated, extremely weak and unable to rise (initially considered as abnormal fearlessness of humans). The female wild deer was also reported to be lying in lateral recumbency and breathing heavily when it was found. Signs of respiratory disease were reported from 6 deer and included dyspnea, polypnea, and productive cough of yellow exudate of several days to one week's duration. Weight loss and emaciation were reported in 4 deer. Lethargy , weakness or inability to rise were described for 3 deer.

Gross lung lesions observed or reported in 6 deer included: severe consolidation of anteroventral regions of the lungs, ranging from 30-75% of lung tissue (n=6); multifocal and coalescing, variably sized nodules of caseous tan material ranging from 1-3 mm to several cm in diameter (n=5); variably sized abscesses containing green purulent fluid (n=2); and severe fibrinous pleuritis (n=5). Other gross lesions included 500 ml of pink fluid in the thoracic cavity (n=1), enlargement of tracheobronchial lymph nodes (n=3); fibrinous pericarditis (n=2) and endocarditis (n=1). Microscopic lesions consisted of severe, chronic fibrinopurulent bronchopneumonia (n=8), variably sized well demarcated zones of caseous necrosis arising within bronchioles, that were surrounded by concentric zones of degenerate neutrophils admixed with karyorrhectic debris, followed by lymphocytes, plasma cells and histiocytes in varying concentrations and combinations, and by fibroplasia and neovascularization. Effacement of pulmonary parenchyma by zones of necrosis was described for 6 deer, and zones of coagulative necrosis of parenchyma were noted in three. Fibrin thrombi within pulmonary vessels and interlobular lymphatics were observed in 5 deer, and pulmonary vasculitis was described in two deer.

Mycoplasma organisms were isolated on solid agar or broth media in all cases. PCR confirmed the isolates as *Mycoplasma bovis* in 5 cases, but was not done in 3 cases. Other organisms isolated from pneumonic lung included *Arcanobacterium pyogenes* (n=3), *Pastereulla multocida* (n=2), *Pseudomonas aeruginosa* (n=4), *E. coli* (n=4), as well as individual isolations of *P. trehalosi, Edwardsiella tarda, Klebsiella oxytoca* and *Clostridium perfringens.* No isolations of *Haemophilus* sp. were made, and F.A. tests for BVD, IBR, BRSV and PI3 were negative in three cases. No evidence of adenovirus infection was noted histopathologically in any case.

There is a single report of pulmonary mycoplasmosis associated with *Mycoplasma bovis* in captive deer, but to our knowledge this is the first report of this condition in wild white-tailed deer. Gross and microscopic lesions were similar in all cases to those of *Mycoplasma bovis* infection in calves and to those previously reported in captive white-tailed deer. Findings in this report indicate that *Mycoplasma bovis* should be included in the differential diagnosis of pneumonic lesions in wild as well as captive white-tailed deer.

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Minneapolis Hilton – Minneapolis, MN

Foot-and-Mouth Disease in Pronghorn (*Antilocapra americana*) and Mule Deer (*Odocoileus hemionus*): Susceptibility, Clinical Signs, and Lesions

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The only time that foot and mouth disease is known to have occurred in wildlife in the United States was an outbreak in California in 1924-25 that was transmitted from cattle to mule deer. FMD has never been reported in pronghorn. In a series of experimental infection studies at Plum Island Animal Disease Center, we examined the susceptibility to, intra- and interspecies transmissibility of, and clinical course and laboratory results of FMD type O virus infection in several North American wildlife species. Here we report the clinical and pathological results of experimental infections in pronghorn and mule deer.

Both species proved highly susceptible to infection with FMDV. Clinical signs developed usually within 2 to 5 days post inoculation or post exposure and included fever, depression, transient inappetence, and lameness. Lesions observed on clinical examination included vesiculation and erosion of the glossal epithelium over the ball of the tongue, and vesiculation and erosion of the coronary bands of the feet in the pronghorn. Deer had more extensive oral lesions often involving much of the dorsal epithelium of the tongue. In both species, foot lesions were severe often resulting in complete undermining of the bulbs of the heels and, occasionally, partial sloughing or separation of the hoof wall. At necropsy, besides the characteristic oral and foot lesions, pronghorn and mule deer had rumen pillar erosions/ulcers. Other lesions seen in some animals included decubital ulcers, pneumonia, and myocarditis. Histopathologic examination of tissues from both species revealed lesions characteristic of FMD in domestic ungulates.

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AAVLD 49th Annual Conference
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An Outbreak of Eastern Equine Encephalomyelitis in Wild White-tailed Deer

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Eastern equine encephalomyelitis (EEE) is endemic in Michigan, causing sporadic outbreaks in horses and humans. During July through October, 2005, there was an outbreak of neurologic disease in both horses and wild white-tailed deer (*Odocoileus virginianus*). These neurologic animals were reported from a 6 county area (Barry, Kent, Ionia, Montcalm, Muskegon and Ottawa counties) in southwestern Michigan. During this period 3 horses and 28 deer were submitted from this area with clinical signs of neurologic disease. Neurologic signs reported in affected deer included ataxia, head tilt, lack of wariness near humans, drooling, as well as poor body condition and hair loss. While 10 deer had gross lesions affecting the CNS (abscesses, fractures, etc.), there were no gross lesions present in the remaining 18 deer or any of the horses. Histopathology was similar in both deer and horses, with all levels of the brain affected (cerebrum, midbrain, brain stem, and cerebellum). Common lesions included moderate to marked perivascular accumulations of lymphocytes, plasma cells, and lesser numbers of neutrophils. Additional lesions encountered in some animals included: diffuse gliosis, multifocal glial nodules, scattered degenerate or necrotic neurons, satellitosis, and perivascular hemorrhage.

The initial differential diagnosis included West Nile virus (WNV) and EEE infection. Additional testing included immunohistochemical staining (IHC) of brain tissue for WNV and EEE, PCR on fixed brains, and virus isolation (VI) and PCR on fresh brain. In addition, fluorescent antibody testing of all three horse brains for rabies virus was negative. Out of the total number of 3 horses submitted, all 3 were positive for EEE on IHC, VI and PCR. Out of 18 non-grossly lesioned deer submitted, 10 had histopathology consistent with encephalitis, 8 were positive by IHC for EEE, 7 were positive for EEE by PCR, and 2 were positive for EEE by VI. In addition, one deer was also positive for WNV by PCR, and one deer with encephalitis had numerous *Toxoplasma gondii* cysts in the inflamed areas of the brain. All deer were negative for Chronic Wasting Disease (CWD) by ELISA testing of the medial retropharyngeal lymph nodes.

In the literature there is only one previous report of clinical EEE in a single white-tailed deer. There was a close geographic and temporal relationship between affected deer and horses, which had not been noted in previous EEE outbreaks in Michigan. We suspect that EEE is more common in deer than may be currently recognized. The close association between diagnostic laboratory personnel in the DCPAH, Michigan State University and Department of Natural Resources Wildlife Disease Laboratory and field staff led to rapid recognition and diagnosis of this infectious disease outbreak.

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Fatal necrotizing fasciitis and pneumonia in a Domestic Shorthair Cat associated with *Streptococcus canis*.

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Since the mid 1980s, there have been reports of the emergence of a rarely occurring but often fatal necrotizing fasciitis or myositis in humans associated with Group A streptococcal infections. In the 1990s, there were several reports of a similar condition in dogs associated with group G streptococci, which were also fatal. There have been only 2 published reports of naturally occurring severe (fatal) necrotizing fasciitis in cats, and these were caused by *S. pneumoniae* and a group G streptococcus respectively. Those affected cats were young (<2 years). A fatal case of *Streptococcus canis*-associated necrotizing fasciitis and pneumonia with septicaemia is described here.

A 9-year-old, neutered male Domestic Shorthair cat weighing 7.2 kg was presented to the Connecticut Veterinary Medical Diagnostic Laboratory for necropsy. On first clinical presentation it was limping, and was dull but afebrile. Anterior cruciate ligament damage was suspected. It was treated with meloxicam but showed no improvement. On the 11th day, it suddenly deteriorated and died.

External examination revealed a 3x5 cm area of complete hair loss from the skin overlying the left mid femur. There was an extensive (9x5 cm) well-demarcated area of gray to yellow discoloration in the underlying subcutis and skeletal muscle fascia, which was confirmed microscopically as necrotizing cellulitis and myositis, with myriad coccoid bacteria. There was approximately 50 ml of brown-red turbid fluid in the pleural cavity and foci of greenish discoloration of the lung, shown histologically to be areas of necrosis with thrombosis of the large vessels. Other histopathologic changes included focal myocarditis, necrotizing pleuritis, nephritis and focal encephalitis. Massive numbers of Gram-positive cocci were observed in all lesions.

Bacteriologic and mycologic culture of affected lung and subcutaneous tissue yielded large numbers of β-hemolytic streptococcal species. These were identified as *S. canis* by the Biolog system. Polymerase chain reaction amplification of genomic DNA of the isolate using primers specific for the *S. canis* CAMP factor *cfg* gene and 16S-23S rDNA intergenic spacer yielded 235bp and 215bp products respectively, confirming the isolate as *S. canis*.

Group G streptococci are considered commensal microflora in cats. They have however been recovered from abscesses, and cases of metritis and mastitis in adults, and septicaemia, tonsillitis and cervical lymphadenitis in kittens. There have been 2 cases of pyothorax associated with group G streptococci. This is the first report of naturally occurring infection with *S. canis* causing myositis, and fasciitis in a Domestic Shorthair cat.

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AAVLD 49th Annual Conference
October 12 – 18, 2006
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Mortality-Associated Lesions of Endurance Sled Dogs

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Little is known about causes of unexpected exercise-associated death in apparently healthy dogs, or the lesions associated with extreme physical exertion in animals. The objective of this study was to characterize the lesions and causes of fatalities in long-distance racing dogs that die while or soon after competing in the Iditarod Trail Sled Dog Race. This information is needed to provide insights into how to clinically recognize working dogs affected with exercise-associated life-threatening conditions.

From 1995-2006, all dogs dying during or following competition while still in the care of the Iditarod Trail veterinary team, were examined by gross necropsy and histopathologic evaluation. Twenty-two dogs were evaluated during the study period, from a population of roughly 13,200 dogs that competed. In most cases, dogs were without premonitory clinical signs, collapsed while competing, and died immediately or while in carriage to the next race checkpoint. Lesions repeatedly observed in the study population included rhabdomyolysis (n = 13), enteritis (n = 10), gastritis (n = 9), aspiration pneumonia (n = 9), gastric ulceration (n = 8), mild centrilobular hepatocellular necrosis (n = 6) or hepatic fibrosis (n = 3), gastric dilation (n = 3), and mild cardiac myodegeneration and necrosis (n = 3). Most deaths were attributed to aspiration of gastric contents (n = 6), aspiration pneumonia (n = 3), or acute blood loss (n = 3) secondary to gastric ulceration. The cause of death was undetermined for seven dogs. All dogs with aspiration pneumonia had concurrent gastric mucosal lesions. No findings suggestive of neglect or abuse were found in any of the dogs.

Unexpected death is a rare event in conditioned sled dogs. Aspiration pneumonia, gastric mucosal lesions, and extensive myonecrosis were conditions recognized by this study that may result in death. Dogs that display clinical signs suggestive of any of these conditions should be released or excluded from strenuous exercise. The significance of hepatic lesions and enteritis is unknown since the prevalence of these lesions in the general population of racing dogs is unknown. Epidemiologic investigations are needed to clarify the risk for death certain factors pose endurance-racing dogs.

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Bacteriology Scientific Session

Sunday, October 15, 2006
Salon C

Moderator: Glen Songer and Loraine Hoffman

10:30am	**Experimental Challenge Model for *Actinobacillus suis* in Pigs** - D. M. Jordan, R. Taylor-Vokes, P. G. Halbur, L.J. Hoffman, R.F. Ross	118
10:45am	**Equine Colitis X Associated with Infection by *Clostridium difficile* NAP1/027** - JG Songer, S Dial, JS Brazier, RD Glock, GE Killgore, A. Thompson, B. Limbago	119
11:00am **GRAD**	**Immunohistochemical, Microbiological, and Molecular Detection of *Brucella abortus* in Rocky Mountain Elk** –A.M. Fluegel, W.H. Edwards, K.W. Mills, T.E. Cornish	120
11:15am	**Preliminary results from *Mycobacterium bovis* surveillance of free-ranging white-tailed deer in Minnesota** - A. Wünschmann, J. Schefers, Susan McClanahan, L . Cornicelli, M. Carstensen Powell, B. Hartmann, N.B. Harris, J.T. Nelson, B.V. Thomsen	121
11:30am	**Vaccination of White-tailed Deer with *Mycobacterium bovis* bacillus Calmette Guerín (BCG)** - M.V. Palmer, T.C. Thacker, W.R. Waters	122
11:45am	**Development of an Immunohistochemistry Assay for the Detection of *Erysipelothrix rhusiopthiae* Antigen in Formalin-fixed Tissues** - T. Opriessnig, L.J. Hoffman, and P.G. Halbur	123

AAVLD 49th Annual Conference
October 12 – 18, 2006
Minneapolis Hilton – Minneapolis, MN

Comparative Genome Sequencing of *Salmonella Enteritidis* Isolates that Vary in Virulence Characteristics

J. Guard-Bouldin[1]

Salmonella enterica subspecies I *enterica* serotype Enteritidis (*S.* Enteritidis) is currently the leading cause of salmonellosis worldwide and the second leading cause in the United States. The Centers for Disease Control (CDC) and the USDA Food Safety Inspection Service (FSIS) have recently described epidemiological trends that suggest that this pathogen could be increasing in incidence in people and in broiler chickens. Research is needed to identify small scale genetic change that correlates with the ability of *S.* Enteritidis to cause food borne outbreaks because methods such as DNA-DNA hybridization microarrays and pulsed field gel electrophoresis (PFGE) have failed to differentiate between strains that vary in virulence phenotype. The objectives of this project are to identify single nucleotide polymorphisms (SNPs) that differentiate the genomes of two isolates that were obtained from a single parent strain but that nonetheless had different pathological outcomes in laying hens.

Comparative genomic sequencing (CGS) is a commercially available service (Nimblegen, Inc). CGS requires that a genomic database be available to generate overlapping primers that resolve sequence to a single base pair. Phage type (PT)4 *S.* Enteritidis genome sequence is available from the Pathogen Sequencing Group at the Sanger Institute (http://www.sanger.ac.uk/Projects/Salmonella/). DNA was extracted from three isolates of *S.* Enteritidis, one of which was a PT4 isolate used as a template to generate the overlapping primers. The other two isolates submitted for CGS were PT13a isolates that varied in their ability to contaminate eggs. One of these isolates could contaminate eggs, grow to high cell density, and produce a capsular LPS molecule at 25°C and it was designated wt *S.* Enteritidis. The other PT13a isolate was orally invasive, produced biofilm but could not contaminate eggs or grow to high cell density or produce much capsular LPS at 25°C. It was designated bf *S.* Enteritidis. Both strains were descended from a single parent strain. The isolates chosen minimize the genetic noise from random SNPs that are unlinked to the phenotype of interest.

Results were that 393 SNPs out of the 4.686 million base pairs in the genome, or less than 0.01% of the genome, differentiated the two PT13a isolates that varied in virulence potential. There was an average of 8 SNPs per 100,000 bp. Areas of the genome that had lysogenic phage could not be compared in this assay, because primers made to the PT13a specific bacteriophage Fels-2 were absent for lack of template in the PT4 genome and primers made to the PT4 specific phage, ST64b, were lacking target DNA in the PT13a strains. Thus, SNPs that occur within phage genes that differ between PT4 and PT13a strains are not included in the total and will require manual sequencing. The virulence plasmid from the two PT13a strains differed by 5 SNPs. All classes of genes had SNPs, although genes involved in metabolism were most heavily represented and included more than 30% of the genes identified. Curvilinear analysis of SNPs with identity to PT4 in every 100kb revealed that the PT4 genome under investigation had SNPs occurring between genes *pps* and *yhjO* (about 2/5ths of the genome) that were preponderantly similar to bf PT13a *S.* Enteritidis; however, the rest of the genome was more similar to the wt PT13a isolate. As compared to the two PT13a strains, the PT4 genomic database was genetically a dimorphic hybrid of the wt and bf PT13a isolates, which agreed with previous results obtained by pan-genomic phenotype microarray (Biolog, Inc.). Thus, the PT4 genome sequenced by the Sanger Institute exhibits a mixture of phenotypes from a single genome in response to environmental signals.

We conclude that very little genetic change is required for *Salmonella* Enteritidis to alter its virulence phenotype and that the ability of bacteria to mutate rapidly obscures identification of those SNPs that are most closely linked to outbreaks of salmonellosis. Furthermore, epidemiological investigations that are based on fingerprinting methodology are inadequate for detecting evolutionary trends due to SNPs that impact the virulence potential of the Salmonellae. The current problem of food borne illness associated with *S.* Enteritidis may have originated when a single bacterial cell was unstably co-infected by two incompatible lysogenic bacteriophage. This single cell may have rapidly split into two phage lines that nonetheless had only slightly different pathogenic potential to begin with and that overtime evolved adaptations to selection pressures present in different regions and niches within the on-farm environment.

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October 12 – 18, 2006
Minneapolis Hilton – Minneapolis, MN

Characteristics of *Mycobacterium paratuberculosis* Super-shedders (SS) in three Northeast USA, Regional Dairy Quality Management Alliance (RDQMA), Dairy Herds

R.H. Whitlock*[1], Y, Schukken[2], J. Smith[3], J. Van Kessel[4], E. Hovingh[5]; J. Karns[4], D. Wolfgang[5], T. Johnson[6], V. Olson[2] and T. Fyock[1]

Over a two year time period of this longitudinal prospective multiyear study, three dairy herds known to have Johne's disease, with a total of more than 530 mature dairy cows were sampled semi-annually for both fecal and blood samples. At the onset of the study, each herd completed a herd risk assessment and implemented best management practices designed to reduce the possibility of fecal oral disease. Additional quarterly herd visits collected blood for Johne's ELISA testing.

The first apparent test prevalence of fecal culture positives for each herd was 3.2%, 6.8% and 14% respectively. During this two-year period of testing 2,138 fecal cultures, 75 (3.5%) was culture positive.
Of those 75 culture positive fecal samples, 46 (61%) were low shedders with less than 10 colonies on four tubes, while 14 (19%) were classified as moderate shedders and 15 (20%) as heavy shedders with more than 50 cfu/tube. These 14 heavy shedder fecal samples originated from 10 different cows. When the fecal samples classified as heavy shedders were serially diluted, the final cfu/gm ranged from 28, 000 to 3,010,000 cfu/gm. If we classify super-shedders as cows with more than 10,000 cfu/gm, then each heavy shedder would be a super-shedder. Eight (57%) of the heavy shedders samples had between 28,000 cfu and 98,000 cfu/gm while 4 (29%) had between 100,000 to 1 million cfu/gm. Only 2 (14%) had more than one million cfu/gm. All heavy shedding cows, including all SS, in this study were ELISA positive concurrently with the classification as a SS.

Five of the 10 (50%) of the SS cows were detected as SS on the first whole herd culture.
Of those SS cows with an initial negative culture, only one cow progressed from culture negative to SS within a 6 month time period. This same cow then progressed from 71,400 cfu to 434,000 cfu over the next 3 months then was culled. Two cows progressed to SS status within one year from a culture negative status. Each herd had two or more cows classified as SS over the two year period of the study. Preliminary observations suggest that most super-shedder cows do not have clinical signs of Johne's disease (weight loss & diarrhea), although they shed as much or significantly more MAP into the environment than a typical cow with clinical Johne's disease. Since these cows are not readily recognized by dairymen, super-shedder cows may be responsible for massive environmental contamination that results in a disproportionate percentage of the new MAP infections that occur in a herd.

Johne's super-shedder cows represent a new dimension in this disease complex that deserves more attention by diagnosticians. These SS cows must be identified promptly and culled quickly. Otherwise they serve as a significant threat to overwhelm even the best management practices designed to safeguard the neonatal calf and growing heifers from MAP infection.

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AAVLD 49th Annual Conference
October 12 – 18, 2006
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Brain heart infusion broth in decontamination process does not have any effect on the recovery of *Mycobacterium avium subsp. paratuberculosis* from broth cultures

Sreekumari Rajeev[1] *, Roy D. Berghaus[2], Beverly Byrum[3],Charles Baldwin[1]

Abstract

The protocol for the recovery of *Mycobacterium avium subsp. paratuberculosis* (MAP) calls for the preparation of hexadecyl pyrinidium chloride (HPC) and antibiotic brew in half strength brain heart infusion broth (BHI). Since our general laboratory experience indicated that increased level of contamination in cultures can result due to the addition of BHI, we decided to examine whether BHI has any influence on the recovery of MAP from broth cultures. National Veterinary Services Laboratory (NVSL) supplied proficiency test samples (n=26) for the year 2005 were used for the comparison. Two sets of samples were processed in the experiment. In one set of samples, the HPC and antibiotic brew were prepared in half strength BHI and for the other set, HPC and antibiotic brew were prepared in sterile distilled water. The samples were processed and inoculated following recommendations of ESP para-JEM culture system. MAP recovery from sample sets processed with BHI/HPC and water/HPC were compared. The result obtained from the comparison study was also compared to the official results obtained from NVSL. Six of the nineteen positive samples were eliminated from the NVSL official results since fewer than 70% of the participating laboratories obtained positive culture results for these samples. Culture of the 26 samples using the BHI/HPC decontamination method identified 13 (50%) positives, while culture using the water/HPC decontamination method identified 14 (54%) positives. The proportions of samples with a positive result did not differ significantly between the two decontamination methods, although both methods identified fewer positive samples than the official NVSL results. If the 6 samples lacking a consensus by participating laboratories were removed, there was no significant difference between any of the testing methods with respect to the proportions of positive results. Although in most cases it took longer to identify a positive sample by the BHI method, the difference between methods with respect to the number of days to a positive culture result was not statistically significant. This study indicates that BHI is not a required component for the recovery of MAP from broth cultures.

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Use of the Trek ESP® Liquid Culture System for the Rapid *Detection of Mycobacterium avium subsp paratuberculosis* and other Acid Fast Bacilli.

Fales, WH[1]; Reilly, TJ[1]; Payeur, JB[2], Harris, BN[2]; Loiacono, CM[2]; and Adams, CA[1]

The TREK ESP Liquid Culture System is designed to detect microbial growth by measuring pressure changes in the head space of the culture bottle. Pressure changes are the result of microbial growth whereby the organisms consume oxygen in the bottle head space or produce gas. The system offers a rapid technique for the detection of *Mycobacterium avium subspecies paratuberculosis* and other AFB from fecal and tissue samples. We have passed all Johne's check tests from NVSL since 2003, when the system was put into service.

All samples are processed via the Cornell Double Enrichment Technique and cultured using the TREK *para*-JEM® bottles, growth solution (GS), egg yolk solution (EYS), *para*-JEM BLUE and antibiotic solution labeled *para*-JEM AS. Other mycobacteria can be recovered and detected from various samples in the system with the use of a different antibiotic solution which is labeled PVNA.

Samples producing a growth signal in the TREK ESP unit are stained for AFB with modified Kinyon cold acid fast stain and subjected to confirmation of *Mycobacterium avium subspecies paratuberculosis* (MAP) with the polymerase chain reaction (PCR). Fecal and other tissue submissions from 2 Sept. 04 to 17 April 06 were reviewed. To date, 799 samples have been processed and 121 or 15% were found to be positive.

Cultures that do not produce a growth signal after 45 days of incubation in the TREK ESP unit are stained with Kinyon acid fast method. Approximately 4% of the samples will demonstrate an AFB positive organism. These isolates do not readily react with the PCR test. However, upon sub-culture to Herrold's Egg Yolk Agar, isolates will grow, demonstrate mycobactin dependency and react with the PCR when tested from the slant.

In addition to detecting MAP from cattle, the TREK ESP Unit has also detected *M. avium*. Samples from exotic species have also been cultured and the following isolates have been recovered: *Mycobacterium haemophilum* from a hedgehog (1) and a *Mycobacterium subspecies* from a 100 year old tortoise (1). The TREK ESP Unit has significantly reduced the culture time for MAP when compared with HEA, which may take up to 16 weeks of incubation. Cattle that are heavy fecal shedders of MAP can be detected in 10 days. Moderate shedders are detected in 25 days and light shedders may take up to 45 days to be detected.

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October 12 – 18, 2006
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Serial Dilutions of TNTC Mycobacterium *avium* subsp. *paratuberculosis* fecal samples

T.L. Fyock[1], R.H. Whitlock[1], R.W. Sweeney[1], E. P. Hoving[2], D.R. Wolfgang[2] and A.C. Monson[1]

The most common method for definitive diagnosis with Johne's disease (JD) is culture of *Mycobacterium avium* subsp. *paratuberculosis* (MAP) from a fecal sample on Herrold's egg yolk media (HEYM). Samples are usually plated on 3 to 4 slants and positive results are reported with a numerical count of colony forming units (cfu) per slant. Standard laboratory practice across the United States has been to report tubes with more than 50 cfu as TNTC (too numerous to count.) The focus of this study was to determine range of shedding in animals that were classed as TNTC by using serial dilutions of the sample and calculating the estimated cfu per gram that each animal was shedding.

The cultures were processed by the standard three day centrifugation method. This method includes an overnight incubation in ½ strength BHI/HPC, a 30 minute centrifugation step followed by an overnight incubation in antibiotic brew. Three samples were processed per animal then combined to create a 3 ml 1:1 sample. Using the 1:1, four additional dilutions were created using nine mls of ½ BHI. The 1:1, 1:100 and 1:1000 dilutions were plated. The original culture was 4 slants plated with 200ul. For this study flasks of HEYM were used instead of slants to provide a flat even surface for growth and to facilitate the ease of counting. Each dilution was plated on 3 flasks and each flask received 100ul of sample. Samples were incubated at 37^0 and read every 2 weeks for 16 weeks. Most of the samples had colonies present at the 6 week reading on the 1:1 dilution and all had visible colonies by week 8. Only samples that yielded a TNTC (50 cfu or more) on the 1:1 dilution were included in the data set.

The estimated cfu was calculated by the following: 2 gm sample in 35 ml water, transfer 5 ml of fecal-water mixture (an estimated/calculated 285 mg of fecal sample) to final BHI-HPC tube that yields 1200 ul of re-suspended pellet in antibiotic brew mixture. Then 100 ul (equivalent to 24 mg of the original fecal sample) is inoculated on each flask. Following incubation for 12 weeks the readable colonies are enumerated and averaged for the three flasks for each dilution. This number is multiplied by 42 to yield cfu/gm then multiplied by the dilution factor to give the final cfu/gm. Of the 166 TNTC samples completed in culture 48 (29%) had an estimated cfu between 1000/gram and 10,000/gram. The range was 1280 to 8400. There were 52 samples (31%) that had >10,000/gram and <100,000/gram. The range was 12,600 to 98,000. The remaining 66 samples (40%) had estimated cfu that were > 100,000/gram. Sixteen (10%) of TNTC samples had over 1 million cfu/gram. The range was 112,000 to 3,675,000.

In conclusion, there is a vast range of shedding in animals classed as TNTC. Standard culture on solid media presents serious limitations to appreciate the spectrum of shedding. Understanding the actual shedding level would be significant to the herd owner that has multiple TNTC animals in the herd. One TNTC animal that sheds 1million organisms per gram would be much more significant to the overall herd than even 100 animals shedding at 1000 cfu/gram. Culture methods and/or practices should be enhanced to define the differences between TNTC animals so that the animals posing the greatest threat can be detected early and removed quickly. Serial dilutions of samples that are positive by week 6 is one option and culture by liquid method using a time to positive scale is an other option.

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AAVLD 49th Annual Conference
October 12 – 18, 2006
Minneapolis Hilton – Minneapolis, MN

Use of a Fecal PCR assay on environmental samples for Johne's disease herd status identification

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Johne's disease is an intestinal disease in ruminants caused by *Mycobacterium avium* subsp. *paratuberculosis* (Map). Map is predominantly spread via the fecal-oral route and can contaminate the environment. It survives in certain environments for over 12 months (Whittington *et al*., 2005) and it can be found easily in many dairy farm environments. Detection of this bacterium in environmental samples can be accomplished by using fecal culture but faster and cheaper detection methods are needed. A previous study (Raizman *et al*., 2004) has shown that targeting common contaminated areas in the farm environment suggest an alternative for herd screening and Johne's infection status assessment. The objective of this study was to evaluate the application of a fecal PCR assay for diagnosis of Map in environmental samples and to determine its sensitivity for detection of dairy cattle herds infected with Johne's disease. Two environmental fecal samples were collected from up to five different locations on each farm: cow alleyways, manure storage (manure push, manure pack or lagoon), calving area, fresh cow area, and sick cow area (for a total of six to ten samples total per herd). 409 environmental fecal samples from 49 Minnesota dairy farms known to be infected from previous testing were tested using Taqman PCR (ISMav2) assay and fecal culture (FC, HEYM using the 72-hours sedimentation protocol) for *Map*. PCR results were compared with solid culture method results at the herd and sample level and by location sampled. Herd level analysis showed that of 49 infected farms, 32 were positive by PCR (65.3%) and 44 by FC (90%). From those 44 herds positive by FC, 31 (70.5%) were also positive by PCR. At the sample level, 202 of 409 environmental samples were positive by FC. Of those 202 positive samples by FC, 22.3% were positive also by PCR. PCR was able to detect 36% of heavy (51-100 CPT) and 59% (13/22) of very high bacterial load samples (>100 CPT) ($p<0.0001$). Within environmental areas, highest detection rates were found in fresh cow area (30%), manure storage (27%) and cow alleyways (21%). Fecal PCR assay can be used as a quick test for detection of high to very high bacterial load samples. In summary, our results demonstrate the applicability of the PCR test as a screening test for detection of high bacterial load environmental samples in herds of unknown status due to the lower costs and quicker turn around time.

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October 12 – 18, 2006
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A sensitive and novel ELISA for bovine paratuberculosis

M.T. Collins, S.J. Shin, D. Cho

A novel ELISA for bovine paratuberculosis (UW-ELISA) was developed and evaluated using a panel of well-characterized bovine sera. This new ELISA is based on novel *Mycobacterium paratuberculosis* antigens. Analytical sensitivity of the UW-ELISA is enhanced using a commercial avidin-biotin conjugate system. The UW-ELISA was evaluated with a panel of previously described bovine sera (Clin. Diagn. Lab. Immunol. 12:685-692, 2005). Paratuberculosis cases (n=444) were fecal culture-positive cows and controls (n=412) were cows resident in seven Midwest dairy herds free of paratuberculosis (test-negative for more than 4 years, i.e., level 4 in the Voluntary Bovine Johne's Disease Program). UW-ELISA results were compared to those produced with paratuberculosis ELISA kits sold by IDEXX, CSL/Prionics, Institut Pourquier, and Synbiotics by ROC analysis. ELISA sensitivity, specificity and area under the ROC curve (AUC) are tabulated below.

Kit / Assay	Sensitivity[a] (95% C.I.)	Specificity[a] (95% C.I.)	Area Under ROC Curve (95% C.I.)
IDEXX	28.9% (24.6 - 33.5)	95.3% (92.53 - 97.22)	0.619 (0.580 – 0.659)
CSL/Prionics	28.4% (24.5 – 33.1)	99.7% (98.46 - 99.99)	0.795 0.741 – 0.808)
Pourquier	28.0% (23.7 – 32.5)	100.0% (99.98 – 100.00)	0.709 (0.673 – 0.745)
Synbiotics	44.5% (39.3 – 50.8)	84.9% (80.48 – 88.46)	0.706 (0.666 – 0.747)
UW-ELISA	56.3% (51.6 – 61.0)	99.0% (97.50 – 99.70)	0.894 (0.873 – 0.915)

[a]Sensitivity and specificity of the four commercial kits listed was previously reported, (Clin. Diagn. Lab. Immunol. 12:685-692, 2005). AUC values were not previously reported.

When compared to four other widely used commercial ELISA kits for bovine paratuberculosis, these data show that UW-ELISA has a significantly higher diagnostic sensitivity with an equivalent specificity giving it the highest AUC of the five assays evaluated. Additionally, the UW-ELISA works effectively on both serum and milk samples (data not shown).

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October 12 – 18, 2006
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Consensus recommendations on diagnostic testing for bovine paratuberculosis.

M.T. Collins[1], F.B. Garry[2], A.J. Roussel[3], S.J. Wells[4], I.A. Gardner[5]

There are multiple diagnostic tests for bovine paratuberculosis and selection of the "best test" for specific situations can be confusing to producers, practitioners and diagnosticians alike. Funded by a USDA Cooperative Agreement, a panel of experts developed consensus testing recommendations for paratuberculosis based on test accuracy, test economics, producer and Johne's disease economics, and epidemiological principles of test interpretation. These recommendations pertain only to bovine paratuberculosis but consider both beef and dairy cattle on either commercial or seedstock production operations. Determination of the best test is driven by these four types of operations and the purpose for testing. Seven testing purposes are considered: 1) classify herds as infected, 2) estimate within-herd *Mycobacterium paratuberculosis* infection prevalence, 3) support a Johne's disease control program, 4) surveillance, 5) eradicate the infection from a herd, 6) confirmation of a clinical diagnosis, and 7) biosecurity (pre-purchase testing). The 28 different scenarios (4 herd types x 7 testing purposes) each have a specific recommendation for the best test, and for some scenarios a second or third choice test is also provided. Among these scenarios, the recommended tests include culture of environmental fecal samples, culture of pooled fecal samples, culture of individual animal fecal samples, PCR on individual animal fecal samples, ELISA on serum or milk samples, histopathology and microbiology on tissues collected by biopsy or necropsy. These consensus recommendations provide diagnosticians a guide for which paratuberculosis testing services veterinary diagnostic laboratories should offer. Hopefully, this also will lead to greater consistency in testing recommendations to producers and practitioners based on fitness of tests for their intended purpose.

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October 12 – 18, 2006
Minneapolis Hilton – Minneapolis, MN

Evaluation of an ELISA for Detection of Botulinum Toxins A, B, E and F in Bulk Tank Milk Samples

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Over the last decade, there have been numerous reports of botulism in dairy cattle and beef cattle exposed to botulinum toxins B, C, or D. Although the risk of transmitting botulism from blood to milk is considered minimal, milk from affected dairies is routinely withheld from the market at significant loss to the producer because of the potential threat to public health. Futhermore, recent work has indicated that clinically-affected mastitic cattle may pass toxin into the milk at detectable levels (Boehnel et al, 2005). It was the goal of the current study to evaluate the ability of a previously-developed ELISA assay to detect toxin in bulk tank milk samples. The assay used was developed as a collaborative effort by CDC for detection of botulinum toxins A, B, E, and F in environmental specimens. The assay has been successfully tested on pasteurized homogenized milk although unprocessed milk samples had not been assessed as a sample matrix. ELISA detection has the advantage of rapid turn-around (hours), a detection limit comparable to bio-assay, and capability for high-throughput screening for large numbers of samples.

One hundred samples of non-pasteurized and non-homogenized bulk tank milk were randomly collected from 100 individual dairies (range 800-2,000 cows). The samples were characterized for protein content, fat content, somatic cell count, and enteric bacterial contamination colony-forming units. Seventeen of the 100 samples were selected to represent the range of the parameters identified (e.g. high or low protein, fat, somatic cell, and CFU). The 17 bulk tank milk samples plus one pasteurized, homogenized whole milk sample were individually spiked with log-dilutions of purified dichain botulinum A, B, E, and F toxins (Metabiologics Inc) in triplicate to assess the toxin detection limit and within assay reproducibility of the ELISA.

In our preliminary trials toxin A was detectable in bulk tank milk at approximately 0.01 ng/ml, 0.1 ng/ml for toxin B and E, and 1 ng/ml for toxin F. These values correspond to <1 mouse LD50 detected for all toxin types. Variability between triplicates was less than 2% for each of the four toxins evaluated, and no significant difference ($p<0.01$) was found between the different milk samples. Cross reactivity between the 4 toxins was not detected.

Preliminary results indicate that the CDC-developed ELISA can be used with milk matrices, and is suitable as a high-throughput format for diagnostic detection of botulinum toxins in milk specimens.

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Experimental Challenge Model for *Actinobacillus suis* in Pigs

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Actinobacillus suis has increasingly been associated with respiratory and septicemic disease in swine. Single agent infections by *A. suis* is commonly seen in field cases through the ISU Veterinary Diagnostic Laboratory. Tabulation of cases involving isolation of *A. suis* indicates a rising trend in disease associated with *A. suis* over the last 10 years, increasing from 16 cases in 1995 to 148 cases in 2005. There are no commercial vaccines nor antimicrobial treatment claims specifically against *A. suis*. A dose-titration study was conducted with intratracheal and intranasal administration of 10^4-10^8 cfu of *A. suis.* Intratracheal administration of high doses (10^7-10^8cfu) of *A. suis* in swine results in acute pleuropneumonia similar to that observed in field cases and resembling pleuropneumonia induced by *A. pleuropneumoniae*. Pigs inoculated intranasally also developed pleuropneumonia when administered high doses *A. suis*, and gross lesions of pneumonia achieved in about 50% of the pigs. Medium dosages of 10^6 cfu of *A. suis* were given intranasally and intratracheally resulted in less extensive clinical disease, fewer gross and microscopic lesions, and dramatic reduction in bacterial recovery from the organs. Pigs receiving low dose inoculations were not different than uninoculated control pigs. This challenge model will provide a basis for further investigation of the pathogenesis, virulence attributes and immunogenicity of *A. suis* infections in swine.

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Equine Colitis X Associated with Infection by *Clostridium difficile* NAP1/027

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In April 2001, a 14-year-old quarterhorse with a 48 h history of colic was euthanized after failure to respond to treatment. At necropsy, the animal was in good overall condition. A large amount of yellow, clear fluid was found in the abdomen, and lungs were somewhat congested. Small intestine contained an excessive amount of fluid contents, but no mucosal lesions were observed. Cecum and colon contained normal amounts of semisolid ingesta, but the mucosa was very congested throughout. Segmental edema was observed, as was significant thickening of intestinal wall. Mucosa appeared to be intact in most areas, but 2 - 4 mm ulcers were found in distal colon.

Submucosa of colon was very edematous, resulting in separation of tissues. Infiltrations of primarily mononuclear inflammatory cells throughout the submucosa and extending into mucosa resulted in excessive numbers of mononuclear cells in mucosal lamina propria. Vascular congestion was common throughout. In some areas, focal hemorrhages were found in submucosa just beneath the muscularis mucosa, while in other areas submucosal hemorrhage was diffuse and extensive. Numerous large Gram-positive rods were observed on mucosa and extending into lamina propria.

Bacteriological findings of significance were isolaton of large numbers of *Clostridium difficile* and demonstration of substantial amounts of toxins A (TcdA) and B (TcdB). A diagnosis of peracute enteritis involving colon and cecum, with lesions and history typical of those attributed to colitis X.

Clostridium difficile colonies were examined by ribotyping, pulsed-field gel electrophoresis (PFGE), toxinotyping, and PCR assays for binary toxins and deletions in *tcdC*. The isolate belonged to North American PFGE type 1 (NAP1), PCR ribotype 027, and toxinotype III. Genes for the binary toxin were present, and *tcdC* contained an 18 bp deletion.

Strains with this specific genotype, referred to as the "epidemic strain," have appeared across North America, Europe, and the United Kingdom over the past several years. They are associated with human *C. difficile*-associated disease (CDAD) of greater-than-normal severity, and have been shown to produce greater amounts of TcdA and TcdB than typical, hospital-acquired ("non-epidemic") strains. Reports of NAP1/027 from domestic animals are rare. However, its occurrence in cases such as described here raises the possiblity of a zoonotic component.

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AAVLD 49th Annual Conference
October 12 – 18, 2006
Minneapolis Hilton – Minneapolis, MN

Immunohistochemical, Microbiological, and Molecular Detection of *Brucella abortus* in Rocky Mountain Elk

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Brucellosis, caused by the bacterium *Brucella abortus*, is endemic in bison (*Bison bison*) and Rocky Mountain elk (*Cervus elaphus nelsoni*) in the Greater Yellowstone Area of Wyoming, Montana, and Idaho. *Brucella abortus* is shed by infected elk during abortion or parturition and most often is transmitted through contact with infected fetuses, fetal fluids, or vaginal exudates. Current diagnostic methods used to identify infection in elk include serological testing and bacterial culture, but the correlation between serology and culture results is poor, serology cutoff values are challenging to determine, and bacterial culture is slow and likely to be positive only later in gestation or following abortion or parturition. There is a need for more informative, sensitive, and rapid diagnostic tests to detect brucellosis in elk. As part of the Wyoming Game and Fish Department's brucellosis surveillance program, elk were trapped using corral traps and serologically tested for brucellosis on the Grey's River elk feedground in 2005 and on the Dell Creek, Muddy Creek, and Grey's River elk feedgrounds in 2006. Twenty-three seropositive cow elk aged ≥ 1.5 years from the feedgrounds were killed and necropsied. Paired tissue samples (routine organs, reproductive tissues including fetal tissues, and a variety of lymph nodes) were collected and frozen at –70 C for bacterial culture and PCR and fixed in 10% neutral buffered formalin for histopathology and immunohistochemistry (IHC). Tissue sections were examined for microscopic lesions indicative of brucellosis and stained with an anti-*B. abortus* polyclonal antibody for IHC. *Brucella abortus* was isolated from 45% (9/20) of the seropositive cow elk and 5% (1/19) of the fetuses. Tissues were collected during necropsy for evaluation with real-time PCR to identify the presence of *B. abortus* in fresh tissue. The assay will be developed further to distinguish *Brucella* biotype, and PCR and immunohistochemistry results will be compared to culture (gold-standard test) results to determine the sensitivity and specificity of each assay.

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AAVLD 49th Annual Conference
October 12 – 18, 2006
Minneapolis Hilton – Minneapolis, MN

Preliminary results from *Mycobacterium bovis* surveillance of free-ranging white-tailed deer (*Odocoileus virginianus*) in Minnesota

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Bovine tuberculosis (*Mycobacterium bovis) was* diagnosed in 5 cattle herds in Minnesota in the summer and fall of 2005 for the first time since 1971. Subsequently, head lymph nodes were collected from 573 hunter-killed white-tailed deer carcasses (*Odocoileus virginianus*) from where the bovine tuberculosis-positive herds were resided (Roseau and Beltrami County). One of the deer carcasses was confiscated and further examined because a granulomatous pleurisy was noted by a DNR official although the deer appeared to be otherwise healthy judged by adequate muscling and fat stores. The head lymph nodes of all deer and the tissues of the confiscated deer were screened for the presence of *M. tuberculosis/bovis* infection by gross examination and culture. *M. bovis* was isolated from two deer during this survey. One deer had *M. bovis* isolated from the pleural lesion and multiple internal and peripheral lymph nodes but not the head lymph nodes. The other deer had *M. bovis* isolated from a small focal mineralization in the retropharyngeal lymph node. Both deer *M. bovis* isolates and all cattle isolates were identical as evidenced by DNA fingerprinting (spoligotyping and restriction fragment length polymorphism (RFLP) typing). The study demonstrated the ability of this particular *M. bovis* isolate to infect white-tailed deer. The infection may have spread from the cattle to the deer population sharing the same habitat. It is interesting to note that the Minnesota *M. bovis* isolate differs from the isolates that are present in white-tailed deer from Michigan and Manitoba, but is similar to strains more commonly found in cattle from the southwestern U.S. and Mexico. Further screening of deer during the hunting season of 2006 is planned to monitor the extent of the *M. bovis* infection in the free-ranging population.

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AAVLD 49th Annual Conference
October 12 – 18, 2006
Minneapolis Hilton – Minneapolis, MN

Vaccination of white-tailed deer with *Mycobacterium bovis* bacillus Calmette Guerín (BCG)

M.V. Palmer[1], T.C. Thacker[1], and W. R. Waters[1]

In 1994, a free-ranging white-tailed deer (*Odocoileus virginianus*) in Michigan was diagnosed with tuberculosis due to *Mycobacterium bovis,* the causative agent of bovine tuberculosis. Subsequent surveys identified a focus of *M. bovis* infection in free-ranging white-tailed deer in northeast Michigan. This represents the first known reservoir of *M. bovis* in free-ranging wildlife in the United States and a significant obstacle to the eradication of bovine tuberculosis in domestic livestock. Current control measures include decreasing deer density through increased hunting and limitations on feeding and baiting of deer. Another possible control measure would be vaccination of white-tailed deer to prevent infection, disease or transmission. Vaccination of captive farmed red deer (*Cervus elaphus*) with *M. bovis* BCG has been shown to decrease infection and/or disease (lesion development). To evaluate the efficacy of *M. bovis* BCG vaccination of white-tailed deer, 31 yearling white-tailed deer were randomly assigned to one of three groups; 2 doses of 10^7 CFU of BCG (Pasteur) administered 6 weeks apart SC (n=11); 1 dose of 10^7 CFU of BCG (Pasteur) SC (n=10) and unvaccinated deer (n=10). All deer were intratonsilarly inoculated with 300 CFU of virulent *M. bovis* 77 days after the 2 dose BCG group received the second dose of vaccine. One hundred thirty days after challenge with virulent *M. bovis* all deer were euthanized and examined. Gross lesion severity scores of the medial retropharyngeal lymph node were reduced ($P < 0.05$) in deer receiving 2 doses of BCG compared to unvaccinated deer. Lesion severity scores of the lungs did not differ between groups. Total pathology scores (sum of scores for medial retropharyngeal lymph nodes and lung) were lower ($p=0.1$) in deer receiving 2 doses of BCG as compared to unvaccinated deer. Microscopic evaluation of the medial retropharyngeal lymph nodes revealed fewer granulomas in BCG-vaccinated deer than in unvaccinated deer. Moreover, medial retropharyngeal lymph node granulomas in deer receiving 2 doses of BCG were smaller, less necrotic with rare acid fast bacilli compared to lesions in lymph nodes from deer receiving a single dose of BCG or unvaccinated deer. *Mycobacterium bovis* BCG (Pasteur) can be effective in reducing lesion severity in *M. bovis*-inoculated deer when initial vaccination is followed by a booster vaccination. Decreased lesion severity with less necrosis and fewer acid fast bacilli would likely decrease the ability of vaccinated deer to shed virulent *M. bovis* thus decreasing intraspecies and interspecies transmission.

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AAVLD 49th Annual Conference
October 12 – 18, 2006
Minneapolis Hilton – Minneapolis, MN

Development of an immunohistochemistry assay for the detection of *Erysipelothrix rhusiopthiae* antigen in formalin-fixed tissues

T. Opriessnig, L.J. Hoffman, and P.G. Halbur

Erysipelothrix rhusiopathiae is pathogenic for both animals and humans. In swine, disease may be either acute (septicemia) or chronic. The chronic form may result in the development of arthritis and endocarditis. During acute erysipelas outbreaks in animals, substantial mortality can occur within days. In the US, diagnosis of erysipelas infection in animals is currently confirmed by culture which is time-consuming since 24-48 hours are required for isolating the bacterium. The Veterinary Diagnostic Laboratory at Iowa State University offers culture and PCR for diagnosis of erysipelas. If requested, further characterization can be done by serotyping and pulsed-field gel eletrophoresis (PFGE). Recently, an immunohistochemistry assay for *Erysipelothrix rhusiopathiae* was developed by using a cocktail of equal volumes of polyclonal antibody against seroypes 1a, 1b, and 2 produced in rabbits. The IHC procedure was tested on 35 cases confirmed to be positive by culture, on 10 negative control cases, and on 5 cases suspected to be erysipelas septicemia but culture negative. The IHC test was found to be specific to *E. rhusiopathiae* antigen in formalin fixed, paraffin embedded tissues and it did not produce staining in cases where other pathogenic swine bacteria were confirmed to be present. We found that the IHC is useful on cases where the animals have been treated with antimicrobials prior to submission and cultures were negative. In addition, the erysipelas IHC was found to be useful to detect erysipelas antigen in skin lesions which are often culture negative.

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Molecular Diagnostics Scientific Session

Sunday, October 15, 2006

Duluth

Moderator: Sharon Hitala and Kathy Kurth

AAVLD 49th Annual Conference
October 12 – 18, 2006
Minneapolis Hilton – Minneapolis, MN

Expanded multiplexed capabilities for the detection of vesicular/ulcerative diseases including bovine and porcine panels and new sample matrices

R.J. Lenhoff, P. Naraghi-arani, B. Hindson, P.J. Hullinger and M. McBride

Last year Lawrence Livermore National Laboratory (LLNL) Bioassays and signatures group (BSG) developed a bead based PCR assay for the detection of four endemic and three foreign animal diseases. The purpose of this panel was to shorten the response time for diagnosis of foot and mouth disease (FMD) and give greater confidence in a negative diagnosis of FMD by providing a differential diagnosis for observed clinical signs/lesions. This panel was developed under DHS funding and in close collaboration with the United States Department of Agriculture (USDA) and the National Animal Health Laboratory Network (NAHLN). The first version of the panel (underwent an interlaboratory comparison in 13 NAHLN labs + FADDL, PI) contained ruminant and porcine diseases and was optimized for use with an oral/nasal swab. In 2006 we are developing bovine and porcine specific panels with additional endemic and foreign animal diseases. We are also optimizing the new panels for additional sample matrices such as whole blood, serum, epithelial tissue and milk (for the bovine panel). This new multiplex PCR approach to disease diagnosis will strengthen the capabilities of the United States to detect and respond to accidental or intentional introduction of foreign animal diseases.

AAVLD 49th Annual Conference
October 12 – 18, 2006
Minneapolis Hilton – Minneapolis, MN

Diagnostics of Emerging Infectious Diseases in Livestock by Microarray.

M.T. McIntosh[1]*, D. Suciu[2], Consuelo Carrillo, and S. Metwally[1]

Emerging infections and foreign animal diseases represent threats to public and animal health and present added challenges to the diagnosis of disease. To address these issues, we previously designed a pan-viral microarray (FADDL V1.0) against the complete viral genomes of more than 1,100 different viruses and phage present in GenBank. The FADDL V1.0 array was found to be useful in the detection of known viruses such as foot-and-mouth disease virus (FMDV), unknown agents such as a genotypic variant of bovine viral diarrhea virus and further proved useful in the detection of Mycoplasma and various tissue culture endogenous viruses. To improve upon this, we have designed a new pan-viral microarray (FADDL V2.0) which enables us to rapidly compare diagnostic specimens against all known animal viruses with genetic representation in the NCBI database. A computational approach was employed that fragments each reference viral genome sequence into segments and then compares each segment against the more than 400,000 viral sequence submissions at NCBI. This process was used to select roughly 11,000 animal virus-specific oligonucleotides which were then synthesized on the microarray. This versatile approach applied specifically to the Aphthoviruses was used to yield a sub-array aimed at genotyping foot-and-mouth disease viruses. The FADDL V2.0 microarray is under design and evaluation against the seven known serotypes of FMD, known foreign animal disease agents and unidentified isolates associated with disease outbreak in the field. For detection of RNA viruses as well as transcription from DNA viruses, mRNA was isolated from infected cell cultures or tissues and reverse transcribed into cDNA. cDNA was made double stranded by incubation with Sequenase and labeled by random primed PCR amplification in the presence of fluorescent Cy3-dUTP (infected) or Cy5-dUTP (controls). Sequence tags were incorporated into the 5' ends of the random primers to facilitate subsequent recovery of viral genetic material hybridized to the arrays. Individual and dual probed hybridizations of the arrays were carried out by previously described methods (Wang, D. et al., 2002) and signals were detected and quantified using an Axon 4000A microarray scanner (Molecular Dynamics Inc.) and GenePix Pro software (Molecular Diagnostics, Inc.).

We conclude that pan-viral microarrays are a useful tool in virus discovery and genotyping.

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AAVLD 49th Annual Conference
October 12 – 18, 2006
Minneapolis Hilton – Minneapolis, MN

Multiplexed Detection of Antibodies to Non-Structural Proteins of Foot-and-Mouth Disease Virus

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Liquid array technology was used to develop a multiplexed assay for the detection of antibodies to viral non-structural proteins (NSPs), raised in cattle in response to infection with foot-and-mouth disease (FMD) virus. Two assays, one based on recombinant NSPs and the other on synthetically produced peptides were developed and compared side-by-side. Serum samples from serial bleeds of cattle, each experimentally infected with one of the seven serotypes (C, A, O, Asia, SAT1, SAT2 and SAT3) of FMD virus were analyzed. A distinct pattern in the detection of NSP antibodies, and a close correlation of the recombinant protein and peptide-based assays was observed. Significantly, all comparative assays in this development phase were carried out in a 48 hour period, demonstrating the high throughput capacity of a multiplexed assay. The detection of antibodies to NSPs is a method to differentiate FMD-infected and FMD-vaccinated animals and a high throughput assay would be an invaluable tool in the case of an outbreak of FMD in North America, when emergency vaccination may be utilized to spare vaccinated, non-infected animals from slaughter and subsequent disposal. Following initial development, NSP assays were optimized and evaluated against large numbers of uninfected cattle, vaccinated cattle and FMD-positive cattle to establish the expected performance of the assay across the different populations.

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AAVLD 49th Annual Conference
October 12 – 18, 2006
Minneapolis Hilton – Minneapolis, MN

An Interlaboratory Comparison of Multiplexed PCR Diagnostics for Foot-and-Mouth Disease and Six Rule Out Diseases

P. Hullinger, B. Hindson, L. Tammero, R. Lenhoff, M. McBride, T. Beckham and B. Martin

Lawrence Livermore National Laboratory (LLNL), in collaboration with the Department of Homeland Security (DHS) and the United States Department of Agriculture (USDA) has developed a multiplexed nucleic acid detection assay which targets three foreign animal and four endemic diseases. The purpose of this assay was to shorten the time for initial diagnosis of foot and mouth disease (FMD) by developing a tool which could provide embedded surveillance for FMD while being used to test for other endemic vesicular/ulcerative diseases of cattle and swine. The first version of assay was developed and optimized for an oral swab-sample in viral transport media Thirteen NAHLN laboratories and the National Veterinary Services Laboratory at the Plum Island Animal Disease Center (PIADC) participated in an interlaboratory comparison of this assay. Participants received equipment, training on how the perform the multiplexed assay, then each laboratory analyzed 192 blinded samples. Blinded samples were prepared in two different sample matrixes (clean VTM and VTM with a clinical bovine swab) individually spiked with one of four infectious domestic viruses; Bovine Herpes Virus-1 (BHV-1, infectious bovine rhinotracheitis (IBR)), Bovine Diarrhea Virus (BVDV), Bluetongue Virus (BTV) and Bovine Papular Stomatitis Virus (BPSV), over four pre-determined concentration ranges for each virus. Each virus-spiked sample was replicated up to 4 times in a single plate to assess assay repeatability. Assay success rate among the laboratories was 92%. Participants successfully detected spiked domestic virus samples over a range of concentrations for each virus. The following performance characteristics of the assays were evaluated: sensitivity, specificity, assay success rate, intra- and inter-laboratory variation. Receiver operator characteristic (ROC) curves for signatures in the multiplex panel will be presented. Follow on testing in the NAHLN laboratories will be conducted to better characterize the diagnostic performance of the domestic disease components of the assay. The diagnostic performance of the foreign animal disease components of the assay will be further characterized in 2006 through collaborations within the US and internationally.

AAVLD 49th Annual Conference
October 12 – 18, 2006
Minneapolis Hilton – Minneapolis, MN

Evaluation of Different Clinical Samples for the Detection of Classical Swine Fever

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Classical swine fever (CSF) is a highly contagious viral disease affecting both domestic and wild pigs, which, if introduced into the United States, would cause severe economic losses to the swine industry. The clinical severity of the disease caused by CSF virus (CSFV) is highly variable, and is often times indistinguishable from many swine diseases present in the U.S. The close proximity to the US of countries in which CSF is endemic, the amount of movement of pork products through international trade, and the amount of international passenger traffic pose a very real threat of CSF introduction into the U.S. Understanding the diagnostic value of different clinical samples plays an important role in the ability to detect the presence of disease. The work described here evaluated the value of different clinical samples for detection of CSF over the course of infection with three isolates of varying virulence.

In order to gain a greater understanding of the clinical course of disease caused by CSF viruses of varying virulence, and to identify the most appropriate diagnostic samples for detection over the course of disease, three isolates of CSFV of varying virulence (low, moderate and high) were inoculated into 6-8 week old swine (27 swine each for the low and moderately virulent strains and 12 swine for the highly virulent strain). Infected and control animals were observed and scored for the severity of clinical signs including fever, anorexia, respiratory distress, neurological signs, and diarrhea/constipation. Samples were collected from live animals at regular intervals over the course of the experiment. For the low virulent strain, animals were sacrificed roughly every seven days during the first 35 days of infection, then roughly every two weeks ending on day 91. For the highly and moderately virulent strains, animals were sacrificed when moribund and/or roughly every seven days. Viremia and virus shedding in nasal and tonsilar secretions were evaluated using blood, nasal swabs, tonsil scrapings, and tonsil samples collected at defined intervals. Samples were evaluated using virus isolation, immunohistochemistry and a real time reverse transcriptase PCR (rRT-PCR) assay. Detection of CSFV and/or CSF viral RNA was correlated with the onset and severity of clinical signs.

Results from this study indicate that irrespective of isolate virulence, blood and tonsil scrapings/tonsil are superior to nasal swabs in detecting CSF virus and CSF viral RNA over the course of CSFV infection.

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AAVLD 49th Annual Conference
October 12 – 18, 2006
Minneapolis Hilton – Minneapolis, MN

Validation of Non-nested and Real-Time PCR for Diagnosis of Sheep-Associated Malignant Catarrhal Fever in Clinical Samples

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Sheep-associated malignant catarrhal fever (SA-MCF), a herpesviral disease caused by ovine herpesvirus 2 (OvHV-2), can pose a significant diagnostic challenge to veterinary clinicians in that its clinical presentation makes it difficult to differentiate from several other common viral diseases. The classical signs of corneal edema, fever, and generalized lymphadenopathy, are not always evident, particularly in acute cases in highly susceptible species such as deer and bison. Although the predominant microscopic lesions of MCF, (lymphoproliferation, mucosal inflammation, and vasculitis) are highly suggestive, they may either be absent or so mild that the diagnostician has difficulty in differentiating them from similar lesions that can accompany bovine virus diarrhea/mucosal disease, infectious bovine rhinotracheitis and epizootic hemorrhagic disease. *In situ* immunohistochemistry or immunofluorescence has been unsuccessful, despite many attempts, for demonstration of viral antigen in tissues.

The previous cloning and sequencing of a fragment of the OvHV-2 genome resulted in the development of a nested PCR assay specific for the virus, which advanced the ability to detect OvHV-2 DNA in animals with clinical MCF. However, the use of this PCR as a routine diagnostic method for clinical MCF in veterinary diagnostic laboratories is problematic because the nested amplification format has the potential for contamination from the amplicons. Recent preliminary data revealed that high levels of OvHV-2 DNA are present in the blood and tissues of various animals, including cattle, bison, deer, and other exotic ruminant species that developed clinical MCF. Based on these data, we hypothesized that OvHV-2 DNA in the blood and tissues of ruminants with clinical MCF could be detected by non-nested PCR and real-time PCR. In this report, we validated non-nested and real-time PCR for detection of OvHV-2 DNA in samples from clinically affected animals. Two sets of tissue or blood samples were collected: one set consisted of 97 samples from naturally-affected animals with clinical or histopathologic, and nested-PCR evidence of MCF, as well as animals with experimentally-induced MCF; the second set consisted of 100 samples from animals without clinical MCF (defined as no MCF clinical signs or histological lesions, and OvHV-2 nested PCR-negative). Among 97 positive samples defined by nested PCR from clinically affected animals, 95 (98%) were positive by non-nested PCR and 93 (96%) were positive by real-time PCR, respectively. Neither non-nested PCR nor real-time PCR resulted in positive signal from any of 100 negative control samples. The data confirmed that both non-nested and real-time PCR maintained the high specificity and had adequate sensitivity for detection of OvHV-2 DNA in clinical samples from animals with SA-MCF.

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AAVLD 49th Annual Conference
October 12 – 18, 2006
Minneapolis Hilton – Minneapolis, MN

Preliminary development of a real-time PCR for all serotypes of Epizootic Hemorrhagic Disease Virus.

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Epizootic hemorrhagic diseases virus (EHDV) has been associated with bluetongue-like disease in cattle. Although US EHDV strains have not been experimentally proven to cause disease in cattle there is serologic evidence of infection in cattle. Bluetongue virus (BTV) causes an estimated $125,000,000 annual loss to the U.S. livestock industry and about $3,000,000,000 annual losses worldwide. Therefore rapid diagnosis and differentiation of BTV and EHDV is required. Our laboratory has been developing the molecular basis for early detection of indigenous and exotic BTV and EHDV disease outbreaks. The foundation for these tests was accomplished by phylogenetic analysis of two conserved target genes; one that is highly expressed in infected mammalian cells, the other is highly expressed in infected insect cells. The analysis of all BTV and EHDV prototype strains indicated that a complex primer design will be necessary for a comprehensive gene amplification diagnostic test. This information has been used as the basis for the development of rapid EHDV real-time PCR that detects all EHDV serotypes. The EHDV detection assay does not cross-react with BTV serotypes; however, this assay is less sensitive than nested-PCR protocols. The sensitivity of 1 pg double-stranded RNA for all eight serotypes is sufficient for diagnostic applications without the contamination problems associated with standard PCR and especially nested-PCR tests.

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AAVLD 49th Annual Conference
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Minneapolis Hilton – Minneapolis, MN

Development of a realtime PCR assay for the detection of foreign and domestic Bluetongue viruses.

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Bluetongue is an important athropod-borne viral disease of ruminants. It is caused by the bluetongue virus (BTV) which is a member of the Orbivirus family of double stranded RNA viruses. BTVs are varied with as many as 24 different serotypes known, 5 of which are known to circulate in the U.S. While outbreaks of BTV threaten livestock and may result in significant animal losses, trade in animals or animal products, particularly exports from the U.S., are also severely curtailed by the incidence of bluetongue. Diagnostic detection of BTV is laborious and difficult. Blood of viremic animals represents the ideal sample for testing and virus isolation of BTV using in vitro culture represents the most definitive method of diagnosis. A complex virus neutralization assay may likewise be employed to distinguish between the 24 distinct virus serotypes. Serum may be tested by complement fixation, AGID, or by various ELISA's though such assays may be confounded by the detection of past infections and by the detection of related Orbiviruses such as African Horse Sickness (AHS), or Epizootic Hemorrhagic Disease (EHD) which typically infects wild ruminants such as deer. While PCR presents a rapid, sensitive and specific testing option for BTV and the OIE recommends a highly sensitive nested RT-PCR assay against the NS1 gene of BTV; this test too is more technically challenging and time consuming than would be a single-step assay. In the detection of foreign animal diseases, it is of particular importance to be able to detect not only the known domestic serotypes of BTV (serotypes 2, 10, 11, 13 and 17) but also all other BTVs known or suspected to exist throughout the world. While serology or virus neutralization can be utilized to accurately determine the serotype (foreign or domestic), a rapid yet sensitive and specific method is needed to quickly diagnose the presence of BTV. To this end we have aligned all available genome sequences for BTV's and have designed degenerate primers to the NS3 gene region of the viral genome. While this represents one of the most conserved regions of the BTV genome, variability was still found to be quite high so inosine was incorporated into the primer designs in order to ensure the efficient hybridization of target and primer sequences. The resultant degenerate primers were tested in a single step RT-PCR assay which was subsequently optimized on a Cepheid Smart Cycler II using a similarly designed inosine-containing Taqman probe. Thus far, this optimized realtime PCR assay reliably detects BTV serotypes 1-20, 22-24. Amplification was not detected among related viruses that included EHD-1 and AHS serotypes 2, 3, 7, 8, and 9.

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Validation of a PCR test for detection of *Haemophilus parasuis* in clinical samples

S. Oliveira[1], J. Tomaszewski[1], R. Gayle[1], J. Collins[1]

Haemophilus parasuis is a Gram-negative bacterium that colonizes the upper respiratory tract of healthy swine. This organism can cause systemic infection depending on the virulence of the strain and the immune status of the affected population. Pigs affected by *H. parasui*s systemic infection may develop high fever, severe respiratory distress, swollen joints, central nervous system signs and occasionally sudden death, which may occur within 48-72 hours post infection. Lesions are characterized by fibrinous exudate on the surface of thoracic and abdominal organs, also know as polyserositis. Diagnosis of *H. parasuis* infection has traditionally been performed by isolating the organism from pigs with characteristic clinical signs and lesions. However, isolation is frequently negative in samples from dead pigs or pigs that were treated with antibiotics prior to sample submission. Considering these factors, a more sensitive test was needed to confirm diagnosis of systemic infection by this organism. A PCR test has been previously developed for detection of *H. parasuis* in clinical samples (Oliveira et al, 2001). This test was initially validated using a limited number of clinical samples, and further validation was needed to access its usefulness in a diagnostic laboratory setting.

Material and methods: Three hundred clinical samples from pigs suspect of *H. parasuis* systemic infection submitted for routine testing at the University of Minnesota Veterinary Diagnostic Laboratory were tested by PCR and bacterial isolation. Only samples from organs containing fibrinous exudate on the surface were included in this study. Samples collected for PCR testing consisted of fibrinous exudate suspended in 2 ml of sterile PBS. Samples were vortexed and DNA was extracted. PCR conditions have been previously described by Oliveira et al 2001. Samples collected for bacterial isolation consisted of swabs from organs containing fibrinous exudate. Swabs were cultured onto blood agar with a *Staphylococcus aureus* nurse streak and incubated at 37ºC during 24 h in a CO_2 incubator. *Haemophilus parasuis* was identified based on the following biochemical tests: V-factor requirement (+), X-factor requirement (-), Catalase (+), Oxidase (+), Hemolysis (-), Urease (-), CAMP test (-), Esculin (-), and Indole (-). Bacterial growth other than *H. parasuis* was also recorded.

Results: Of the 300 samples tested, 146 (48.6%) were positive for *H. parasuis* by PCR and 37 (12.3%) were positive by isolation. Most PCR and isolation positive results originated from samples with acute lesions (104 by PCR and 22 by isolation), based on histopathological evaluation. PCR was also positive for 9 samples with subacute lesions and 20 samples with chronic lesions, compared with 3 and 7 samples positive by isolation, respectively. The PCR test detected *H. parasuis* in samples containing mixed bacteria, such as *Actinobacillus. suis, Actinobacillus. pleuropneumoniae, Actinobacillus. indolicus, Actinobacillus. minor, Actinobacillus porcinus, Bordetella Bronchiseptica, Pasteurella multocida, Escherichia coli, and Streptococcus suis.* PCR testing of these bacterial species generated negative results, confirming the specificity of the test for detection of *H. parasuis*. PCR was also successful in detecting *H. parasuis* from samples of acute lesions with negative isolation results.

Conclusion: PCR was far more sensitive than traditional bacterial isolation for the diagnosis of *Haemophilus parasuis* systemic infection and greatly improved the detection of this underdiagnosed swine pathogen.

Reference: Oliveira S, Galina L, Pijoan C. (2001) Development of a PCR test to diagnose *Haemophilus parasuis* infections. J Vet Diagn Invest. 13(6):495-501.

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Identification of O Groups of Non-serotypable *Escherichia coli* Strains by PCR-RFLP Analysis of O Antigen Gene Cluster

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E. coli serotyping is conventionally performed by agglutination reactions using antisera raised in rabbits against 175 different O standard strains. O serotyping is laborious and often generates equivocal results due to cross reactions between different serogroups. At the Gastroenteric Disease Center, out of a total of 49,754 *E. coli* strains that have been serotyped over the last thirty years, 12,265 strains (24.6%) are O negative as they did not react with any of the standard antisera. One thousand one hundred and twenty five strains (2.2%) were rough and could not be typed. Another 1234 isolates (2.5%) were autoagglutinating, mucoid and sometimes cross reacted with antisera from multiple O types .Therefore, about 29% of the isolates could not be designated O types by conventional serotyping. A PCR-RFLP based method was developed for all *E. col*i serogroups where 10-14 kb O-antigen gene clusters of 175 O-serogroups were amplified by long-range PCR method using the flanking conserved sequences in the "JUMPstart" region and the *gnd* gene. The amplicons were digested with restriction enzymes *EcoRV, EcoR*1 and *Hae* III for RFLP analysis. Fifty non-typable isolates that could not be designated O groups by conventional serotyping were typed by this method. Sixty percent of the isolates tested could be assigned an O group. However, RFLP profiles of some isolates did not match with any known serogroup. The PCR-RFLP based method will not only be useful for *E. coli* diagnostics but can eventually detect new O types that have not be designated serologically for epidemiological and other studies. The method can also identify the serogroups that are very similar with respect to O antigen cluster sequence. Furthermore, it will not require use of animals for antigen production. The molecular method is rapid, cost-effective, highly reproducible and easy to interpret as compared to the conventional serological method.

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Impact generated by viral recombination on molecular epidemiology of PRRS viruses

Sang-Ho Cha[1*], Kyoung-Jin Yoon[1,2]

Porcine reproductive and respiratory syndrome virus (PRRSV) has demonstrated a high degree of genetic and antigenic variability, which imposes difficulties in controlling the disease. Sequential pig-to-pig passages of a virus demonstrated that: a) continuous point mutations contributed to this variability; and b) genetic changes of the virus over times were in a predicted and steady manner, supporting "molecular clock" theory. However, it is not uncommon that multiple strains of PRRSV co-circulate in a herd/site. Hence, recombination between 2 different strains can be expected besides random point mutation of each strain. In the present study, we examined the recombination event by inoculating pigs simultaneously with 2 North American strains (13B6 and 13C8) that were derived from the same parental virus and its impract on sequencing and phylogenetic analysis.

The animals were randomly divided into 3 treatment groups (2 single infection groups and 1 dual infection group). Each group consisted of 3 pigs at each passage. At the first passage, 3 pigs each were inoculated with single virus (13B6 or 13C8), or both viruses (mixed viruses) and kept individually in HEPA-filtered isolation units for 60 days post inoculation (DPI). One pig served as the negative control. At the end of 60-day observation period, various specimens (15 tissues and organs) were collected from each pig and used as a mixed tissue homogenate for subsequent inoculation of corresponding pigs. This study represented a total of 180 days *in vivo* replication of each virus. During the observation period, all pigs were bled periodically. Virus isolation was attempted by plaque assay using MARC-145 cells on 7, 14, 21, 28 and 35 DPI sera from dual infection group in the first passage, and 21DPI sera from mixed or single infection groups in first and second passage. A total of 760 plaque-cloned viruses (20 viruses per pig at each sampling) were obtained from sera and subjected to sequence analyses for all structural protein genes (ORFs 2-7). Phylogenetic analysis was conducted based on each ORF or all ORFs. Phylogetic analysis was also carried out on field isolates from submissions to Iowa State University Veterinary Diagnostic Laboratory or ones available at GenBank to elucidate the presumable roles of the recombination in field studies on PRRSV evolution.

Five recombinants, representing five types of recombination, arose in all pigs of dual infection group throughout this study and appeared as early as 14 DPI. The dominant recombinant virus varied in each pig and over time, resulting in transfer of different recombinants to pigs in the subsequent passage. Crossover sites were random and varied among the observed recombinants. Phylogenetic analyses revealed that the recombinants could be classified into different groups depending on ORF of choice. Combined with phylogenetic results using field isolates, this study suggested inconsistent genetic classifications among the selected regions of field isolates occurred by random recombination among multiple strains in the fields. Therefore, recombination among PRRS viruses occurs and can lead to inaccurate phylogenetic results when partial gene (ORF5 or/and ORF7) was used, which should be taken into consideration when molecular epidemiology is conducted.

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Mycoplasma haemolamae in camelids: Diagnosis, experimental infections and treatments

Susan J. Tornquist[1]

Mycoplasma haemolamae (*M. haemolamae*), is an organism that infects the red blood cells of llamas and alpacas. Infection may be subclinical or it may be associated with clinical signs including fever, mild to marked anemia, depression, icterus, infertility, edema, poor growth rate.

Most of the organisms formerly known as *Haemobartonella spp*. and *Eperythrozoon spp*. have been reclassified as mycoplasmas and are in the group of hemotropic mycoplasmas. The hemotropic mycoplasmas are small bacteria that lack a cell wall. They are generally < 1.0 □n and usually appear as coccoid, rod-shaped, or ring-shaped organisms on the erythrocyte plasma membrane. The haemoplasmas have not been successfully cultivated *in vitro*, so study of these organisms requires maintenance in an infected animal.

In 2001, a PCR-based assay to amplify the 16S ribosomal RNA gene of *M. haemolamae* was developed using primers based on GenBank sequence accession AF306346 and blood from a naturally-infected alpaca. Specificity was shown by failure of these primers to amplify related *Mycoplasma* species including *M. haemosuis*, *M. haemofelis*, and *M. genitalium.* This assay has been useful in diagnostic testing and in study of transmission, epidemiology, and treatment of infections. PCR analysis of asymptomatic and/or treated animals, have shown that many infected animals are inapparent, probably chronic carriers of these organisms

Using the PCR assay, studies have been performed to determine the kinetics of infection and response to treatment for this infection. An initial study of experimentally-infected alpacas showed that all developed parasitemia detectable by PCR at least two days before organisms were seen on blood smears. Some alpacas became mildly to moderately anemic. None developed a fever nor hypoglycemia. Some infected alpacas were treated with a commonly-used tetracycline regime (20 mg/kg subQ every 3rd day, for 5 treatments) while others received no antibiotic treatment. The alpacas were monitored for the following 6 months with weekly body temperatures, packed cell volumes (PCV) and detection of *M. haemolamae* by both blood smear examination and PCR performed. After 6 months, all alpacas in the study were treated with immunosuppressive doses of dexamethasone. Both antibiotic-treated and non-treated groups included alpacas that were PCR negative at some time points during the study and PCR positive at others. The study showed that some healthy camelids may clear parasitemia without antibiotic treatment, and that the standard tetracycline regime used in camelids often does not clear the infection as detectable by PCR, though organisms are no longer seen on blood smears.

A similar study of experimentally-infected llamas followed a similar protocol, but tested the efficacy of injectable florfenicol in treatment of *M. haemolamae* infection. Florfenicol was less effective in clearing infection as detectable by PCR than was tetracycline. In fact, untreated infected llamas often cleared the infection faster than those treated with florfenicol.

A third study examined the use of both oral and injectable enrofloxacin to clear experimental infection in llamas. Ten llamas were infected, and 5 were treated with oral enrofloxacin; the other 5 were treated with injectable enrofloxacin. Neither oral nor injectable enrofloxacin showed efficacy in clearing the infection and there was no significant difference between the two formulations of the drug.

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October 12 – 18, 2006
Minneapolis Hilton – Minneapolis, MN

A Streamlined Integrated Process for Viral RNA Isolation and qRT-PCR for Detection of ALV

Q. Hoang[1*], A. M. Burrell[1], I. Moon[1], W. Xu[1], K. Evans[1], R. C. Willis[1], M. A. Bounpheng[1], and X. Fang[1]

The need for rapid detection of viral nucleic acid is important for the identification and control of many animal pathogens. The current gold standard for most virus pathogen detection is virus isolation (VI) followed by ELISA. This process is labor intensive, time consuming (2-4 weeks), and costly. As a result of this cumbersome method and the multiple steps involved, the data is inconsistent and not 100% reliable. Multiple steps lead to increases in human error and sample cross-contamination. Moreover, the lengthy turn-around time associated with this method makes surveillance, eradication, and control of many animal pathogens challenging. To overcome these challenges, we offer an alternative process for pathogen detection based on molecular detection technology. Viral RNA isolation using Ambion's MagMAX™ magnetic beads is coupled to real-time quantitative RT-PCR (qRT-PCR), providing a streamlined integrated process for a simple, rapid, and highly sensitive method for viral pathogen detection. Highly reliable results can be obtained in less than 3 hours using our method.

To minimize manual sample processing and reduce labor of this process, we have optimized protocols for the automation of Ambion's MagMAX™ magnetic bead-based viral RNA isolation technology on the KingFisher® Magnetic Bead Processors. The KingFisher Processor automatically transfers MagMAX™ particles containing nucleic acid through binding and washing steps. This particle transfer method provides highly effective binding and washing processes and shorter incubation steps. Viral RNA isolation from 96 samples requires less than 30 minutes. The purified RNA is of high quality and is suitable for qRT-PCR using Ambion's optimized AgPath-ID™ Detection Kits and Applied Biosystem's 7500 Real-Time PCR System. This workflow provides the complete solution for a well integrated system for viral pathogen detection.

To validate our integrated process, we analyzed 250 blood samples from a poultry breeder flock for identification of Avian Leukosis Viruses (ALV). ALV in chickens is known to cause a variety of erythroid, lymphoid, and/or myeloid leukoses resulting in significant economic losses in the poultry industry by diminishing feed conversion, inciting neoplastic disease, and increasing mortality. The gold standard for ALV detection is virus isolation followed by antigen capture ELISA. We analyzed these samples using the gold standard and our integrated molecular detection approach using MagMAX™ particles and AgPath-ID™ ALV Detection Assay. Our results demonstrated high correlation with the gold standard. More importantly, our method was faster and less labor intensive. Molecular detection of ALV by qRT-PCR provided better detection sensitivity than ELISA. Our process provides a better method for ALV identification which can expedite the control of ALV infection.

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Detection of canine distemper virus in canine blood samples by real-time reverse transcription polymerase chain reaction

B. Podell[1]*, K. Pabilonia[1], C. Duncan[1], C. Gerhard[1], H. VanCampen[1]

Canine distemper virus (CDV) causes severe multi-systemic disease in dogs and other carnivores. Variable clinical presentations of the disease can make diagnosis of CDV challenging. Thus, a rapid and sensitive diagnostic test will aid in the diagnosis of canine distemper virus. CDV spreads rapidly to multiple organ systems through extensive infection of the host lymphocyte population, making whole blood an ideal diagnostic sample that is easily attainable by the submitting veterinarian.

A real-time reverse transcription-polymerase chain reaction (RT-PCR) assay using a dual-labeled fluorogenic probe on a Smart Cycler platform was developed for rapid detection of CDV in canine whole blood samples. The primers and probe were designed to target the conserved central region of the CDV nucleoprotein (NP) encoding gene using Primer Express software (Applied Biosystems, Foster City, CA) and sequence information available on NCBI GenBank.

Analytical sensitivity was evaluated by 10-fold serial dilution of a CDV Onderstepoort strain to determine the minimum copy number detectable by the real time RT-PCR assay. Analytical specificity was evaluated by melting curve analysis of diagnostic samples and a CDV Onderstepoort strain. Sequence analysis of the amplification product of real-time RT-PCR positive samples indicated that canine distemper virus was specifically amplified. Standard curve analysis allowed for evaluation of amplification efficiency and reproducibility of the assay.

The real time RT-PCR assay was compared to a conventional gel-based RT-PCR assay for the detection of CDV to evaluate agreement between the assays. 108 out of 110 samples that were positive for CDV by conventional RT-PCR were also detected as positive by real time RT-PCR. The 2 contradicting results may have been due to inherent problems with conventional PCR, including contamination and misinterpretation in gel analysis. 211 out of 220 samples that were negative for CDV by conventional RT-PCR were also found to be negative by real time RT-PCR. The 9 samples that were negative for CDV by conventional RT-PCR but found to be positive for CDV by real-time RT-PCR were subjected to sequence analysis. The sequence analyzed was consistent with CDV, showing that the results determined by conventional RT-PCR were falsely negative.

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October 12 – 18, 2006
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Proteomics-Based Diagnosis of Canine Lymphoma:
Affinity Selection and Stable Isotope Labeling with HPLC/MALDI-TOF MS Peptide Profiling

C.R. Wilson[1,2], K.A. Meyerholtz[1], F.E. Regnier[3], and S.B. Hooser[1,2]

Lymphoma is a relatively common type of leukemia in dogs with many similarities to non-Hodgkin's lymphoma in humans. Increases in fucosylated proteins have been shown to occur in various types of neoplasia, including lymphoma. Using targeted proteomics techniques, a method has been developed to isolate and quantify changes in fucosylated serum peptides from dogs with lymphoma compared to normal canine serum or serum samples from the same dog post-chemotherapy. This procedure involves: 1) trypsinization of serum proteins, 2) differentially labeling peptides in serum samples using a stable isotope-coded labeling agent, 3) isolating fucosylated serum peptides using lectin affinity chromatography, 4) separating the serum peptides via reverse phase liquid chromatography, and 5) identifying and quantifying relative changes in these peptides using MALDI-TOF mass spectrometry. Using this approach, 46 fucosylated peptides were elevated in serum from a dog with lymphoma compared to normal serum. The same 46 peptides decreased when the dog was in clinical remission (after chemotherapy) and subsequently increased during relapse of the cancer. When the pretreatment serum from this and two other dogs with lymphoma were analyzed and compared to normal serum, there were 22 similar peptides identified in all three animals, of which 19 peptides were increased with lymphoma. These results suggest the utility of proteomics for diagnosis of lymphoma and monitoring response to treatment.

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Toxicology Scientific Session

Sunday, October 15, 2006

Rochester

Moderator: Catherine Barr and Patricia Talcott

10:45am	**Presumptive Zinc Toxicosis in a Dog Following Ingestion of a Brassiere** - K. Meyerholtz, C. Wilson, R. Everson, J. Weitzel, , J. Ellis, E. Whitcomb, and S. Hooser	153
11:00am	**Exposure of California Mountain Lions (*Puma concolor*) to Anticoagulant Rodenticides** - R. H. Poppenga, F. Uzal, S. Riley, W. Boyce, P. Swift, and R. Sauvajot	154
11:15am	**Two Cases of Barium Poisoning in Cattle** - T. Richards, D.L.Erickson, P.A. Talcott and T.V. Bazler	155
11:30am **GRAD**	**Toxicity of the Lichen Substance (+)-Usnic Acid in Ruminants** - R.N. Dailey, M.F. Raisbeck, D.L. Montgomery, R.S. Siemion, T.E. Cornish, and M.J. Vasquez	156
11:45am	**Evaluation of the Toxicity of *Adonis aestivalis* in Calves** – L. W. Woods, L. W. George, M. L. Anderson, D.M. Woods, M.S. Filigenzi, B. Puschner	157

AAVLD 49th Annual Conference
October 12 – 18, 2006
Minneapolis Hilton – Minneapolis, MN

Chemical Agroterrorism Surveillance -
The last 28 years in Minnesota.

T.D. Arendt,[1] C.M. Stowe,[2] and **M.J. Murphy**[1]

The Center for Disease Control has identified 13 Categories of Chemicals that may be used in a terrorism event. These Categories are: Biotoxins, Blister Agents or Vesicants, Blood Agents, Caustic Acids, Choking/Lung/Pulmonary Agents, Incapacitating Agents, Long-Acting Anticoagulants, Metals, Nerve Agents, Organic Solvents, Riot Control Agents/Tear Gas, Toxic Alcohols, and Vomiting Agents. The Biotoxins include: Abrin, Brevetoxin, Colchicine, Digitalis, Nicotine, Ricin, Saxitoxin, Strychnine, Tetrodotoxin, and Trichothecenes. The Blister Agents or Vesicants include: Mustards, Distilled mustard (HD), Mustard gas (H) (sulfur mustard), Mustard/lewisite (HL), Mustard/T, Nitrogen mustard (HN-1, HN-2, HN-3), Sesqui mustard, Sulfur mustard (H) (mustard gas), Lewisites/chloroarsine agents, Lewisite (L, L-1, L-2, L-3), Mustard/lewisite (HL), and Phosgene oxime (CX). The Blood Agents include: Arsine (SA), Carbon Monoxide, Cyanide, Cyanogen chloride (CK), Hydrogen cyanide (AC), Potassium cyanide (KCN), Sodium cyanide (NaCN), and Sodium monofluoroacetate (compound 1080). The Caustic Acids include: Hydrofluoric acid (hydrogen fluoride). The Choking, Lung or Pulmonary Agents include: Ammonia, Bromine (CA), Chlorine (CL), Hydrogen chloride, Methyl bromide, Methyl isocyanate, Osmium tetroxide, Phosgene, Diphosgene (DP), Phosgene (CG), Phosphine, Phosphorus, elemental, white or yellow, and Sulfuryl fluoride. The Incapacitating Agents or Drugs include BZ, Fentanyls & other opioids. The Long-Acting Anticoagulants. The Metals include: Arsenic, Barium, Mercury, Thallium. The Nerve Agents include: G agents, Sarin (GB), Soman (GD), Tabun (GA), V agents, and VX. The Organic Solvents include: Benzene. The Riot Control and Tear Gas agents include: Bromobenzylcyanide (CA), Chloroacetophenone (CN), Chlorobenzylidenemalononitrile (CS), Chloropicrin (PS), and Dibenzoxazepine (CR). The Toxic Alcohols include: Ethylene glycol. The Vomiting Agents include Adamsite (DM).

One target for terrorists is the food supply in the United States (US). One means of targeting the US food supply is through livestock and poultry prior to slaughter. Livestock and poultry are routinely examined in Veterinary Diagnostic Laboratories (VDL) across the US. Toxicologists in these VDLs are uniquely qualified to distinguish those diseases that are routinely seen in domestic livestock and poultry from known terrorism agents. These toxicologists have established relationships with the appropriate individuals at both the state and federal level if a laboratory finding in a food animal may adversely affect the health of the public.

The number of samples from food animals analyzed by the Toxicology Section of the Minnesota VDL will be presented. The potential for expanded surveillance testing of these samples will be discussed. Cases illustrating effective interaction with appropriate state and federal officials in the disposition of cases will be presented. Relevant information learned from toxicoses of non-food animals will also be presented.

We conclude that toxicologists in VDL's have a unique opportunity to recognize chemical agroterrorism events and take appropriate action to maximize protection of the health of the public while simultaneously minimizing the terror impact.

1 Department of Veterinary Population Medicine, College of Veterinary Medicine, University of Minnesota.
2 Deceased.

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Overcoming Analytical Challenges in a Veterinary Toxicology Laboratory by Utilizing LC/MS/MS

G. Yang,* M. Johnson, W. Rumbeiha, M. Huang, W. E. Braselton

Analytical test requests for medication are becoming more common in veterinary toxicology laboratories. Some of these requests deal with the analysis of compounds which possess either unique properties or are present in unique sample matrices. These are of great challenge to analytical laboratories, especially when method development is required for a case that is time sensitive.

Mass spectrometry is a robust detection technology that is both sensitive and confirmatory. GC/MS, the technique most commonly employed in veterinary toxicology laboratories, has a number of limitations and drawbacks. For example, it usually requires a procedure that includes several steps to extract the analyte(s) from different biological matrices into an organic solution and several steps to clean up interfering substances. For compounds with poor volatility or with some specific functional groups, GC/MS is not a good analytical choice because these compounds require a derivatization step before analysis. Hence, method development for GC/MS may be time consuming. The addition of a LC/MS/MS instrument enables a toxicology laboratory to solve those analytical cases which otherwise would have been challenging. A couple of case studies are presented to highlight the versatility of the LC/MS/MS in the veterinary toxicology laboratory.

Metronidazole is an antibiotic which is commonly used to treat protozoal and anaerobic bacterial infections. A request to determine the amount of the drug in a tablet was sent to this laboratory in October, 2005. A spectrophotometric method has been reported[1], but steps of chemical reactions to reduce metronidazole were required to perform the test. The test was instead performed on LC/MS/MS in our laboratory. The tablet was simply dissolved in the mobile phase solution, acetonitrile/water (30/70), and was quantified under multiple reaction monitoring (MRM) (m/z: 172 → 128, 111, 82; APEI, positive). The recovery rate of a 2.5 ppm spike (on sample) was 88%. The tablet, which was labelled as 250 mg/tablet, was found to contain 335 mg of metronidazole.

A liver sample and a piece of tissue from the injection site of a dead performance horse was presented to our lab in September, 2005. Malicious injection of fluphenazine and/or acepromazine was suspected and a GC/MS screen was initially requested. After our routine GC/MS analysis on the liver sample turned out negative, we decided to analyze the samples by LC/MS/MS. A quick method development, which demonstrated that the method could easily detect the compound at 1 ppm in tissue, was completed in two days. Fluphenazine was analyzed under MRM (m/z: 438 → 248, 173, 143; positive mode). Both the injection site and the liver were positve for fluphenazine.

In the past year, we have done many unique analyses by utilizing LC/MS/MS, which has proved to be a powerful tool. Other examples include analysis of oxycodone in bait, alprazolam in bait, acepromazine maleate in liver, levothyroxine tablet, albendazole in serum, and yohimbine in liver. The approach in developing assays for these unique complex cases by LC/MS/MS will be presented.

Reference

1. Atta-ur-Rehman, A.S.Ijaz and A. Raza, Spectrophotometric Determination of Metronidazole in Pharmaceitical Pure and Dosage Forms using p-Benzoquinone, Journal of the Iranian Chemical Society, 2(3), September 2005, 197-202.

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October 12 – 18, 2006
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Quantitative Analysis of Lasalocid, Monensin, Salinomycin and Narasin in Feed by Liquid Chromatography Triple Quadrupole Tandem Mass Spectrometry (LC/MS/MS)

M. Huang, Z. Yang, W.K. Rumbeiha*, W.E. Braselton, M. Johnson

The widespread use of ionophores antibiotics in veterinary medicine has raised concerns about their effects on animal health and food safety. Diagnostic laboratories are often called upon to determine the quantity of these compounds in feeds. A method with single step of solvent extraction was developed for rapid quantification of monensin, lasalocid, salinomycin, and narasin in feeds by liquid chromatography tandem mass spectrometry (LC/MS/MS). The ionophores were extracted by methanol/water (90/10). With the high specificity and high sensitivity of tandem mass spectrometry, the extract could be introduced without further separation after the extraction and centrifugation. In order to avoid contamination of the mass analyzer and suppression on analyte signal by dirty matrices, the elution procedure for liquid chromatography was carefully determined. With the use of acetonitrile/water mobile phase and optimization of the elution program, reasonable retention times for the ionophores on the column allowed us to lead the elution of the first two minutes to the waste and avoid introducing the dirty matrixes to the mass spectrometer. The effect of particle size of feed on extraction efficiency was investigated. It was found out that feed passing through a 1 mm filter allows quantitative extraction. Nigericin was used as internal standard for the measurement. The method was validated by fortification of the ionophores in horse feed at different concentration levels. In addition, various inter-laboratory check samples with different matrices were also measured for the validation. A good agreement was found between the LC/MS/MS results and the expected values.

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Determination of microcystins by LC-MS/MS

E.R Tor, B. Puschner, R.H. Poppenga

Microcystins (MC) are a group of hepatotoxins produced by cyanobacteria (blue-green algae) such as *Microcystis aeruginosa*, *Anabaena spp, Oscillatoria spp, Planktothrix spp. and Nostoc spp.* Microcystins are cyclic peptides, containing 7 amino acids. About 75 different microcystins have been identified so far. The most common in the world is Microcystin-LR (LD_{50} 25-50 µg/kg bw in mice i.p.). Cyanobacteria are photosynthetic organisms which occur naturally in most bodies of water. Optimal combinations of nutrients, light, temperature and stagnant water often result in blooms and production of toxins. This creates a worldwide problem for liver disease in domestic animals, wildlife and humans. Because microcystins are also considered tumor-promoting substances, the detection at extremely low concentrations is critical for drinking water quality assessments. While the US has not developed a maximum allowable contaminant level for microcystins in water, the World Health Organization established a drinking water guideline level of 1 µg/L (1 ppb) of total MC-LR (intracellular and extracellular).

A rapid, sensitive and selective analytical method for screening, confirmation and quantitation of MC-LR, MC-RR, MC-YR and MC-LA in pond water samples using liquid chromatography coupled with triple quadrupole/linear ion trap mass spectrometry (LC-MS/MS) has been developed with a method detection limit of 1 ppb for each of the microcystins. Water samples (100 g) were sonicated to facilitate cell wall breakage and release of the toxins into the water. An aliquot of each sample was loaded onto a C18 SPE cartridge. Microcystins were eluted with methanol. The extract was then filtered through a 0.45 µm filter and injected onto an Agilent Model 1100 (binary) high performance liquid chromatograph coupled with a hybrid triple quadrupole/linear ion trap mass spectrometer, model 4000 Q TRAP (Applied Biosystems/MDS SCIEX, Concord, Canada). The analytical column was a 150 mm x 4.6 mm Synergi Polar-RP (Phenomenex^{R}). The mobile phase consisted of: (A) 0.1% formic acid in water (v/v); (B) 0.1% formic acid in methanol (v/v) at a flow rate of 500 µL/min under a linear gradient of 75% B to 95% B over 15 min. MS data for this screen were acquired in the positive ion electrospray ionization (ESI) mode. The MS/MS acquisition program consisted of a single period with four experiments using full scan MS/MS in Enhanced Product Ion (EPI) mode. In this mode of operation, the third quadrupole stage functions as a linear ion trap, providing a high level of sensitivity with full scan data. In EPI experiment 1, MC-LR was analyzed using the following parameters: Precursor ion = *m/z* 995, collision energy (CE) = 40, declustering potential (DP) = 50, product ion scan range = *m/z* 500-1000. In EPI experiment 2, MC-RR was analyzed using the following parameters: Precursor ion = *m/z* 520, CE=30, DP=50, product ion scan range = *m/z* 500-1040. In EPI experiment 3, MC-YR was analyzed with precursor ion = *m/z* 1045, CE= 40, DP= 50, product ion scan range = *m/z* 800-1050. In EPI experiment 4, MC-LA was analyzed with precursor ion = *m/z* 910, CE= 40, DP = 50, product ion scan range = *m/z* 550-920. Twenty microliters of standards in matching matrix or sample extracts were injected into the system described above. Each set of samples contained a reagent blank, control and fortified samples. Quantification was by comparison with a five-point calibration curve using external standards in matching matrix and non-weighted linear regression. Five replicate fortifications of stagnant pond water at 0.001 µg/g (1 ppb) each of MC-LR, MC-RR, MC-YR and MC-LA gave average recoveries of 110% with 5% CV (relative standard deviation), 77% (19%), 109% (8%) and 94% (11%), respectively. This method was applied to confirmation and quantitation of microcystins in water and biological samples**.**

California Animal Health and Food Safety Laboratory System – Toxicology Laboratory, University of California, Davis, CA

AAVLD 49th Annual Conference
October 12 – 18, 2006
Minneapolis Hilton – Minneapolis, MN

Prolonged Sublethal Microcystin Dosing Related To Altered Hepatic Gene Expression In Mice

S.P. Clark[1,2*] and S.B. Hooser[1,2]

Microcystin-LR (MCLR) is an environmental toxin causing acute hepatotoxicity in various species of domestic animals and humans, and with chronic, low-level exposure is a suspected carcinogen due to its tumor promoting activity. In this study, the chronic sublethal effects of MCLR in mice were characterized. Saline or 40µg MCLR/kg was given daily by intraperitoneal injection for up to 28 days. At 4h, 24h, 4d, 14d and 28d after the first MCLR dose, liver tissue was collected in formalin for histology and in RNA Later for RNA analysis. Heparinized blood was collected for plasma chemistry evaluations. The RNA from the 28 day study was processed and hybridized onto Mouse Genome 430A 2.0 GeneChip arrays. The RNA from the other time points was processed for quantitative-PCR. Statistically significant increases in plasma ALT and AST activities related to hepatocyte damage, and statistically significant decreases in plasma total protein, albumin and glucose concentrations related to decreased liver function were identified in the MCLR-treated groups at 14 and 28 days. Histologically, marked hepatokaryomegaly was identified in the 14 day MCLR-treated group with giant cells also present in the 28 day MCLR-treated group. Using toxicogenomics, we were able to identify genes that participate in molecular pathways involved in chronic sublethal MCLR exposure. Major gene expression changes were identified in the actin organization, apoptotic, cellular redox pathways and the organic anion transport pathway (OATP) system. These findings assist in our understanding of the molecular mechanisms of MCLR toxicity as well as provide novel biomarkers in pathways associated with hepatic carcinogenesis.

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October 12 – 18, 2006
Minneapolis Hilton – Minneapolis, MN

Effectiveness of three commercial adsorbents for binding oleandrin and oleandrigenin

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Oleander (*Nerium oleander*) exposure and intoxication is the most common poisonous plant problem encountered in California. Oleandrin and oleandrigenin are the primary cardiotoxins in oleander and are associated with the clinical signs in the intoxicated animal. Activated charcoal (AC) is a potentially useful decontaminating agent in plant poisonings, however, specific studies involving oleander toxins are lacking. A relatively new clay product, Bio-Sponge™ (di-tri-octahedral smectite), recommended for adsorbing bacterial toxins in the GI tract, has also been used by some practitioners in cases of oleander intoxicated animals. However, the relative efficacy of this product to adsorb oleander toxins compared to AC has not been evaluated.

We designed a simple *in vitro* experiment to compare the oleandrin and oleandrigenin adsorption efficacy of three commercially available adsorbents, Bio-Sponge™, ToxiBan™ (containing AC and kaolin), and a generic grade activated charcoal available through the Veterinary Teaching Hospital pharmacy at UC Davis. Briefly, 0.1 g of the adsorbent was added to 10 ml aqueous oleander leaf extract containing 300 ppm oleandrin and 150 ppm oleandrigenin. After 1 hr of incubation at room temperature, and centrifugation at 2000 rpm for 10 min, the supernatant was collected and quantitatively analyzed for oleandrin and oleandrigenin using LC/MS/MS method.

Results from this study demonstrate that ToxiBan™ has the highest adsorptive capacity among the three adsorbents, followed by the generic AC and then Bio-Sponge™. Based on this *in vitro* study, products containing AC would be preferred over Bio-Sponge™ in cases of oleander exposure. However, the ability of these adsorbents to alter the clinical outcome in exposed animals remains to be assessed.

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AAVLD 49th Annual Conference
October 12 – 18, 2006
Minneapolis Hilton – Minneapolis, MN

A Method for the Analysis of Ricinine in Canine Stomach Content

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The castor bean plant (Ricinus communis) has been implicated in numerous poisonings in humans and animals. Its toxicity is primarily derived from ricin, a lectin with a molecular weight of approximately 66,000. The oral LD50 in humans for ricin is 30 µg/kg, and it has been reported that the amount of ricin present in a single bean can be enough to cause serious toxicity in adult humans. Castor beans are also known to contain the alkaloid ricinine (3-cyano-4-methoxy-N-methyl-2-pyridone). Ricinine is not considered to contribute significantly to the toxicity of the castor bean, but its presence in biological samples such as stomach content, urine, or serum would infer exposure to castor beans. Diagnostic analysis for ricin is complicated by its high molecular weight, so analysis for ricinine is preferred in the diagnosis of castor bean poisoning.

Our laboratory recently received a sample consisting of the empty stomach of a dog suspected to have died of castor bean poisoning. The objective of the work presented here was to develop a method for the analysis of ricinine in canine stomach content and to use that method to determine the presence of ricinine in the stomach sample.

The stomach sample and control samples consisting of one gram aliquots of a surrogate stomach content (negative control) matrix were extracted by sonication and vortexing in methanol. The methanolic extracts were evaporated dry and reconstituted in water. The aqueous extracts were cleaned up using Waters Sep-pak VAC C-18 solid phase extraction cartridges. The cleaned up extracts were evaporated dry, reconstituted in water, and fortified with the internal standard 13C-ricinine. These extracts were then injected on a Thermo LTQ ion trap HPLC-MS/MS system equipped with a Phenomenex Synergi RP-Polar column. Ricinine and its associated internal standard were monitored in MS/MS mode. Five point calibration curves at levels from 0.01 to 50 ng/mL of ricinine were used for quantification. The method was validated by analysis of seven replicates of negative control matrix fortified with ricinine at a level of 1 ng/g. The average recovery of ricinine at 1 ng/g was 85% (after subtracting background), with 5.9% RSD.

The most challenging aspect of this method has been the consistent presence of a low level of ricinine in negative control sample extracts. A number of potential sources for contamination have been evaluated, without success. The background level is well below the 1 ppb level in fortified samples, but at present it must be used to define the detection limit of ricin. The detection limit based on a response 5x greater than the background peak would be approximately 0.4 ppb.

Ricinine was detected in the stomach sample at a level approximately 50x higher than background. Since the stomach was empty when received, the detected ricinine represents what was present in the gastric mucosa. This work represents the first known confirmation of fatal castor bean poisoning in a dog.

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AAVLD 49th Annual Conference
October 12 – 18, 2006
Minneapolis Hilton – Minneapolis, MN

Fatal ricin toxicosis in a puppy confirmed by liquid chromatography/mass spectrometry

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Ricin, a lectin from castor bean plant (*Ricinis communis)*, is considered one of the most potent plant toxins known and is listed as a Category B bioterrorism agent by the Center for Disease Control and Prevention. Castor beans are cultivated to produce castor oil and are ubiquitous ornamental plants. The toxin, released when the seed coat is cracked during mastication, inhibits protein synthesis leading to cell death. Ricin poisoning may result in high morbidity with vomiting and watery to hemorrhagic diarrhea. Prognosis varies with number of seeds ingested, degree of mastication, individual susceptibility, and delay prior to treatment. Low mortality restricts assessment of histologic lesions, and literature on toxicologic analysis for ricin is limited. This report describes the microscopic lesions in a fatal case of castor bean ingestion in a puppy, with confirmation of ricin exposure by liquid chromatography/mass spectrometry (LC/MS).

A 12-week-old, 14.6 kg, female Mastiff puppy presented with vomiting and diarrhea of less than one day duration. The puppy was lethargic with pale mucous membranes and delayed capillary refill time. Results of laboratory tests included negative fecal ELISA for parvovirus (IDEXX Laboratories, Westbrook, ME), no parasites on direct fecal analysis, packed cell volume of 51%, and elevated serum alkaline phosphatase, BUN, and phosphorus. The puppy was hospitalized and treated with intravenous fluids, activated charcoal gel, corticosteroids, antibiotics, and vitamin K. It remained depressed and lethargic, with hemorrhagic diarrhea, and died several hours after admission. Approximately 30-60 cm of dark red to purple jejunum with swollen, congested mesenteric lymph nodes was found on necropsy examination. Subsequently, the owners reported finding castor beans, consumed from ornamental plants at the residence, in the dog's feces. Formalin-fixed stomach, small intestine, large intestine, liver, spleen, kidney, and lymph node samples were submitted along with fresh stomach and kidney samples to the Animal Disease Diagnostic Laboratory at Purdue University.

Histopathologic findings in the jejunum included acute, superficial necrotizing enteritis, with moderate eosinophilia and smooth muscle vacuolization in the tunica muscularis. The liver had occasional, random foci of coagulative necrosis, with minimal inflammatory response. Lymphoid apoptosis was identified in splenic white pulp and lymph node follicles were depleted. Mild renal medullary mineralization was also present. A LC/MS method for ricinine, an alkaloid marker for castor bean extracts, was developed. Ricinine was identified in the stomach sample and its presence confirmed exposure to castor beans. Confirmation of exposure through LC/MS is important to support a clinical diagnosis of ricin toxicosis, since ingestion of castor beans is not always fatal, histologic lesions are nonspecific, and degree of mastication can influence effective dose of ricin.

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AAVLD 49th Annual Conference
October 12 – 18, 2006
Minneapolis Hilton – Minneapolis, MN

A Case of Suspected Anatoxin Toxicosis in a Dog

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An 8 year old spayed female German Shorthair dog whose owners lived near the shoreline of private lake located along the Platte River in Saunders County, Nebraska, had been outdoors for about an hour on a Thursday afternoon in late May 2006 and had had access to the lake where there had been a thick, green scum along the shoreline recently. About 5 hours later, the owners noticed salivation, bubbles coming from her mouth, and body tremors and she was presented to a veterinary clinic about an hour later.

The attending veterinarian found the dog to be unresponsive with a fair amount of salivation, a heart rate of 25 – 30 bpm, and a rectal temperature of 102.4 ^{o}F. Introduction of the thermometer elicited a bowl movement that was green in color and contained spots of blood. She died approximately 10 minutes after presentation.

At necropsy the following day, the carcass was in good condition. There was evidence of vomitus around her mouth, but no evidence of algal scum on her coat. Cecum and large intestine contained a small amount of green mucoid contents. Liver and spleen were moderately congested, but no other gross lesions were noted.

Histopathologically, varying amounts of congestion were observed in all tissues examined. Small aggregates of macrophages containing black intracytoplasmic granular pigment, interpreted as pneumonconiosis, were seen around bronchioles. Small siderotic plaques were noted in association with larger caliber arteries in the spleen. Mild vacuolar degeneration was observed in hepatocytes. There was a mild to moderate thickening of Bowman's capsules and mild mesagnial thickening in glomeruli. No other significant lesions were noted.

Anabena sp. was identified in a specimen of the scum taken from the lake, vomitus collected at the veterinary clinic, and stomach content collected at necropsy. Only *Anabena* spp. were found in the scum.

A water specimen and vomitus from the dog were analyzed for cyanobacterial toxins by LC/MS/M. The water was found to contain 585 ng anatoxin-a/mL and 5,000 ng microcystin LR/mL. The vomitus was found to contain 5,980 ppb anatoxin-a and 307 ppb microcystin LR. No detectable amounts of other microcystins were found.

This case of suspected anatoxin-a toxicosis is supported by the following evidence:

1. Identification of *Anabena* spp. in the scum taken from the lake.
2. Presence of *Anabena* spp. in vomitus and stomach content of the dog.
3. Presence of anatoxin-a in vomitus/stomach content and lake water specimens.
4. Clinical signs, time to death, and post mortem and histopahtological results consistent with an anatoxin-a and not a microcystin toxicosis. The vacuolar degeneration observed in the hepatocytes may be an early manifestation of microcystin toxicosis, but the damage is not sufficient to cause the death of the dog.

The time of occurrence for this case is odd. Veterinary toxicology literature says that cyanobacterial toxicoses mostly occur in late summer or early fall in temperate zones. This was the second time cyanobacterial toxicoses have occurred in the spring of the year in Nebraska. Three dogs died in the Spring of 2004 and the deaths were attributed to cyanobacterial toxicoses.

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AAVLD 49th Annual Conference
October 12 – 18, 2006
Minneapolis Hilton – Minneapolis, MN

Diagnosis of *Amanita* toxicosis in a dog

Birgit Puschner[1], Heidi Huffman Rose[2], Pat McCoy[2], Michael S. Filigenzi[1]

A fifteen-week-old, female Dachshund was presented to the referring veterinarian for acute onset of lethargy. At presentation, the dog had progressed to sternal recumbency and demonstrated generalized twitching. The owner indicated that the dog ingested unidentified mushrooms approximately 12 hours earlier. Initial diagnostic work-up revealed marked hypoglycemia, an increased alanine aminotransferase activity and moderate neutrophilic leukocytosis. Fecal floatation and parvovirus ELISA tests were negative. Supportive care was implemented, but the dog remained lethargic and developed diarrhea. Despite clinical signs, the dog was discharged the same day and died approximately 12 hours after initial presentation. A full necropsy was performed 3 days after death that revealed a slightly swollen, pale yellow liver but no other specific findings. Microscopic examination of the liver showed diffuse, massive coagulative necrosis of hepatocytes.

The frequency of reported mushroom poisonings in animals is low because of the lack of methods to confirm exposure to toxic mushrooms. In the past, a presumptive diagnosis of amanitin poisoning was based on history of exposure, clinical signs and pathological findings. The objective of this case report was to determine whether the acute onset of disease followed by death described in this dog was caused by the ingestion of hepatotoxic *Amanita* mushrooms. A sensitive and highly specific liquid chromatography/mass spectrometry methodology was developed for amanitin analysis in the liver with the positive identification of α-amanitin. Liver samples were homogenized in a blend of acetonitrile and phosphate buffer. The resulting extracts were cleaned up using mixed-mode solid phase extraction cartridges. The extracts were analyzed by LC-MS^3 on a linear ion trap mass spectrometer. Liquid-chromatography/mass spectrometry of liver for amanitins is a powerful diagnostic tool to establish a definite diagnosis of hepatotoxic *Amanita* toxicosis.

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AAVLD 49th Annual Conference
October 12 – 18, 2006
Minneapolis Hilton – Minneapolis, MN

Presumptive Zinc Toxicosis in a Dog Following Ingestion of a Brassiere

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A 9 month old, neutered male mixed breed dog was presented with a history of vomiting, diarrhea, and red-colored urine. The owners reported that this animal had, approximately one month previously, ingested various non-food items such as pennies, packets of silica gel, small amounts of ant bait containing boric acid, and possibly paint chips from home remodeling. On examination, the dog had pale oral mucus membranes (PCV at presentation = 12.5%), hemoglobinuria, and was lethargic. Abdominal radiographs revealed the presence of radiopaque objects in the stomach which appeared similar to brassiere clasps. A gastrotomy was performed and pieces of a woman's brassiere were removed from the stomach. No other metallic objects or money were found in the stomach or GI tract. On the day following the surgery, the PCV had decreased to 5%. The dog was given a transfusion of whole blood and stabilized. Following the transfusion, the PCV increased to 24% and the hemoglobinuria resolved. Two days following removal of the brassiere clasps from the stomach, serum and whole blood in EDTA were obtained by venipuncture. Serum was collected by centrifugation, placed in a plastic tube, and frozen prior to analysis. The serum zinc concentration was 0.87 ppm (normal = 0.7 – 2.00 ppm) by flame AAS. The blood lead concentration was below the limits of detection by furnace AAS. The dog was released from the hospital and approximately one month later was doing well. The pieces of the brassiere, with clasps attached, were submitted for zinc analysis. This sample was positive for zinc. A clasp from another brassiere was weighed, digested and analyzed for zinc by flame AAS. It was found to contain 7221 ppm of zinc. Further investigation revealed that some white bra clasps are made of "white bronze", an electroplating alloy combination of tin, copper and zinc which doesn't tarnish, is more amenable to washing & drying, and eliminates the use of nickel which can be associated with skin allergies. Although the serum zinc concentration two days following removal of the clasps from the stomach was within normal limits, the combination of adverse clinical signs of vomiting, diarrhea, hemolytic anemia and hemoglobinuria with the presence of a zinc-containing object in the stomach, lead to a presumptive diagnosis of zinc intoxication. In addition, one month previously this dog had been found with pennies in its mouth, suggesting that it had a predisposition to eat non-food items including money and may have had previous zinc exposure without demonstrating severe adverse signs related to poisoning. Its rapid recovery following transfusion and removal of the brassiere also support the presumptive diagnosis of zinc intoxication. This case emphasizes that zinc is used in many applications and needs to be included a list of differential diagnoses when ingestion of metal objects is correlated to the appropriate adverse clinical signs.

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AAVLD 49th Annual Conference
October 12 – 18, 2006
Minneapolis Hilton – Minneapolis, MN

Exposure of California Mountain Lions (*Puma concolor*) to Anticoagulant Rodenticides

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A frozen whole carcass from a male mountain lion (*Puma concolor*) and the head, pelt, liver and reproductive tract from a female mountain lion were submitted to the San Bernardino Branch of the California Animal Health and Food Safety Laboratory System (CAHFS) for necropsy after being found dead. A field necropsy had been performed on the female, since the whole carcass could not be retrieved safely. Grossly, the male had severe hemorrhages in the thoracic, pericardial and peritoneal cavities and the female had extensive free blood in the pericardial cavity. There was no evidence of trauma in either animal. Both mountain lions had severe skin lesions consistent with mange. *Notoedric cati* was subsequently identified. Toxicologic testing, including measurement of brain cholinesterase activity (male lion) and liver metal and anticoagulant rodenticide screening (both the male and the female) was performed. The anticoagulant rodenticides, bromodiolone and brodifacoum were detected in the liver from the male at 1.27 ppm and 0.57 ppm, respectively. The same two rodenticides were detected in the female at 0.51 ppm and 0.31 ppm, respectively. The extensive hemorrhages noted in the animals, in combination with the detection of two anticoagulant rodenticides at high concentrations in their livers, were consistent with a diagnosis of anticoagulant rodenticide intoxication. Unfortunately, the circumstances of exposure were not determined.

Based upon the detection of the two rodenticides in both mountain lions and concern that mountain lions might be exposed more widely to anticoagulant rodenticides, archived serum or plasma (N = 73) and liver (N = 6) samples collected from mountain lions throughout California were analyzed for the presence of anticoagulant rodenticides (bromodiolone, brodifacoum, chlorophacinone, coumachlor, difethialone, diphacinone, and warfarin) by both HPLC and LC-MS techniques. Two of the six liver samples were positive for brodifacoum. One of the 73 serum sample also contained a trace amount of brodifacoum. A search of the CAHFS database identified five other mountain lion submissions since April of 2003. Liver samples from four of the five mountain lions were positive for one or more anticoagulant rodenticides. Brodifacoum was detected in one animal, brodifacoum and bromodiolone in two animals and brodifacoum, bromodiolone and diphacinone in two animals. All four mountain lions were from Southern California (Los Angeles, San Bernardino, San Diego and Riverside Counties). The one negative sample was from a mountain lion that originated in less populated Tulare County. Results suggest that mountain lions are exposed to anticoagulant rodenticides and that exposure appears to be more frequent in heavily urbanized areas. The effect of exposure on mountain lion populations is unknown, but continued surveillance is warranted.

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AAVLD 49th Annual Conference
October 12 – 18, 2006
Minneapolis Hilton – Minneapolis, MN

Two Cases of Barium Poisoning in Cattle

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Approximately 30 Hereford, cow/calf pairs were moved over a 48 hour period from a high mountain range to a lush green valley pasture in late August. While moving the cattle, the animals were trailed through a restricted area that contained an old, abandoned lead, silver and zinc mine. The cattle had potential access to a tailings pond in this restricted mine area.

Shortly after arrival, 6 animals died over the next 3 days (4 adult cows, 1 calf). Clinical signs included excessive salivation, protrusion of the tongue, drooping ears, and watery diarrhea rapidly progressing to recumbency and death. No seizures were reported. One adult cow was submitted for necropsy. Widespread ecchymotic hemorrhages were observed in multiple tissues and moderate quantities green/gray, granular, clay-like material was present in the abomasum. Mild pulmonary edema and congestion were the only histologic changes reported. Liver and kidney tissues contained normal concentrations of molybdenum, zinc, cadmium, lead, manganese, iron, copper, selenium, strontium, cobalt, vanadium, nickel and arsenic.

Two years later, the same owner reported a similar case scenario. Ten cow/calf pairs were being moved down to the valley pasture along the same route as two years prior. The move took approximately 48 hours, and the cows trailed through the same restricted abandoned mine site. Clinical signs of illness were first recognized in 8 cows shortly after arriving in the valley pasture. These included protruding tongues, excessive salivation, mild muscle tremors, hypermotile rumens, watery mucoid diarrhea and elevated respiratory and heart rate. All affected cows were afebrile and were noted to have very little muscle tone. All affected cows died with no signs of seizures or violent struggling. No calves were affected. No significant gross or histologic changes were observed in 3 necropsied cows, but all cows had similar clay-like material in the abomasum as observed in the previous case two years earlier. However, this time additional history obtained indicated that the abandoned ore mine had also at some point been processing barium.

Serum samples collected from 2 cows antemortem, along with tissues and the clay-like material, were submitted to the toxicology laboratory for mineral testing. Barium was found to be high in all samples tested: serum samples (12 ppm and 16 ppm), liver (9.7 ppm) and kidney (20 ppm). The liver and kidney samples did not contain excessive concentrations of any other heavy metals. The clay-like material was found to contain approximately 69,000 ppm barium by ICP. The clay-like material was analyzed by X-ray diffraction and microprobe analysis by two separate laboratories, and was found to contain quartz (SiO_2), barite ($BaSO_4$), barytocalcite ($BaCa[CO_3]_2$), and calcite ($BaCO_3$). The water from the tailings pond contained 2.2 mg/L barium and the bottom of the pond was covered with this same clay-like material.

Barium poisoning has been rarely reported in the veterinary literature. Barium acetate, carbonate, chloride, nitrate and sulfide are all considered to be highly toxic due to their acid solubility, whereas barium sulfate is considered to be relatively nontoxic. The toxicity of barium is related to its direct stimulation of smooth, striated, and cardiac muscle. Blockage of voltage gate potassium ion channels leads to a severe hypokalemia and a digitalis-like effect. Due to the acute nature of fatal barium toxicity, no gross or microscopic cardiac lesions are described, consistent with the cattle examined in this report.

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AAVLD 49th Annual Conference
October 12 – 18, 2006
Minneapolis Hilton – Minneapolis, MN

Toxicity of the Lichen Substance (+)-Usnic Acid in Ruminants

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Identifying *Xanthoparmelia chlorochroa* as the culprit behind the 2004 Red Rim-Daley Wildlife Habitat Management Area (WHMA) elk die-off resulted in many questions regarding the toxicity of this lichen species from ranchers, wildlife managers, and concerned citizens. Unfortunately, most questions could not be answered with any certainty because the toxin(s) within the lichen remained unknown. A reference from 1939 attributes *X. chlorochroa* toxicity in domestic cattle and sheep to usnic acid, a lichen secondary metabolite. In both the historical report and the recent episode, clinical signs were recumbency, weakness, and paralysis. To test the hypothesis that usnic acid causes the clinical syndrome seen in elk, domestic sheep were dosed with (+)-usnic acid utilizing an up-and-down procedure for acute toxicity testing. Sheep were examined for congruence of signs and lesions with the Red Rim elk, and clinical pathology was used to monitor for evidence of subclinical disease.

Prior to dosing ewes with (+)-usnic acid, a lichen feeding trial was conducted to ensure domestic sheep were a valid model. Three adult Rambouillet ewes were fed *X. chlorochroa* collected from the Red Rim-Daley WHMA. All three ewes showed clinical signs in varying degrees of severity matching those seen in the Red Rim-Daley elk. Following the lichen feeding trial, nine adult Rambouillet ewes were dosed with (+)-usnic acid resulting in an ED_{50} between 485 and 647 mg/kg/day. The two ewes that received the highest doses (776 and 647 mg/kg/day) were the only ewes to display clinical signs, abnormalities in clinical pathology, and have appreciable lesions. The remaining seven ewes were not clinically affected, nor were any changes observed in clinical pathology or under the light microscope. The clinically affected (+)-usnic acid ewes displayed markedly different clinical signs compared to those seen in lichen affected elk and ewes. Clinical signs included anorexia, appendicular stiffness, and signs of a belly-ache. Serum creatine kinase, aspartate aminotransferase, and lactate dehydrogenase were considerably elevated in the two high dose (+)-usnic acid ewes. Similarly these were the only ewes with significant lesions, which consisted of a degenerative appendicular skeletal myopathy. Based on clinical signs, clinical pathology, and light microscopy, it was determined that (+)-usnic acid is not the toxic component of *X. chlorochroa* directly responsible for the syndrome seen in the Red Rim-Daley elk or lichen affected ewes. However, it is possible that (+)-usnic acid is working synergistically or antagonistically with other compounds within the lichen to induce the specific syndrome.

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AAVLD 49th Annual Conference
October 12 – 18, 2006
Minneapolis Hilton – Minneapolis, MN

Evaluation of the Toxicity of *Adonis aestivalis* in Calves

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Toxicosis of *Adonis aestivalis* is well documented in horses but adonis toxicity is unknown in cattle, and in fact, cattle have been suggested as an alternate consumer of adonis-contaminated hay based on incomplete information obtained in a study performed in 1942. *Adonis aestivalis* (summer pheasant's eye) was fed to six calves in acute, high dose and chronic, low-to-high dose feeding trials in order to establish the toxicity of *Adonis aestivalis* in cattle. Four 300 lb Holstein and two 90 lb, pre-ruminating Jersey calves were administered 1% body weight of ground *Adonis aestivalis* via a stomach tube and monitored for clinical signs for two weeks and one week, respectively. The Holstein calves were then fed 0.2-1% body weight *Adonis aestivalis* either ground or as a 50% adonis:alfalfa pellet daily for 4-5 weeks. The Holstein calves exhibited no clinical signs during the feeding trial. No gross or microscopic lesions were seen on necropsies performed at the end of the study. The 90 lb, pre-ruminating Jersey calves developed diarrhea 2-3 days post-dosing and mild signs of cardiac disease, both of which were transient and had no gross or microscopic lesions on necropsies performed 1 week post-dosing. Based on this feeding trial, cattle do not appear to be susceptible to toxicosis from *Adonis aestivalis* when fed up to 1% body weight daily for up to 5 weeks.

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USAHA/AAVLD Scientific Session

Monday, October 16, 2006
Salon AB

Co Chairs: Barbara Powers President-Elect AAAVLD; Lee Myers President-Elect USAHA

		Page
7:30 am	**WELCOME AND ANNOUNCEMENTS –** Barb Powers President Elect AAVLD Lee Myers President-Elect USAHA	
7:45 am	**Global Animal Surveillance for Public Health** – Juan Lubroth, United Nations Food and Agriculture Organization	159
8:15 am	**Zoonotic Disease Surveillance for Public Health** - Tracee Treadwell, Centers for Disease Control and Surveillance	160
8:45 am	**Integration of Food Surveillance Systems** – Pat McCaskey, Food Emergency Response Network	161
9:15 am	**Questions**	
9:30 am	**BREAK**	
9:45 am	**Transforming Animal Health Surveillance** – Brian McCluskey, USDA APHIS VS National Surveillance Unit	162
10:15 am	**Monitoring the health of wildlife and implementing the Global Avian Influenza Network for Surveillance of Wild Birds (GAINS) –** Robert Cook, Wildlife Conservation Society	163
10:45 am	**The Future of Bio Surveillance Systems** – Kimothy Smith, US Department of Homeland Security	164
11:15 am	**Questions**	
11:25 am	**Briefing on FAD Rapid Test Technology** – Barbara Powers, Colorado State University	
11:30 am	**Closing Remarks and Adjournment**	

Global Animal Surveillance for Public Health

Juan Lubroth, United Nations Food and Agriculture Organization

Zoonotic Disease Surveillance for Public Health –

Tracee Treadwell, Centers for Disease Control and Surveillance

Integration of Food Surveillance Systems

Pat McCaskey, Food Emergency Response Network

AAVLD 49th Annual Conference
October 12 – 18, 2006
Minneapolis Hilton – Minneapolis, MN

Transforming Animal Health Surveillance

Brian J McCluskey

Animal health surveillance is essential to the protection and improvement of the health, welfare, quality, and marketability of the nation's livestock, animal products, and veterinary biologics. A comprehensive, coordinated, and integrated surveillance system is the foundation for animal health, public health, food safety, and environmental health. The National Animal Health Surveillance System (NAHSS) is a U.S. Department of Agriculture-Animal and Plant Health Inspection Service-Veterinary Services initiative to integrate existing animal health monitoring programs and surveillance activities into a comprehensive and coordinated system.

Veterinary Services has historically conducted animal health surveillance through a number of systems, focused primarily on disease eradication programs and passive reporting of foreign animal diseases. While these systems have been effective in meeting their established goals, they have not maximized the ability to appropriately allocate resources and to inform international trading partners regarding the safety and health of U.S. Animals and animal products. In addition, under current world conditions of heightened security concerns, the previous system may no longer be adequate for rapid detection and appropriate, timely response to a foreign animal disease incursion. An all-encompassing surveillance system is vital to ensuring efficient animal production agriculture, food, security, and food safety.

Veterinary Services has assumed the role of leader and coordinator in building the NAHSS. Through its infrastructure, expertise, and array of established agency partnerships, Veterinary Services is positioned to respond effectively to Homeland Security Presidential Directive HSPD-9 (Defense of United States Agriculture and Food) and to meet national needs for improved food security and enhanced economic viability.

The transition from current surveillance activities to a comprehensive, coordinated, and integrated NAHSS requires institutional and cultural changes in both Veterinary Services and the animal health community. The new system necessitates a shift from conducting compartmentalized surveillance efforts surrounding one disease to viewing animal disease surveillance as an overall system, which will entail development and integration of many activities and partnerships.

Partnerships with organizations and agencies within and outside of APHIS are essential to the success of the surveillance system. NAHSS partners include, among others: private veterinarians, industry, state animal health officials, Vet Services Units at headquarters and in the field, National Veterinary Services Laboratories (NVSL), Wildlife Services, and the Food Safety Inspection Service (FSIS). Such collaborations allow the NAHSS to be more effective in protecting the U.S. livestock inventory from the threat of domestic, foreign, and emerging diseases. Partnerships with industry stakeholders also provide an opportunity to break down barriers and improve surveillance plan design and efficiency.

Under a more effective and efficient structure, the new surveillance system is designed to provide greater protection from endemic, emerging, and foreign animal diseases (FADs) that could affect the nation's livestock, poultry, and wildlife populations.

USDA Centers for Epidemiology and Animal Health
National Surveillance Unit Fort Collins, CO 80526

AAVLD 49th Annual Conference
October 12 – 18, 2006
Minneapolis Hilton – Minneapolis, MN

Monitoring the Health of Wildlife Worldwide and Implementing the Wild Bird Global Avian Influenza Network for Surveillance

Robert A. Cook, V.M.D., M.P.A.[1] and William B. Karesh, D.V.M.[2]

The Wildlife Conservation Society (WCS) operates 5 parks in the City of New York including the Wildlife Centers at Central Park, Queens and Prospect Park, the New York Aquarium and the Bronx Zoo. In addition WCS manages some 400 conservation projects in 60 countries around the world. WCS is committed to the well-being of animals in its zoological parks and to the conservation of wildlife and wild lands around the world. The expertise gained from over one hundred years of animal handling experience as well as disease surveillance of hundreds of species of animals in zoological parks and from critical landscapes around the globe provides a unique and robust set of competencies to provide critical health care programs that work to ensure the health of people, domestic animals and wildlife. This was clearly demonstrated in 1999 when veterinary pathologists of the WCS identified a link between the deaths of people, wild free-ranging birds and zoo birds and were integral in the identification of the emergence of a new disease in the Western Hemisphere. With samples from the zoological park wildlife health surveillance program the disease was confirmed to be West Nile virus.

The Field Veterinary Program (FVP) of the Wildlife Health Sciences Division of WCS is active on 4 continents and performs community-based wildlife population health monitoring and surveillance. This on-the-ground commitment to assessing the long term health of wild populations provides critical information that can serve as an early warning system for the emergence of new and renewed pathogens at the domestic animal - wildlife – human interface from remote rural settings to urban marketplaces. WCS FVP trained teams provided the observations and samples that confirmed that gorilla's were dying of Ebola hemorrhagic fever virus in Central Africa and demonstrated the link to human outbreaks due to consumption of infected animals. Over the last many years the broad disease surveillance techniques of the FVP have provided a baseline of information on the health of mammals, birds and reptiles in various parts of the world.

Low pathogen avian influenza (LPAI) is endemic in wild migratory waterfowl populations. Best theories suggest that a LPAI moved from wild birds to domestic fowl and in 1997 highly pathogenic avian influenza (HPAI) H5N1 was first identified as a disease of domestic fowl that spread to people in close contact with them in Hong Kong. The resurgence of this disease in Southeast Asia in 2004 brought a new wave of poultry and human deaths. One must study not only the molecular genetics of avian influenza but also the cultural and agricultural practices of the Asia region to understand how this disease might mutate to a highly pathogenic form in domestic fowl, how it might spread into humans and how it might jump back into wild migratory waterfowl. Religious practices such as merit bird releases bring people and passerine birds into intimate contact after mixing them with domestic poultry and other animals. The popular practice of cockfighting results in people moving these birds across vast regions and caring for injured cocks results in human contact with bird blood and body fluids. It is common practice for people in rural areas to live in close proximity to their domestic animals, often times sharing room in the home or close by. It is a typical scene to see cattle, ducks, chickens and pigs living in or very near a family dwelling. The methods in which domestic poultry are raised includes providing domestic ducks access to recently harvested rice paddies where mixing with wild birds is commonplace and positioning chicken houses over aquaculture ponds to utilize chicken excrement for fish food. These aquatic habitats are excellent environments for the persistence of viable avian influenza virus and provide the opportunity for infectious agents to spill over to wild bird populations.

The trade in wild animals is a multi-billion dollar global operation. The animal markets or "wet markets" of Asia are a mixing bowl of domestic animals, wildlife from near and far and people. Most often sanitation and hygiene are very poor to non-existent and both people and animals are under a tremendous amount of stress, lowering immunocompetency. People in the marketplace are handling live birds and butchering others without any personal protection and often live, eat and sleep in their shops amongst their animals for sale. This serves as an excellent environment in which pathogens can mutate and jump into novel species. While it is uncertain that palm civets were the source of Severe Acute Respiratory Syndrome (SARS) in the Guandong markets of China it is clear that they carried the disease. Since initial findings implicated palm civets, other species, such as domestic cats and fruit bats have been shown to harbor the same or closely related viruses.

The FVP has been performing surveillance of wild bird populations for 15 years in selected landscapes around the globe. These efforts have demonstrated low pathogenic strains of avian influenza as far south as the Falkland Islands. In August of 2005 a multi-disciplinary team comprised of FVP field veterinarians and Mongolian scientists performed field surveillance research in 9 different sites along the migratory waterfowl flyways in Mongolia, including Erhel Lake where a bird die-off was reported. No virus was detected in live bird fecal culture samples. 6 dead birds were necropsied. HPAI H5N1 was isolated from a whooper swan on Erhel Lake. The organism was isolated from samples provided to the USDA/ARS Poultry Laboratory in Athens, Georgia. The H5N1 isolate was determined to be a Clade II strain and was selected by the WHO and CDC to be included in the research efforts to produce a human vaccine.

More than 60 percent of the approximately 1,400 infectious diseases currently known to modern medicine are shared between people and animals. Current global disease surveillance efforts are focused primarily on human populations and domestic livestock. No federal or international agency is currently responsible for monitoring and preventing the full array of diseases that cross borders and can be transmitted between domestic and wild animals and people. One of the needed components in controlling avian influenza and preventing outbreaks is a global surveillance and monitoring system that gathers information on diseases in wild birds, shares this information openly and facilitates the development of appropriate responses prior to outbreaks.

The aim of the Wild Bird ***Global Avian Influenza Network for Surveillance*** (GAINS) program is to expand operational field capabilities, improve the understanding of viral strains and transmission of influenza viruses in wild birds, and to disseminate information to all levels of governments, international organizations, the private sector and the general public. GAINS will establish a global surveillance network of wild birds by: improving the collection and coordination of samples from wild birds in order to identify locations of avian influenza viral strains; noting species affected; identifying genetic changes in virus isolates; enhancing links with wild bird distribution and migration information, and providing an early warning system for global spread of HPAI that threatens domestic poultry and human health as well as biodiversity (particularly avian). The GAINS program and partners will work in or travel to areas of importance in key migratory routes, as well as work with wild species which may serve to link migratory birds with domestic poultry. These individuals and organizations will not only work in an advisory capacity to host governments and local/national organizations by providing technical input into wild bird surveillance programs, but will emphasize transfer of technical capacity to local staff where needed. GAINS will also make available information related to wild bird avian influenza surveillance and migratory bird activity through a comprehensive database which will also include agency reports, scientific publications and news. The site can be accessed at WWW.GAINS.ORG .The US Agency for International Development has committed significant funding to expand the operational scope of GAINS and has coordinated with the US Centers for Disease Control and Prevention to provide additional financial support for the GAINS system. Other agencies and organizations, such as the U.N. Food and Agriculture Organization, USDA/ARS, and the U.S.G.S. have provided both monetary and in-kind support.

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AAVLD 49th Annual Conference
October 12 – 18, 2006
Minneapolis Hilton – Minneapolis, MN

The future of biosurveillance systems

Kimothy L. Smith[1], DVM PhD

The future of biosurveillance is global in nature. Biosurveillance cannot be limited by borders or species; public or private. Our world is a global village; a global farm. As eloquently said by Dr. Roger Wahr, – One world; One health; One medicine, and to this I must ad, one biosurveillance. The future of biosurveillance systems will not be confined to the boundaries of our country or the efficacy of deployed countermeasures. The strategies based on a defensive perimeter does not address the dynamic effect globalization has when dealing with infectious diseases. Our nation must continue to improve information-sharing partnerships to enhance situational awareness, as well as set the standard for gathering and sharing information and intelligence. Since human health is inextricably linked to animal and agricultural health, we must also continue to advocate the importance of enhanced animal, agricultural, and food surveillance. As technology continues to penetrate developing countries, comprehensive biosurveillance will be determined by our ability to build a system that can fully integrate all forms of surveillance throughout our nation, and the world. The National Biosurveillance Integration System (NBIS) will provide our nation the first capability to start down the path of future surveillance systems in an effort to achieve global biosurveillance. It will integrate the critical surveillance activities of the U.N., CDC, USDA, Wildlife Conservation Society, and several other Federal, private sector, and non-governmental organization partners in painting a biological common operating picture.

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Poster Session

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Multiple polypoid uterine leiomyosarcomas in a sheep

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Uterine leiomyosarcomas are uncommon tumors in ruminants, with a general incidence of 10% among all neoplasms of the female genital tract in these species. Leiomyosarcoma was diagnosed in the uterus surgically removed from a 3-year-old pet Suffolk ewe with a history of bleeding from the vulva. The uterus had multiple, well circumscribed, soft, intraluminal polypoid masses of variable size (0.5 – 4 cm). The masses were red, with white, smooth and glistening cut surface. Histologically, these masses were comprised of variably dense sheets of moderately pleomorphic, plump spindloid cells embedded in richly vascularized stroma. The mitotic index was usually low (0-1/high power field), but in some polyps there were up to 10 mitoses/high power field. Neoplastic cells stained positive for alpha smooth muscle actin (α-SMA) by immunohistochemistry. Ultrastructural features of neoplastic cells included the presence of basal lamina, scant microfilaments, contracted nuclei with blunt ends, and flat intercellular junctions. Leiomyosarcoma was diagnosed based on cellular morphology and atypia, and positive immunohistochemistry for α-SMA.

Reports of smooth muscle tumors in the female genitalia of ruminants are generally sporadic. A significantly higher incidence of genital leiomyosarcomas has been described in the Saanen doe, but only a few cases of genital smooth muscle tumors have been reported in other caprine breeds, in sheep and in cattle. A hormonal basis for their occurrence has been suggested in humans and some animals. In this sheep, the ovaries were histologically normal and no known history of hormone usage was reported. The leiomyosarcoma in this case was considered to be a low-grade malignant tumor based on the presence of multiple masses, moderate cellular atypia and generally low mitotic index. To the authors' knowledge, this is the first case of multiple uterine leiomyosarcomas in a sheep. The animal in this case is alive and doing well three months after surgery.

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AAVLD 49th Annual Conference
October 12 – 18, 2006
Minneapolis Hilton – Minneapolis, MN

Hemangiosarcoma of the Urinary Bladder in a Dog.

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A six-year-old, spayed female Labrador retriever presented to the Purdue University Veterinary Teaching Hospital for evaluation of hematuria, dysuria, and pollakiuria of two months duration. Abdominal radiographs revealed a soft tissue mass in the left mid abdomen, which appeared to be an enlarged cranial pole of the left kidney. An additional mass, approximately 6 cm in diameter, was caudal to the left kidney. The urinary bladder appeared markedly distended, but normal in shape. Thoracic radiographs revealed multiple nodules, ranging from 0.5 cm to 5 cm in diameter in the caudal, middle and cranial lung fields. Due to the poor prognosis the pet owner requested euthanasia of the dog.

Post mortem examination revealed numerous dark red to black, soft, round, and multilobulated masses throughout the mesentery. On cut section these masses were dark, red, soft and often had a tan, cavitated, friable center. A 2.0 cm in diameter mass was adhered to the diaphragm. The left ureter was enlarged, filled with fluid, and measured 1.0 cm in diameter. Within the lumen of the urinary bladder, at the level of the trigone, was a multilobulated (botryoid), 4 cm long red to black mass, similar in appearance to the mesenteric masses. The serosal surface of the urinary bladder at the level of the trigone was also covered by similar masses. One mass appeared to compress the left ureter at its termination in the urinary bladder resulting in hydroureter and hydronephrosis of the left kidney.

Microscopically, the urinary bladder wall (full-thickness) at the trigone was effaced by a large multilobular neoplastic proliferation of pleomorphic, spindle-shaped to polygonal cells lining vascular channels filled with erythrocytes. Neoplastic cells had poorly defined cell margins and moderate amounts of pale eosinophilic cytoplasm. Nuclei were hyperchromatic and variable sized, containing 1-3 nucleoli. There were 5-7 mitotic figures per high power field. Multifocal hemorrhage and necrosis were present within the mass. The areas of necrosis were characterized by an eosinophilic coagulum admixed with cell debris and neutrophils. Multifocally, vessels within the muscularis contained tumor emboli. Similar neoplastic masses were present in the liver, lung and spleen. Tumors in the urinary bladder and elsewhere were positive for CD 31 antigen and factor VIII-related antigen, confirming a diagnosis of hemangiosarcoma (HSA).

Urinary bladder cancer accounts for only 2% of all canine cancers. The majority of bladder cancers are epithelial, with transitional cell carcinoma being the most common. Primary HSA of the urinary bladder is rare in dogs with fewer than five reported cases. Human primary urinary bladder HSA is also rare. The location of the bladder tumor in our case is also typical for transitional cell carcinomas and rhabdomyosarcomas. The location of two other reported canine bladder HSA was the trigone, occluding ureteral orifices, and the dorsal wall of the urinary bladder. Neither of these tumors metastasized. The metastatic nature of HSA in our case is similar to the aggressive biological behavior of human urinary bladder HSA.

The clinical history and pathologic findings in this case suggest primary HSA of the urinary bladder with metastasis to the liver, lung, mesentery, and spleen. Tumors metastatic to the urinary bladder are extremely rare other than lymphoma or other tumors extending from a primary prostatic, rectal or uterine neoplasia. The authors acknowledge, however, that the precise site of the primary tumor in this dog could not be determined with certainty.

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AAVLD 49th Annual Conference
October 12 – 18, 2006
Minneapolis Hilton – Minneapolis, MN

Alimentary T-cell Lymphoma in a Horse with Pituitary Invasion

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Equine alimentary lymphoma involves the small intestine more commonly than the large intestine. Other organs that may be affected are the liver and mesenteric lymph nodes. Equine lymphoma involving the central nervous system is rare and, to our knowledge, pituitary involvement has not been reported.

A 13-year-old Quarterhorse mare with a 6-month history of diarrhea, progressive weight loss, and lethargy was presented to Purdue University Veterinary Teaching Hospital. An initial diagnosis of protein-losing enteropathy was made. A diagnosis of T-cell lymphoma was made from a rectal biopsy with immunohistochemistry for CD3 (T-cell marker), CD20 and CD79a (B-cell markers). Because the mare had not shed its winter haircoat the previous summer (hypertrichosis), a diagnosis of pituitary pars intermedia dysfunction (PPID) was considered. The clinical condition (lymphoma) did not improve with immunosuppressive therapy and the horse was euthanized.

Necropsy findings included hypertrichosis, focal duodenal mucosal hyperemia, and pituitary gland enlargement (2.9 x 2.5 x 2.3 cm), which was homogeneously tan. Histologically, neoplastic lymphocytes infiltrated the lamina propria and submucosa of the stomach, small intestine, and large intestine. Neoplastic lymphocytes also diffusely infiltrated mesenteric lymph nodes and the pituitary gland. Neoplastic lymphocytes in the pituitary gland and other organs were CD3-positive (T-cell lymphoma).

Hypertrichosis is a consistent clinical feature of PPID. The exact pathogenesis of PPID-related hypertrichosis is unclear. Several hypotheses include: 1) excessive adrenocortical production of sex hormones; 2) increased secretion of melanocyte stimulating hormone from the pituitary pars intermedia through loss of dopaminergic inhibition; 3) mechanical pressure of the pituitary gland on the this marecould have been triggered by enlargement of the pituitary gland secondary to lymphoma and mechanical pressure on the hypothalamic thermoregulatory center.

Primary lymphoma of the pituitary gland has rarely been reported in humans. Human cases are mostly of large B-cell type. The present case is the first report of lymphoma affecting the pituitary gland of horses or other domestic species.

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Acute Disseminated Toxoplasmosis in American Red Squirrels

D. S. Bangari, H. L. Thacker, and M. A. Miller

Toxoplasmosis is a systemic protozoal infection of zoonotic importance, caused by the apicomplexan protozoan *Toxoplasma gondii*. Domestic cats and wild felids are the only known definitive hosts whereas a variety of warm-blooded animals including humans, domestic animals, wild mammals, and birds serve as intermediate hosts. *T. gondii* infection has been reported in a variety of squirrels including eastern gray squirrel, Korean squirrel and the California tree squirrel. This is the first report of toxoplasmosis in American red squirrels (*Tamasciurus hudsonicus*). American red squirrels, also known as Chickaree or the Hudson's Bay Squirrel, are distributed in the Rocky Mountain regions of the western United States, Appalachian Mountains, northeastern United States, Alaska and Canada.

Two juvenile male red squirrels were found dead and submitted for necropsy examination. At necropsy, the squirrels were lean with moderate ectoparasite infestation. Gross lesions were only observed in lungs from both squirrels. The lungs were diffusely mottled dark red to tan and consolidated. Numerous pale tan to creamy-white, oval to circular, up to 2 mm in diameter, nodules were scattered throughout the lungs. Microscopically, both lungs had diffuse necrotizing lymphohistiocytic pneumonia with intracellular as well as free protozoal organisms, morphologically consistent with *T. gondii*. There was mild hyperplasia of type II pneumocytes. There was multifocal lymphoplasmacytic encephalitis with intralesional protozoal organisms in both squirrels. Additional histologic lesions included lymphoplasmacytic periportal hepatitis and lymphoplasmacytic interstitial nephritis. Protozoal organisms in the lung tested positive for *T. gondii* by immunohistochemistry. Transmission electron microscopy of the lung demonstrated intracellular protozoa with morphologic features consistent with *T. gondii.*

On the basis of histopathology, immunohistochemistry and transmission electron microscopy, disseminated *T. gondii* infection was determined to be the cause of death of these squirrels. The source of infection is not determined but is speculated to be through contamination of premises with oocysts from wild and/or domesic felids. Since red squirrels are common near urban settlements, this finding underscores their role as important intermediate hosts for *T. gondii.*

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AAVLD 49th Annual Conference
October 12 – 18, 2006
Minneapolis Hilton – Minneapolis, MN

Blastomycosis in a Grizzly Bear (*Ursus arctos horribilis*)

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A sixteen-year-old female grizzly bear at the Little Rock Zoo presented for malaise for a period of at least three weeks. Exploratory laparotomy revealed two large masses involving both ovaries and oviducts. Additional mass lesions in the abdomen were not observed. Both masses were encapsulated and contained lobules of yellow-tan fatty tissue separated by regions of dense fibrous tissue.

Histologically, the dense fibrous regions correspond to areas of marked granulomatous to pyogranulomatous inflammation with numerous 10-20µm yeasts compatible with *Blastomyces dermatitidis*. Fungal organisms reacted strongly with both periodic acid-Schiff and Gomori methenamine silver stains. Fatty tissue observed grossly contained regions of fat necrosis and granulomatous inflammation with a large number of multinucleated giant cells surrounding enlarged cholesterol clefts. These regions did not contain fungal organisms.

Serum obtained at surgery tested positive for antibodies to *Blastomyces dermatidis* using the Agar Gel Immunodiffusion Test (AGID). Subsequent testing of a series of banked serum samples indicated that this animal was positive for antibodies against *Blastomyces dermatidis* at least seven months prior to surgery. Antibodies were not present in the exhibit mate.

Swab cultures taken from the tissue masses were plated for aerobic bacteria and fungal isolation. Aerobic cultures were negative for the presence of bacteria. Fungal cultures were positive for growth after 11 days of incubation on Sabouraud Dextrose C-G Medium.

The bear responded very well post-surgically and continues to improve with anti-fungal therapy. The source of infection has not been identified, however ongoing construction at the zoo near this bear's enclosure is considered the most likely source. To the authors' knowledge this case report is the first to document Blastomycosis in a grizzly bear.

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AAVLD 49th Annual Conference
October 12 – 18, 2006
Minneapolis Hilton – Minneapolis, MN

Escherichia coli genotyping and relation to histopathology

T. V. Koynarski, D. M. Jordan

Post-wean diarrhea due to *Escherichia coli* continues to be a significant concern in many swine production practices. In the 2005 at the ISU Veterinary Diagnostic Laboratory, there were approximately 350 cases diagnosed as enteritis related to *E. coli* in post-wean pigs. Based on the diagnostician's assessment, practitioner, and producer concerns, these isolates were further analyzed by PCR for the presence or absence of virulence toxin (LT, Sta, Stb, Stx2) and adhesion genes (K88, K99, F18, F41 and 987P). Isolates from one hundred cases were categorized based on the diagnosis, presence of bacilli and PCR positive results. Upon histopathologic examination, adherent bacilli were present along otherwise normal small intestinal villi in small intestinal sections in 69 of the cases. The most common gene combinations for the *E. coli* isolates associated those cases were Stb:Lt:K88 (19%), followed by Sta:Stb:F18:Stx2 (13%). There were thirty-one cases in which the pathologist thought the isolated *E. coli* was important in disease but lacked bacilli present along the villi. The most common gene combinations were Stb:Lt:K88 (19%) and Stb:Sta:F18 (13%). F18 was the most common single gene present in both groups: bacilli present (6%) and bacilli absent (16%) in the intestinal sections. The retrospective evaluation of data provides a basis for a prospective study to determine further trends of correlating histopathologic lesions with colony morphology and virulence genes in cases of post-wean diarrhea in pigs.

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AAVLD 49th Annual Conference
October 12 – 18, 2006
Minneapolis Hilton – Minneapolis, MN

Infections caused by Pathogenic Free- Living Amoebae (*Balamuthia mandrillaris* and *Acanthamoeba sp.)* in Horses.

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Granulomatous amoebic encephalitis (GAE) is a rare clinicopathologic entity in humans and animals produced by several species of free-living amoebas belonging to the genera *Acanthamoeba*, *Naegleria* and *Balamuthia.* Amoebic infections in four horsesfrom Southern California was diagnosed. GAE was caused by *B. mandrillaris* in one horse; GAE and pneumonia was associated with *A. culbertsoni* in a second horse; and in two other horses, pneumonia and systemic infection caused by *Acanthamoeba* sp. was associated with bacterial and fungal agents. Microscopically, the lungs had severe subacute bronchointerstitial pneumonia with predominant neutrophilic infiltrates, multifocal to coalescing necrosis, and fibrin thrombi of vessels. Involved lung had perivascular inflammation consisting of infiltrations of lymphocytes, plasma cells and neutrophils and numerous intralesional amoebae with a diameter size of 15-20 µm. The cerebral corticies grossly were malacic with locally extensive granulomatous inflammation consisting of macrophages, lymphocytes, and few neutrophils. The intralesional amoebae were identified by their reactivity with immunofluorescence and/ or immunohistochemistry staining with rabbit anti-*B. mandrillaris* and anti-*Acanthamoeba* serums and/ or culture (*B. mandrillaris, A. culbertsoni*). Additional laboratory findings include polymerase-chain-reactions (PCR) and ultrastructure characteristics of amoebic organisms from culture aspirates. Though rare, the occurrence of amoebic infections in horses is reportedly unique to southern California and it should be considered in the differential diagnosis of other neurological diseases.

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AAVLD 49th Annual Conference
October 12 – 18, 2006
Minneapolis Hilton – Minneapolis, MN

Pulmonary Pneumocystosis in Immunodeficient Calves

D.E.B. Knudsen[1]

The genus *Pneumocystis* is comprised of obligate parasitic fungi inhabiting the lungs of mammals. An often lethal pneumonia occurs in humans, mice, and rats colonized by *Pneumocystis* sp. as a result of chronic, sustained loss of CD4+ T lymphocyte function from a variety of causes, including immunosuppressive viral infections, high dose chemotherapy, and spontaneous mutation. This pneumonia is characterized by proliferation of the organism and diffuse filling of alveoli by foamy macrophages and multinucleate giant cells which represent an unsuccessful attempt by the host at clearing the infection in the absence of competent T cells. Here we report on diffuse alveolar pneumonia associated with *Pneumocystis* sp. in two immunodeficient calves. Both calves were heavily colonized by the organism throughout the lung; this proliferation was accompanied by marked, diffuse hyperplasia of alveolar macrophages and formation of multinucleate cells. As livestock species increasingly become animal models and subjects of biomedical research, the appearance of unusual opportunistic agents may become a more regular feature of diagnostic practice.

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Salmonella typhimurium var. *copenhagen* septicemia as a cause of meningoencephalomyelitis/encephalitis in neonatal foals

M.R.S. Ilha[*1], H. Burgess[1], A. Rodriguez[3], D. Slavic[2], B. McEwen[2], J. Baird[3]

We report here two cases of septicemic salmonellosis in neonatal foals with involvement of the central nervous system. Foal number 1, a 14-day-old, Thoroughbred, female horse was presented with a history of diarrhea, dehydration, respiratory distress, lethargy, depression, anorexia, hyperesthesia, and seizures. Blood cell count revealed marked leukocytosis (74.7 x 10^9 /l) with neutrophilia (69.47 x 10^9 /l), and left shift (1.49 x 10^9 band neutrophils/l). CSF analysis revealed remarkably high nucleated cell count (246 x 10^9/L) and an abundant monomorphic population of intracellular rods. *Salmonella* sp. was isolated from feces and CSF. At necropsy, there was pulmonary edema, mild enterocolitis, suppurative urachitis and meningoencephalomyelitis. Purulent exudate was deposited on the leptomeninges of the cranial and ventral aspects of the cerebrum, cerebellum, and entire length of the spinal cord. There was subtentorial and cerebellar herniation (cerebral edema). Multifocal areas of malacia were observed on the cut surface of the cerebrum and mid thalamus. *Salmonella* sp. was isolated from the urachus, meninges, brain stem, cerebellum, spinal cord, and mesenteric lymph node. On histopathology, there were diffuse and severe suppurative meningitis and multifocal areas of malacia in the brain with vasculitis, thrombosis and gram-negative bacilli around blood vessels. Fibrinosuppurative bronchopneumonia with intralesional gram-negative bacteria was also noted. IHC for *Salmonella* sp was positive in brain and lung.

The second foal, a 5-day-old, Thoroughbred, female horse was presented with severe diarrhea, and arthritis of the right hock. This foal presented with a mild neutropenia (2.60 x 10^9 /l), and left shift (1.24 x 10^9 band neutrophils/l). The neutrophil numbers recover over the following four days producing a neutrophilia (18.84 x 10^9 /l). *Salmonella* sp. was isolated from the blood, feces and synovial fluid of this foal during the clinical course of the disease. Necropsy findings included pulmonary edema, splenomegaly, mild enlargement of the mesenteric lymph nodes, suppurative omphalophlebitis, fibrinous arthritis of the right hock, and fibrinous enterotyphlocolitis. *Salmonella* sp. was isolated from the umbilicus, small intestine, and colon. Histopathologic findings included fibrinosuppurative synovitis, ulcerative colitis, necrotizing hepatitis, and a single focus of malacia infiltrated with neutrophils and surrounded by microglia and reactive blood vessels in the cuneate nucleus of the medulla oblongata. Further salmonella serotyping of the isolates identified *Salmonella typhimurium* var. *copenhagen* as the causative agent in both foals.

The most important feature in these cases was the central nervous system involvement which is unusual for salmonellosis. Meningoencephalomyelitis and meningoencephalitis caused by *S. agona* and *S. typhimurium* respectively have been described in foals only occasionally (Patterson-Kane JC et al. N Z Vet J 2001; 49:159-161; Stuart BP et al. JAVMA 1973; 162:211-213). Both foals had umbilical infection from which *Salmonella* was isolated. The umbilicus was considered as the potential portal of entry for the bacteria in these cases. Septicemic neonatal salmonellosis affecting animals that had umbilical infection suggests environmental contamination of the foaling area with *Salmonella*. An outbreak of equine salmonellosis has been described in neonatal foals in which the asymptomatic carrier mares were the presumed source of infection for the offspring (Walker RL et al. J Vet Diagn Invest 1991; 3:223-227). A similar situation might have occurred in our cases. The initial source of infection in an outbreak of equine salmonellosis can be birds, rodents, contaminated water or feed, and recently introduced horses.

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AAVLD 49th Annual Conference
October 12 – 18, 2006
Minneapolis Hilton – Minneapolis, MN

An Intersex Female Llama *(Llama glama)* with Bilateral Ovotestes

M.T. Bouljihad [1] , S.L. McClanahan1[2] , J.E. Romanao[2]

An intact female llama *(Llama glama)* aged 2.5 years had a 1-year history of aggressive behavior and inability to breed. Examination of external genitalia revealed an elongated vulva and enlarged clitoris. Except for a relatively small left ovary, no other abnormalities were detected by ultrasonic reproductive examination. At necropsy, the vulva was mildly elongated and the clitoris was moderately enlarged and devoid of urethral orifice. The gonads had yellow to red cystic structures, and the vagina, cervix, uterinetube, uterine horns, broad ligaments, and Fallopian tubes appeared to be within normal limits. Microscopic examination revealed that each gonad was composed of both ovarian and testicular tissue. To our knowledge, this is the first recorded case of hermaphroditism with bilateral ovotestes in llama.

Key words: Camelids; ovotestes; hermaphroditism, intersex.

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AAVLD 49th Annual Conference
October 12 – 18, 2006
Minneapolis Hilton – Minneapolis, MN

Clinical and pathological aspects of osteogenesis imperfecta in newborn Angus calves in Brazil

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Osteogenesis imperfecta (OI) is a genetic disease that affects the connective tissue of bones, tendons, dentin and sclera causing increased bone fragility, joint hyperlaxity, pink teeth, and blue sclerae. Mutations in the genes encoding for type I collagen result in defective formation of this type of collagen, which is the predominant type of collagen in these tissues. OI has been reported in human beings and in a variety of animal species including cattle (in the Charolais, Friesian, Holstein and Holstein-Friesian breeds), sheep, dogs, cats, a llama, a fox, a tiger, and a Rhesus macaque. Transgenic/knockout mice have been used as animal models for this disease. In the State of Nebraska, USA, this disease was observed in Angus calves on one occasion[I], and was presumptively diagnosed in Angus cross calves on another occasion[II]. In Angus/Angus cross calves, however, this condition is not as well characterized as in other bovine breeds in which this disorder is described.

The present report documents the clinical and pathological aspects of a severe form of OI in Angus calves in Brazil. This disease was observed in 12 of 59 newborn purebred Angus calves from a beef herd in the State of Rio Grande do Sul. The calves were derived from 77 unrelated Angus cows all of which were artificially inseminated using semen from the same clinically normal, purebred Red Angus bull born in Brazil. The bull's dam was derived from an embryo imported from Canada and its sire was also of Canadian origin, the cow having been inseminated with imported semen. Affected calves were observed on the first occasion that semen from the Brazilian bull was used. All clinically affected calves were identified at birth and euthanized 2-20 days after calving. There were also 6 abortions, and 10 animals had bilateral lens opacity since birth. Clinical signs included marked joint laxity, curved hindlimbs, domed forehead, prognathia, inability to stand/walk, and subnormal body size. Post-mortem radiographs of 3 affected calves revealed multiple healing rib fractures, complete long bone fractures, and luxation/subluxation of some limb joints. Necropsy findings included several complete fractures in the axial/appendicular skeleton with hemorrhage/edema of the subcutis/skeletal muscles, multiple bony calluses on the ribs, reduced length of long bones, poorly aligned, fragile, pink incisors, presence of eponychium, and absence of the ligaments and foveae of the femoral heads resulting in hip luxation. Microscopic findings were osteopenia with thin, poorly mineralized trabeculae/marked reduction in the amount of trabecular bone, hypoplastic/hypercellular tendons, and thin dentin. There was also Wallerian degeneration of the spinal cord white matter with axonal spheroids. Immunohistochemistry for BVDV on skin was negative. Semen/blood of the progenitor bull was not available for further investigation. Teratogenic drugs, agrochemical substances, or poisonous plants were not found in this farm. After this diagnosis of OI, the Brazilian bull was removed from artificial insemination. The disease has not been observed in that country since then.

In this study, the diagnosis of OI was based on the history, clinical signs, radiology, necropsy, and histopathology. As in previous reports of OI in cattle/sheep, the inheritance is almost certainly dominant, and the defective gene would have developed as a new germ-line mutation during testicular development of the Brazilian bull rather than having been imported from Canada with genetic material from its sire or dam. This would explain the normal phenotype of this bull and the occurrence of the disease in the offspring of cows unrelated to each other or to the sire/dam.

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AAVLD 49th Annual Conference
October 12 – 18, 2006
Minneapolis Hilton – Minneapolis, MN

INTEGRATED SYSTEMS FOR THE SAFE MANAGEMENT OF BIOLOGICAL AND BIOHAZARDOUS INFECTIOUS WASTE AND EFFLUENT FROM BIOCONTAINMENT ANIMAL FACILITIES

Gordon Kaye, Ph.D. and Marty Wetzel, B.A.

Until recently, disposal of solid wastes from BSL-3, BSL-3ag and BSL-4 facilities, specifically biological waste (animal tissues and carcasses, cultures, etc.) and biohazardous waste, required special packaging and autoclaving of materials being removed from within the containment barrier prior to treatment and disposal. Introduction of the WR^2 Process, i.e., alkaline hydrolysis at elevated temperature, and the WR2 Tissue Digestors™, allow for the treatment of such wastes within the containment barrier with subsequent release of the hydrolyzate of digested biological material directly from within the barrier to a sanitary sewer and the removal of treated non-organic materials through a port located outside the barrier. Simply put, the treatment system forms part of the containment barrier.

It is now standard practice to sterilize all liquid effluent from BSL3ag, and BSL-4 and most new BSL-3 facilities prior to release of such effluent to a sanitary sewer. Effluent Decontamination Systems (EDS) of various sizes and complexity can now be installed either within the containment barrier or in a subcontainment location. Both independent EDS and EDS that collect the waste effluent for fill water for the Tissue Digestors have been included in the design of several new BSL-3 and BSL-3ag facilities. Examples of recently constructed facilities at Kansas State University, University of Georgia, and Albany Medical College, that incorporate these modalities will be presented and the bases for choosing the specific modalities for each facility will be discussed.

Traditionally, other solid, non-organic wastes were bagged and passed through the barrier using a double-door autoclave. While an autoclave will certainly adequately disinfect the surfaces of solid material and the bags, it is well known that many factors such as closed containers inside the bags, suction canisters in the bags, and material becoming wrapped in Tyvek® protective clothing can prevent adequate decontamination of the significant portions of the contents of the bags. For this reason, the WR^2 STI Medical Waste Processing Systems may be employed to comminute and decontaminate the waste. The STI System shreds the bagged waste as an integral first step so that the disinfecting steam can effectively reach all surfaces of the confetti-sized pieces produced. Further, the STI treatment produces a final product that meets the specifications of those jurisdictions that require unrecognizability..

Finally, double-door combination pass-through autoclave/Tissue Digestors are being developed for the NIH at Fort Detrick and for the University of Pittsburgh. These units would be able to serve both as autoclaves to pass material through the barrier into or out of the containment facility or as Tissue Digestors to destroy biological materials that must be removed from containment.

WR^2, Inc., Indianapolis, IN 46241

WITHDRAWN

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October 12 – 18, 2006
Minneapolis Hilton – Minneapolis, MN

Occurrence of Caprine Arthritis Encephalitis in Korea

In-Soon Roh, Kyung-Hyun Lee, Young-Hwa Jean and O-Soo Lee

Caprine arthritis-encephalitis (CAE) is a disease of domestic goats caused by a lentivirus of the family Retroviridae. CAE has been identified in most goat-rearing countries around the world. Most goats naturally infected with CAE virus are largely asymptomatic and remain life-long carrier. Only some of the infected goats, less than 10%, develop clinical disease characterized by chronic persistent arthritis, mastitis, respiratory distress and encephalitis. A disease characterized by arthritis and lameness, especially carpal joints, was seen on goat farm in Chunbuk province of Korea in the February 2006. We investigated the cause of the disease by pathological examination and agent detection by PCR and DNA sequence analysis.

Ninety seven goats were bred in the farm and clinical abnormality with arthritis of carpal joint has been occasionally observed in some goats since the October 2005. One of them, 3 years old, showing severe arthritis in carpal joints was euthanized and necropsied. Brain, spinal cord, lungs, synovial membranes and carpal joints were collected for pathological observation, and peripheral blood and joint fluids were collected for agent detection by PCR and DNA sequence analysis.

Grossly, the goat appeared emaciation and lameness by swelling of carpal joint. The tendon sheaths and joint capsule were thickened by fibrous tissue. The right carpal joints was filled with yellow to bloody fluid which contains fibrinous masses. Microscopically, lesion of the brain was mild nonsuppurative perivascular cuffing, involving the white matter. There observed focal lymphoid nodules in lung. The major joints, especially carpus and stifle, were synovial villi hypertrophy, edema, and infiltration of plasma cells, lymphocytes and macrophages. By the PCR, a 296bp fragment was amplified from the DNA of the PBL and joint fluid by the first PCR, and a 184bp fragment was produced by nested PCR. The nested PCR product was sequenced. The sequence homology in gag region of that to CAEV-Br/URFGS-2/C767 strain (Genebank No. AJ305042) were 96%.

Based on the pathological examination PCR and DNA sequence analysis, we diagnosed as the first case of Caprine Arthritis Encephalitis in Korea.

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AAVLD 49th Annual Conference
October 12 – 18, 2006
Minneapolis Hilton – Minneapolis, MN

Optimization of Sample Collection and Automated Immunocytochemical Staining of Lymphomas

A.D. Aulbach[*1], C.L. Swenson[1], M. Kiupel[1]

Distinguishing B vs. T-cell lymphoma directs clinicians toward more appropriate treatment protocols and aides in predicting prognosis for these neoplastic entities. Performing this procedure on cytologic specimens provides a less invasive, rapid cost effective alternative to surgical biopsy and histopathologic examination of these tissues. A variety of techniques have been employed over the past decade in an attempt to obtain accurate, consistent immunocytochemical staining of cytologic specimens. Both manual and automated methods have been utilized with varying degrees of success. Inherent difficulties in collection and preservation of cytologic specimens with sufficient numbers of intact cells contribute to the unpredictable nature of these methods. The practice of manual staining has been touted as more reliable; however, when compared with the automated method, increased labor, turnaround time, technical expertise, and batch variation remain formidable obstacles for many institutions. Traditional fine needle aspiration techniques generally have a high degree of variability in concentration and hemodilution. Starting with a well-preserved, cellular, "clean" cytologic specimen remains one of the most important factors to the success of these staining methods. The purpose of this study was to optimize a technique for obtaining, preparing and preserving high quality cytologic preparations for antibody labeling using an automated immunostainer.

Pre-prepared vials of collection media were made using 3 mol K_3EDTA tubes with 1 ml of 0. 9% phosphate buffered saline (PBS) plus 2 drops of 22% bovine serum albumin (SSigma Chemical Co., St. Louis, MO) added. Collection media tubes were stored at 3-8°C. Fine needle aspirates obtained from lymph nodes of animals presented to Michigan State University Veterinary Teaching Hospital for lymphadenomegaly were used. Lymph node aspirates were introduced into the collection media and assessed for cellularity and hemodilution. Concentrated preparations were made by placing 25-50 µL of sample plus sufficient 0.9% PBS for a total volume of 100 µL into a cytospin cup and centrifuging at 530RPM for 10 minutes (Cytospin 3. Shandon Scientific Ltd., England). One slide was stained with Wright's stain and examined under light microscopy for cellularity and sample quality. Based upon the survey slide, additional cytospin preparations were made incorporating any needed adjustments in sample volume until a desired neoplastic cell concentration was obtained. Multiple (2-10) additional unstained cytospin preparations were then prepared using the appropriate concentration and stored at 3-8°C. The final preparations were stained using a Bond-max automated immunostainer (Vision Biosystems, Boston, MA) and a Bond polymer detection kit (DS9713 – Vision Biosystems). CD3e (clone CD3-12, P. Moore) and CD79a (clone HM57 – Dako, Carpenteria, CA) antibodies were used in these studies to distinguish B versus T cell subtypes of lymphoma.

Cytologic specimens prepared in this way:

1. Had optimal cellularity and preservation without significant hemodilution or interference by cellular/proteinaceous debris.
2. Remained stable for up to 4 weeks when stored at3-8°C.
3. Had excellent morphology and staining quality resulting in consistent diagnostic utility.

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AAVLD 49th Annual Conference
October 12 – 18, 2006
Minneapolis Hilton – Minneapolis, MN

Further observations on spironucleosis (hexamitiasis) in gamebirds and turkeys: Light microscopic and ultrastructural morphology of the cysts of *Spironucleus meleagridis*, and genotyping of this flagellate from turkeys, pheasants and red-legged partridges.

A.M. Wood[1], A. Jorgensen[2]

Spironucleosis (syn. hexamitiasis) due to intestinal infection with the diplomonad flagellate *Spironucleus meleagridis* continues to be a significant problem in the rearing of gamebirds (pheasants and red-legged partridges) in the United Kingdom, and although uncommon in young turkeys, can also cause serious disease in this species. Transmission experiments in the USA in the 1940s indicated that hexamitiasis in turkeys, pheasants and other gallinaceous birds was caused by a single protozoan species *Hexamita meleagridis*. More recently, morphological studies indicated that *H. meleagridis* should be reclassified as a member of the closely related but distinct diplomonad genus, *Spironucleus*.

The life cycle of *Spironucleus* alternates between a motile trophozoite in the host, and a resistant cyst which is important in transmission of the infection. The cystic stage of *S. meleagridis* was reported from the UK in the 1950s but not confirmed until recently (1). Cysts are most numerous within thick intestinal mucus and are best demonstrated in stained smears of compressed mucus. The first part of this presentation illustrates the morphology of cysts and the process of excystation as demonstrated by light and electron microscopy.

Genetic studies on diplomonad flagellates have shown that organisms which are indistinguishable by light microscopy and even by scanning and transmission electron microscopy can be genetically very different, and can actually consist of several distinct species. This raised the possibility that the *Spironucleus* flagellates parasitic in different species of gallinaceous birds, previously regarded as all being *Hexamita (Spironucleus) meleagridis* might consist of several different genotypes.

We have therefore sequenced most of the small subunit ribosomal RNA gene from *S. meleagridis* isolated from turkeys, pheasants and partridges with spironucleosis. The results confirm the morphological observation that these organisms belong to the genus *Spironucleus*, and that the organisms from these 3 different species of bird are essentially genetically identical.

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AAVLD 49th Annual Conference
October 12 – 18, 2006
Minneapolis Hilton – Minneapolis, MN

Respiratory Herpesvirus infection in Parakeets

T. Lazic[1], J. S. Haynes[1], J. M. Drahos[2], J. Stasko[3]

A flock of Indian Ringneck Parakeets (Psittacula krameri manillensis) was imported to United States from Australia. Soon after, two parakeets died after a two day course of illness consisting of anorexia, lethargy, emaciation and dyspnea. Necropsy revealed diffuse consolidation and red discoloration of the lungs as well as thickened, congested air sacks. The microscopic examination revealed multifocal, necrotizing bronchitis, parabronchitis and interstitial pneumonia. The lumen of affected airways contained numerous, large syncytial cells with up to 15 nuclei. The nuclei of these syncytial cells often contained large, eosinophilic inclusion bodies, consistent with herpesvirus. The epithelium of the trachea and air sacks was hypertrophied and contained syncytial cells with intranuclear inclusion bodies similar to the bronchi. In addition, a few intranuclear inclusion bodies were also present in the epithelial cells lining the air capillaries. These lesions are characteristic of those caused by respiratory herpesvirus of parakeets.

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AAVLD 49th Annual Conference
October 12 – 18, 2006
Minneapolis Hilton – Minneapolis, MN

Accuracy of commercially available paratuberculosis ELISA kits and an in-house ELISA on bovine milk samples

H.F. Cushing*[1], M.T. Collins[1], S.J. Shin[1], E.J.B. Manning[1], Y. Chander[2], N. Cernichiaro[2], S.J. Wells[2], S. Goyal[2], J.E. Collins[2]

Antibody produced during *Mycobacterium paratuberculosis* infection in cattle appears in both blood and milk. However, milk is a more convenient and cost-effective clinical sample. The goal of the study was to evaluate ELISA specificity by correlating ELISA results on serum and milk samples using three commercial paratuberculosis ELISA kits (A, B, and C) and a new in-house ELISA (ELISA D). To estimate ELISA specificity, milk samples from non-infected adult Holstein cows (n=213) resident in herds that annually tested negative for >4 years and qualified for level 4 in the Voluntary Bovine Johne's Disease Control Program were tested. Serum and milk ELISA results for 82 cattle residing in *Mycobacterium paratuberculosis*-infected dairy herds and suspected of being infected were also correlated. All four ELISAs used serum or milk absorption with *Mycobacterium phlei* antigens. Milk was tested at a dilution of 1:2 with *M. phlei* –containing diluent for all four ELISAs. All other procedures were performed according to manufacturers' instructions.

Dichotomous results were compared by ROC analysis. The specificity of ELISA A, B, C, and D was 99.5%, 99.5%, 99.0%, and 99.4%, respectively. Interestingly, the specificity of a commercial milk ELISA service on these same milk samples was only 95.3%. The sensitivity of detecting cows that tested positive for serum antibodies using the same diagnostic kit was 14.1%, 88.0%, 72.5%, 91.1% for ELISA A, B, C, and D, respectively. Within-ELISA kit agreement and kappa value between assays on serum and milk from the same cows collected on the same day for ELISA A, B, C, and D was 32.9% (kappa = 0.07), 92.7% (kappa = 0.85), 92.7% (kappa = 0.85), and 93.6% (kappa = 0.87), respectively. Linear regression analysis of serum and milk results within kit gave r^2 values of 0.28, 0.70, 0.67, and 0.78 for ELISAs A, B, C, and D. The area under the curve (AUC) for ELISAs A, B, C, and D were 0.748, 0.911, 0.982, and 0.845.

We conclude from these data that some ELISA kits for bovine paratuberculosis can effectively be used on bovine milk samples with limited loss in overall diagnostic accuracy. However, not all assays perform equally well on both serum and milk. ELISA kits B and C and in-house ELISA D performed comparably well.

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AAVLD 49th Annual Conference
October 12 – 18, 2006
Minneapolis Hilton – Minneapolis, MN

Comparison of BACTEC and MGIT systems for detection of *M. paratuberculosis*

G. Thomas, E.J.B. Manning, and M.T. Collins

The BACTEC 460 system (modified 12B medium and 460 instrument) was the first liquid culture system adapted for detection of *M. paratuberculosis*. It has proven to be fast and reliable for both detection and quantification of the organism from clinical samples. This system is no longer in commercial production and has been replaced with a non-radiometric system; a culture medium designed specifically for *M. paratuberculosis* growth called the Mycobacterial Growth Indicator Tube (MGIT) paraTB and an instrument, the MGIT 960. The purpose of the present study was a head-to-head comparison of the BACTEC 460 and MGIT 960 systems on 1,128 consecutively submitted clinical samples (feces and tissues). The samples originated from domestic and wild animals from diverse locations (21 U.S. states). Samples were split into two portions and processed using the previously described HPC-filter concentration protocol. All cultures that gave a positive signal in their respective instrument were subcultured on a blood agar plate to detect contaminating microbes and stained (Ziehl-Neelsen) for detection of acid-fast bacteria (AFB). AFB-positive cultures were subcultured to HEY with and without mycobactin and also tested by multiplex PCR to identify *M. paratuberculosis*. In total, 921 (81.6%) of samples were signal-negative by both systems. Contamination rates (no AFB detected) were 6.8% for MGIT cultures and 5.9% for BACTEC cultures. AFB were detected by one or both systems from 102 (9.0%) samples. Of these 102 AFB-positive samples, from 23 (22.6%) both systems detected *M. paratuberculosis*, from 16 (15.7%) only MGIT detected *M. paratuberculosis*, from 5 (4.9%) only BACTEC detected *M. paratuberculosis*, and for 58 (58.9%) the AFB detected were mycobacterial other than *M. paratuberculosis*. The MGIT system was more sensitive ($p<0.05$) and faster (23 versus 33 days to detection; $p<0.009$) than the BACTEC system. MGIT technology is an improvement over BACTEC system for *M. paratuberculosis* detection.

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AAVLD 49th Annual Conference
October 12 – 18, 2006
Minneapolis Hilton – Minneapolis, MN

Contamination Suppression and *M. avium* subsp. *paratuberculosis* Recovery by the BD BACTEC™ MGIT™ Para TB System with High Nalidixic Acid, Frozen Inocula, and Malachite Green.

M. T. Warns[1], V. Parvu[2], and **R. F. Pfeltz**[1]

Introduction and Objectives. The bovine fecal processing protocol for the BD BACTEC™ MGIT™ 960 Para TB System (BD Diagnostic Systems, Sparks, MD) greatly reduces liquid culture contamination rates in *M. avium* subsp. *paratuberculosis* (MAP) detection for Johne's disease diagnosis. However, contamination may still occur from very highly contaminated samples. This study assessed nalidixic acid at 200 µg/ml (high NAL) versus the standard 18-19 µg/ml in culture medium to suppress contamination, use of 0.05% malachite green as a decontaminant, and storing inocula at -70°C or -20°C.

Materials and Methods. In experiment #1, four samples were processed with and without 0.05% malachite green in the HPC decontamination solution, and inoculated into culture medium with standard or high NAL. Six samples were all processed with malachite green and inoculated into standard NAL in experiment #2. Excess inocula were frozen at -70°C in experiment #1 and -70°C and -20°C in experiment #2, then inoculated into standard and high NAL media after thawing. All experimental conditions employed 5-10 replicate culture tubes. MINITAB Release 14.1 Statistical Software (Minitab, Inc.) was used for descriptive statistics, to analyze MAP times-to-detection (TTDs) by means of ANOVA-type general linear model, and to analyze contamination frequencies and system performance (odds of agreement) in correctly calling samples positive or negative for MAP by means of binary logistic regression. Colony counts from inocula were made on Herrold's Egg Yolk agar ± mycobactin J.

Experiment #1 Results. Use of either malachite green or high nalidixic acid significantly improved instrument performance (odds of agreement) and reduced the odds of contamination across all samples ($P < 0.001$). Malachite green had no significant impact on MAP TTDs ($P = 0.058$), while high NAL generally added 1-3 days to TTDs from the moderate shedder with little effect on the high shedder sample. Freezing inocula at -70°C resulted in significant contamination increases ($P = 0.015$), primarily for the two highly-contaminated MAP-negative samples. TTDs were significantly longer after freezing inocula ($P < 0.001$), 1-6 days for the high shedder and 6-9 days for the moderate shedder sample. Of 21 high shedder cultures lost to contamination, 20 were with standard NAL and without use of malachite green.

Experiment #2 Results. High NAL use significantly reduced the odds of contamination ($P = 0.001$) and improved instrument performance (odds of agreement, $P = 0.002$) across all samples. Fresh versus frozen inocula had no significant impact on contamination rates or instrument performance ($P > 0.05$). TTDs were not significantly different from fresh vs. -20°C frozen inocula ($P = 0.9995$), but were longer for inocula frozen at -70°C ($P = 0.0054$). Mean TTDs were all within 2.5 days of each other regardless of inoculum or NAL from the high shedder sample also used in experiment #1. A second high shedder sample had TTDs more negatively impacted by both high NAL and/or -70°C. Compared with fresh inoculum in standard NAL, mean TTDs for this sample in high NAL were more than eight days longer after -70°C, but only 1.4 days longer after -20°C storage. Freezing at -70°C added six days to the mean TTD for the low shedder sample with standard NAL, but less than one day with inocula stored at -20°C. Colony counts indicated that freezing at -70°C reduced the recovery of viable MAP vs. fresh inocula.

Conclusions. High NAL reduced contamination but not MAP recovery, although it added several days to TTDs for some strains. Malachite green use reduced contamination with no impact on MAP. Reculturing with frozen inocula and high NAL is an effective rescue procedure when initial cultures are lost to contamination, although TTDs may be longer and recovery rates impacted for lower shedders. MAP recovery was better from inocula stored at -20°C than -70°C. Laboratories that consistently receive very highly contaminated fecal samples have the option of employing 200 µg/ml NAL routinely for all cultures.

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October 12 – 18, 2006
Minneapolis Hilton – Minneapolis, MN

Counting *Mycobacterium paratuberculosis* cells: a comparison of MGIT, Flow Cytometry and ATP Assays

A.S. Yuroff[1], E.J.B. Manning[1], R.M. Hoffman[2], M.T. Collins[1]

Enumeration of *Mycobacterium paratuberculosis* is crucial to studies on survivability of the agent in the face of antibiotics, disinfectants, environmental stressors, etc. The goal of these experiments was to determine accurate, reliable, and efficient methods to count *M. paratuberculosis* that avoid the problems seen by traditional plate counts (clumping) and the BACTEC 460 counting algorithm (reliance on ^{14}C incubation vials that must be read daily). The Mycobacterial Growth Indicator Tube System (MGIT®; BD Biosciences) is an automated non-radioactive culture system for mycobacteria. An objective of this study was to evaluate the relationship between *M. paratuberculosis* inoculum cell number and time to detection (TTD) signal from the BACTEC MGIT 960 system. In addition, we evaluated the accuracy of flow cytometry and ATP assays (BacTiter-Glo®, Promega) to quickly quantify viable *M. paratuberculosis* cells. For these experiments, log-phase *M. paratuberculosis* cells were serially diluted. The number of cells in each dilution was determined by flow cytometry and standard plate counts. Tubes of MGIT paraTB medium were inoculated with each dilution; each dilution was also evaluated by the ATP assay. The TTD signal recorded by the BACTEC MGIT 960 instrument was linear ($r^2 < 0.98$) with respect to the log cell number (as measured by plate counts) from 10^6 to 10^1 cells per tube. The lower limit of detection in the MGIT system was less than 10 cells per tube. Furthermore, there was good agreement of *M. paratuberculosis* cell numbers generated by flow cytometry and plate count methods between 10^6 and 10^3 cells / ml. In quantitative ATP assays, the amount of ATP measured was linear ($r^2 < 0.99$) with respect to cell number between 10^6 and 10^3 cells. In conclusion, TTD in the MGIT liquid culture system, flow cytometry and ATP measurements provide useful and accurate methods for quantification of viable *M. paratuberculosis* cell numbers. These enumeration methods can provide valid data to support a variety of experimental systems, such as comparing organism concentration methods and the effects of decontaminants and antibiotics on *M. paratuberculosis* bacteria.

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Isolation and Identification of Mycobacteria from Selected Animal Cases: A Summary of Fiscal Years 2001 through 2005

J.T. Nelson, N.B. Harris, and J.B. Payeur

One of the primary missions of the National Veterinary Services Laboratories (NVSL) is to assist the National Bovine Tuberculosis Eradication Program by identifying animals and herds that are infected with bovine tuberculosis. This is accomplished through histologic examination, mycobacterial culture, and mycobacterial identification of submissions from domesticated and wild animal species. Over the past five fiscal years (October 2000 through September 2005) 28,633 cattle, 535 swine (feral and domestic), and 1601 selected cervid cases (antelope, caribou, deer and elk) have been received for mycobacterial culture. The number of cattle samples received and processed has increased every year from 2001 through 2005 and has doubled during this time period (4023 and 8047, respectively). Of the 1532 cattle cases that were identified as positive for mycobacteria, *Mycobacterium bovis* had been identified 40.0% of the time (611/1532). The *M. bovis* isolates cultured in fiscal years 2001 through 2005 confirmed infection in cattle herds in the states of Arizona (2 herds), California (3), Michigan (26), Minnesota (3), New Mexico (2), and Texas (7). Other mycobacterial species identified from cattle include *M. paratuberculosis* (320/1532, 20.9%), *M. avium* complex (180/1532, 11.7%) and 31 other mycobacteria species (421/1532, 27.4%). Mycobacteria isolated from swine consisted of *M. avium* complex (64/118, 54.2%), *M. avium* (31/118, 26.3%), *M. fortuitum* (3/118, 2.5%), *M. bovis* 1.7% (2/118, 1.7%) and 12 other mycobacteria species (18/118, 15.3%). Mycobacteria isolated from selected cervids consisted of *M. avium complex* (50/174, 28.7%), *M. terrae* complex (13/174, 7.5%), *M. avium* (10/174, 5.7%), *M. bovis* (6/174, 3.4%), and 19 other mycobacteria species (95/174, 54.6%). New technology such as 16s rDNA sequencing and molecular techniques has been incorporated into the Mycobacteria and Brucella Section of NVSL within the last few years. This has increased the number of mycobacterial species identified from cattle submissions compared to a similar survey done from 1985 through 1994 (34 versus 19). The new technologies have also enhanced the National Bovine Tuberculosis Eradication Program through better epidemiologic understanding of the isolates and strains in order to control the potential spread of disease across state and national borders.

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October 12 – 18, 2006
Minneapolis Hilton – Minneapolis, MN

PROTOCOL CHANGES FROM THE STANDARD *M. PARATUBERCULOSIS* CULTURE METHODS TO INCREASE THE SENSITIVITY OF JOHNE'S DIAGNOSIS IN BEEF CATTLE

B. E. Mamer, J. E. England, B. C. Anderson

Johne's Disease is caused by *Mycobacterium avium* subspecies *paratuberculosis* (MAP). This disease affects cattle and other ruminants. After animals become infected, this bacterium will eventually target the mesenteric lymph nodes and intestines of animals. Animals infected with MAP develop chronic wasting, diarrhea with shedding of these bacteria in the feces, and, eventually death. More is known about Johne's infection in dairy cattle than beef cattle. The majority of serology and culture assays used to identify MAP positive cattle are from dairy herd studies.

Blood and fecal samples from adult beef cattle were collected from cooperator herds at the yearly fall pregnancy check. Tested subjects included some with symptoms suggestive of Johne's Disease. Historically, these herds have had two to four cows positive for Johne's disease that are culled each year. We also tested individual clinical animals for Johne's using tissues from dead animal's feces and sera. We tested the serum samples for antibodies with the ELISA test. The fecal samples were assayed with two culture media: Herrold's Egg Yolk agar (HEYA) and MGIT liquid culture media that has a fluorometric indicator for detection of positive growth, and is enriched to enhance the growth of MAP. We used the MGIT fluorometric manual read method using a Woods lamp table to signal positive growth in the tube. Because of the difficulty in finding culture positive animals from seropositive beef cattle fecal samples, we increased the fecal culture assay concentrations and length of incubation time in culture media during these studies.

There were approximately 250 cows (*Bos taurus - Bos indicus*) in the first herd. In the first year of testing, we identified five serologically positive cows. Two of the cows were not pregnant and were culled after the samples were taken. None of the 250 cows was culture positive after 18 months using standard culture methods. The following spring, blood and fecal samples were taken from the three remaining sero- positive cows. One of them was positive by fecal culture for MAP after 6 months culture in the MGIT tube, with no growth on the corresponding HEYA slant with standard culture methods. In the second year of the study we identified seven sero- positive cows from samples taken in the fall of the year. The fecal samples from all of these cows were culture negative after 18 months in culture even after the doubling of sediment inoculum during the first stage of the fecal procedure.

The second herd of 500 *Bos taurus* cows had 21 seropositive cows the first year. There were 42 confirmed MAP fecal culture positive cows with the MGIT tubes after 14 months in culture. These liquid culture tubes were positive after two to seven months in culture. The MAP bacteria also grew on HEYA for 16 of the 42 culture positive cows. Eight of the 42 culture positive cows were also seropositive. Eleven of 42 culture positive samples were set-up using the standard inoculum method; however, the majority of the culture positive cows (31) were identified using the increased sediment inoculum. In the second year of the study we identified 14 seropositive cows. The fecal samples from the second year are in the process of set up with all of these fecal samples being set up with the fecal culture assay that doubles the sediment inoculum and all media will be incubated for one year.

We have also found that if fecal samples from individual Johne's seropositive beef animals from other herds are cultured with the increased inoculum, MAP bacteria will grow in the liquid culture media and confirm a Johne's Disease positive animal more often than the standard inoculum will confirm a positive animal.

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Minneapolis Hilton – Minneapolis, MN

Using molecular typing to characterize bacterial pathogens associated with the dairy farm environment

James A Higgins[1], Christina M Hohn[1], and William R Hare[2]

We are interested in applying several molecular biology-based methods to trace the epidemiology of select bacterial pathogens at the dairy farm operation at the Beltsville Agricultural Research Center (BARC). Because these organisms are often ubiquitous in the dairy farm environment, differentiating them at the subspecies or strain level can play an important role in determining sources of the bacteria, and consequently advising infection control measures on the part of attending veterinarians and animal caretakers.

For isolates of *Bacillus* spp. and *Klebsiella pneumoniae*,enterobacterial repetitive intergenic consensus PCR (ERIC-PCR) is being used to create isolate-specific molecular fingerprints that revealed differences at the species and strain levels. For some (but not all) strains of *K. pneumoniae*, ERIC-PCR profiles were different between antibiotic-resistant and antibiotic-susceptible isolates, a level of discrimination not achievable with conventional biochemical analyses. For *Bacillus pumilus*, ERIC-PCR permitted us to identify two different strains simultaneously present in the internal organs of a transgenic animal that died from an iatrogenic infection.

We are using *spa* gene typing, a technique showing promise in tracking human outbreaks with *S. aureus*, to monitor mastitis in our dairy herd. *spa* typing relies on PCR amplification and sequencing of hypervariable region of the protein A gene; the resultant sequence can be compared with those in an international database to determine the genetic similarity between *S. aureus* isolates. When applied to nine *S. aureus* isolates, associated with mastitis in six animals over a five-month period, only two *spa* types were demonstrated to be circulating among the BARC herd. Both types originated from isolates of *S. aureus* used in vaccine challenge studies; thus, *S. aureus* from sources outside the dairy were not responsible for causing mastitis in our animals.

E. coli is an important pathogen in dairy cows, as a cause of mastitis, and enteric disease. Clinical microbiologists have been relying on genotyping of *E. coli* as a useful method of categorizing isolates from different sites of infection (i.e., perianal region Vs urinary tract). We are now applying genotyping, via a triplex PCR assay, to *E. coli* from the feces, internal organs, and milk of dairy cows. We have found that genotypes A, B1, and B2 are all represented in mastitis, indicating that a genetically diverse assemblage of *E. coli* participates in this infection. Genotype D (which contains *E. coli* O157:H7) represents < 5% of more than 30 typed isolates, regardless if the source is feces, tissues, or milk.

We conclude that molecular biology-based methods, while more expensive and labor-intensive than traditional microbiologic assays, can offer new insights into the epidemiology and ecology of bacterial disease among dairy herds.

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AAVLD 49th Annual Conference
October 12 – 18, 2006
Minneapolis Hilton – Minneapolis, MN

Isolation of *Nicoletella semolina* from a horse imported from Germany

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The Animal Disease Diagnostic Laboratory isolated a bacterium from clitoral swab samples of a warm-blood horse imported to Ohio from Germany. The bacterium was fastidious in growth. The optimum growth condition was on chocolate agar in a 5% CO_2 atmosphere. After 24-48 hours of incubation, the colonies were 1 to 2 mm in diameter, gray, odorless, waxy, and non-adherent. The bacterium could be sub-cultured on TSA agar containing 5% sheep blood and did not produce haemolysis. The organism was gram-negative, non-motile, pleomorphic rods that were positive for catalase, oxidase, and urease tests. It was inactive in other biochemical reactions. DNA sequence analysis of the 16S rDNA indicated that the organism was *Nicoletella semolina*. This is the first time that *Nicoletella semolina* has been reported in the United States of America.

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Minneapolis Hilton – Minneapolis, MN

MIC Interpretive Criteria for Tulathromycin Bovine and Swine Respiratory Disease

CJ Lindeman, ES Portis, JL Watts

Tulathromycin is a new macrolide antimicrobial agent characterized by a 15 membered ring with three amine groups. Due to the tribasic nature of this compound, tulathromycin belongs to a new subclass of macrolides termed triamilides. Tulathromycin is currently approved in the US and EU for the treatment of bovine respiratory disease (BRD) associated with *Mannheimia haemolytica*, *Pasteurella multocida*, and *Histophilus somni* and for the control of BRD in cattle at high risk of developing respiratory infections with these pathogens. In swine, tulathromycin is indicated for the treatment of swine respiratory disease (SRD) associated with *Actinobacillus pleuropneumoniae*, *P. multocida*, *Bordetella bronchiseptica* and *Haemophilus parasuis*.

Tulathromycin demonstrates good *in vitro* activity against a wide range of Gram-positive and Gram-negative bacteria including the label indicated pathogens. The MIC_{90} value for tulathromycin against *M. haemolytica* (n=701) was 8.0 μg/mL. For *P. multocida* isolated from cattle (n=223), the MIC_{90} value was 2.0 μg/mL, and for P. multocida isolated from swine (n=68), the MIC_{90} value was 4.0 μg/mL. The MIC_{90} value of tulathromycin against *H. somni* (n=41) was 1.0 μg/mL. Against the other swine pathogens, *A. pleuropneumoniae* (n=138), *H. parasuis* (n=22), and *B. bronchiseptica* (n=42), the MIC_{90} values were 32.0, 4.0, and 8.0 μg/mL, respectively.

A single dose of tulathromycin at 2.5 mg/kg BW delivers a full course of therapy in both cattle (SC) and swine (IM). The drug is rapidly absorbed, extensively distributed, has an extended half life and high availability (cattle 91%, swine 88%) making it an extremely effective single dose agent. Tulathromycin readily penetrates lung tissues, achieving high lung concentrations approximately 73 times greater than plasma in cattle and 61 times greater than plasma in swine. Additionally, tulathromycin accumulates in neutrophils and macrophages, so that high levels of drug are directly delivered to bacterial pathogens. Using the pharmacodynamic parameter for azithromycin, a closely related compound, of AUC/MIC ≥25, tulathromycin lung concentrations meet or exceed this value for all labeled pathogens.

In multi-location field studies, 314 calves and 266 pigs with naturally occurring BRD and SRD received a 2.5 mg/kg BW, single dose of tulathromycin. Responses to treatment were compared to saline treated controls. Success was defined as animals with normal attitude, normal respiration, and a rectal temperature of ≤ 104°F on Day 14. The treatment success rate was significantly greater ($P \leq 0.05$) in tulathromycin-treated animals compared to control animals. In the calf study, 78% of the tulathromycin-treated calves were considered successfully treated and 24% of calves receiving the placebo showed resolution of clinical symptoms. In the pig study, 70.5% of the tulathromycin-treated pigs were considered successfully treated while 46.1% of the control pigs showed resolution. These studies have shown that tulathromycin administered as a single 2.5 mg/kg dose is effective in the treatment of BRD and SRD.

Taking into consideration the *in vitro* activity, clinical pharmacology, and the high efficacy for treating naturally occurring respiratory infections, this data supports a susceptible breakpoint of ≤16.0 μg/mL, intermediate breakpoint of 32 μg/mL, and a resistant breakpoint of ≥64.0 μg/mL.

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AAVLD 49th Annual Conference
October 12 – 18, 2006
Minneapolis Hilton – Minneapolis, MN

Tulathromycin *In Vitro* Susceptibility of Bovine and Swine Respiratory Disease Pathogens

ES Portis, CJ Lindeman, JL Watts

Tulathromycin is a novel triamilide antimicrobial that has been approved for use in the treatment and prevention of bovine respiratory disease (BRD) and the treatment of swine respiratory disease (SRD) in the European Union and the United States. Tulathromycin has shown high efficacy against the common bacterial causes of respiratory disease in cattle and swine. The minimal inhibitory concentrations (MICs) of tulathromycin against cattle and swine respiratory bacterial pathogens in the United States were evaluated using microbial isolates collected during clinical development studies. MICs were also determined for tulathromycin against cattle and swine respiratory bacterial pathogens received in 2004 as part of the Pfizer Animal Health BRD/SRD Susceptibility Monitoring Program.

Pfizer Animal Health (PAH) obtained the BRD and SRD isolates originally submitted to Dr. Don Bade (Microbial Research, Inc., Ft. Collins, CO; MRI) from PAH clinical studies conducted from 1999 to 2001. All MIC determinations were performed using the reference microdilution broth method (1). All MIC panels were prepared in-house, tested for quality control, and frozen prior to use.

The Pfizer Animal Health BRD/SRD Susceptibility Monitoring program requested BRD and SRD pathogens isolated between January 1, 2004 and December 31, 2004. Seventeen veterinary diagnostic laboratories (15 US, 1 Canadian, 1 Australian) were requested to send no more than one strain from a herd during each three-month period. Minimal inhibitory concentrations were determined using a commercial broth microdilution system (Trek Diagnostic Systems, Inc.) that conforms to the standards of the Clinical and Laboratory Standards Institute (CLSI) broth microdilution method [1].

Results from both of these studies yielded MIC_{90} values of 4.0 μg/mL for tulathromycin against the strains of *Mannheimia haemolytica* and bovine *Pasteurella multocida* and 8.0 μg/mL against *Histophilus somni.* Against porcine P. multocida, the MIC_{90} was 4.0 μg/mL for 1999-2001 isolates and 1.0 μg/mL against the 2004 isolates. Tulathromycin against *Streptococcus suis* isolates yielded an MIC_{90} value of >64 μg/mL for both sets of isolates. Tulathromycin is not indicated for *S. suis*.

The effect of CO_2 incubation on media pH and its detrimental effect on the *in vitro* activity of macrolides are well known. Based on the data generated, the activity of tulathromycin is also reduced under standardized test conditions when incubating *H. somni*, and *A. pleuropneumoniae* in a 5-7% CO_2 atmosphere. The *A. pleuropneumoniae* isolates from the 1999-2001 studies were incubated in a 5-7% CO_2 atmosphere, whereas the *A. pleuropneumoniae* isolates from the 2004 study were incubated in ambient air. The tulathromycin MIC_{90} value for the *A. pleuropneumoniae* incubated with the addition of CO_2 was 32 μg/mL and the MIC_{90} value for the *A. pleuropneumoniae* incubated without the addition of CO_2 was 8.0 μg/mL.

The proposed **susceptible** breakpoints for tulathromycin are ≤16.0 μg/mL for *M. haemolytica*, *P. multocida*, *H. somni*, and ≤ 32 μg/mL for *A. pleuropneumoniae.* Reviewing the *in vitro* antimicrobial susceptibility testing of BRD and SRD pathogens isolated from clinical studies from 1999-2001 and diagnostic cases in 2004, tulathromycin shows good *in vitro* activity against bovine *M. haemolytica*, *P. multocida*, *H. somni*, and porcine *P. multocida* and *A. pleuropneumoniae.*

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AAVLD 49th Annual Conference
October 12 – 18, 2006
Minneapolis Hilton – Minneapolis, MN

Hookworm Enteritis/Bactermia Complex As A Cause Of Mortality In California Sea Lion Pups.

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As a follow-up to previous studies conducted in 2002, samples were collected from sea lion pups during the summer of 2005 from 2 islands, San Nicolas and San Miguel, off the coast of California. The objective of this study was to evaluate the cause of mortality in California sea lion pups as a result of hookworm infections. Freshly dead sea lion pups (60) were necropsied, and tissues from 47 of those animals which had gross lesions were sampled for culture (101 samples total). Small intestines were submitted on all 47 animals. Other tissues submitted for culture included peritoneal cavity (23), brain (8), heart (8), lung (5), joint (4), lymph node (3), kidney (2), and abscess (1). Small intestine samples were cultured aerobically and anaerobically, all other tissue samples were cultured for aerobes only. Traditional culture methods were used to detect and identify all bacteria present in each sample. Of the 47 animals sampled, all had a moderate to severe hookworm infestation. The most prevalent pathogenic organisms recovered were *Salmonella* sp. group B (10 animals), group C_2 (9 animals), group C_1 (6 animals), *Clostridium perfringens* (14 animals), hemolytic *Escherichia coli* (37animals), *Vibrio* sp. (4 animals), and *Klebsiella terrigena* (16 animals). Interestingly, some of the pathogens (such as *Salmonella* sp.) were found in samples from both islands, while others were found in samples from a single island (such as *Klebsiella terrigena*). This sampling was part of a multi-year study and the findings support the hypothesis that a hookworm enteritis/bacteremia complex is causing mortality in sea lion pups off the California coast.

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AAVLD 49th Annual Conference
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Effects of pre-incubation holding time and temperature on interferon-γ responses in whole blood cultures from *Mycobacterium bovis*-infected cattle

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The Bovigam™ assay is approved for use within the US as a complimentary diagnostic test for tuberculosis. The in vitro assay detects interferon (IFN)-γ produced in whole blood cultures stimulated with specific antigen. Stimulants commonly used in the assay are purified protein derivatives derived from *Mycobacterium avium* (i.e., PPDa) and *M. bovis* (PPDb), *M. bovis*-specific proteins (e.g., rESAT-6:CFP10), and pokeweed mitogen (PWM, an indicator of functional capacity). Prior to whole blood culture and the ensuing ELISA to detect IFN-γ, blood samples are subjected to various holding times and temperatures due, in part, to practical constraints associated with the shipment of samples to diagnostic laboratories. To investigate effects of holding conditions on the response, 2.5 month-old Holstein calves received 10^3 cfu *M. bovis* via aerosol delivery into a mask covering the mouth and nasal passages. Two months after inoculation, blood was collected into heparinized tubes and held at 4°C or 22°C for 0, 8 or 24 hr prior to set-up for whole blood culture. Pre-incubation at 37°C for the initial 2 hr of each of the 8 and 24 hr holding treatments was also evaluated. Under each time and temperature condition, pre-incubation of the sample at 37°C for 2 hr diminished the response to stimulation with antigen and PWM. In general, responses to each of the stimulants were decreased in all samples held for 8 or 24 hr at each of the temperature variables as compared to immediate set-up; however, the response to rESAT-6:CFP10 held for 8 hr at 4°C did not differ from that of samples set-up immediately. Responses to PPDb, PPDa, and PWM did not differ when held at 8 hr as compared to 24 hr, regardless of the temperature. Responses to rESAT-6:CFP10 decreased when held for 24 hr as compared to 8 hr, regardless of the temperature. Pre-incubation of samples at 37°C for the initial 2 hr of holding resulted in decreased responses to all stimulants held at 4°C or 22°C for the remaining 6 hr (i.e., 8 hr hold) as compared to responses of samples held for an additional 22 hr (i.e., 24 hr hold). Conclusions include: (1) delays in set-up of blood cultures for stimulation negatively impact the IFN-γ response to mycobacterial antigens and mitogen, (2) pre-incubation of samples at 37°C for 2 hr diminishes the response, and (3) increased holding periods (i.e., 8 hr versus 24 hr) may interfere with the response to certain antigens, particularly when samples are warmed to 37°C for the initial 2 hr. Sample quality is particularly crucial for the Bovigam™ assay as this test relies on the maintenance of the functional capacity cells for the production of the readout parameter.

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Detection of *vspL* Gene of *Mycoplasma bovis* Field Strains Using a PCR Assay

S. Heller and B. C. Lin

Mycoplasma bovis is an important bovine pathogen that can evade the host immune system using an extensive antigenic variation of the highly immunogenic surface lipoprotein antigens (Vsps). A family of 13 *vsp* genes was recently found in the chromosome of *M. bovis*. Among them a lympho-inhibitory peptide (Mb-LIP) encoded by the *vspL* gene of *M. bovis* was found to be able to inhibit naïve lymphocyte activation during the respiratory infection. In this study, a PCR assay was developed to detect the genes that encode VspL, VspA, VspO, and VspC from some 70 *M. bovis* strains. The VspL protein could also be detected by western blotting with VspL-specific antiserum. Test results by PCR assays indicated that the most common *vsp* genes among 70 *M. bovis* field strains isolated in the U.S. were *vspA* and *vspL*. The less common gene was *vspC* gene.

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October 12 – 18, 2006
Minneapolis Hilton – Minneapolis, MN

Identification of *Mycoplasma bovis* from Bovine Clinical Cases Using Polymerase Chain Reaction and RFLP Assays in Alabama

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Mycoplasma bovis is an important veterinary pathogen causing pneumonia, arthritis and mastitis in cattle production. Prevalence of *M. bovis* appears to be increasing throughout the world. Principally, on large dairy farms *M. bovis* can cause mastitis, on smaller farms the typical diseases are calf pneumonia and arthritis. Since 1998, 1,488 bovine tissue samples were collected and tested for *M. bovis* by PCR and RFLP methods in the Alabama State Diagnostic Laboratory. Two PCR tests were used for the identification of *M. bovis*; one PCR procedure was specific for *M. bovis* species (primers from *M. bovis* 16SrRNA); another PCR/RFLP procedure was for general *Mycoplasma* detection (primers from the 16SrRNA-end part and the 23SrRNA-beginning part). The specimens consisted of 755 lungs, 546 milk samples, 126 nasal swabs, and two joint fluids. Two-hundred-eighty (18.81%) were positive for *M. bovis*. Positive samples included 200 lung tissue (71.4%), 21 nasal swabs (7.5%) 36 milk samples (12.8%), and one joint fluid. The positive cases involved 83 female and 43 male cattle. The molecular characterization and comparison of 16SrRNA of field *M. bovis* species will be presented.

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AAVLD 49th Annual Conference
October 12 – 18, 2006
Minneapolis Hilton – Minneapolis, MN

High Throughput Nucleic Acid Isolation and Quantitative Real Time PCR Assay for Detection of Johne's Disease in Cattle

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Mycobacterium avium sbsp*. paratuberculosis* (MAP) is known to be the causative agent of Johne's disease (chronic granulomatous enteritis of the small intestine) in cattle. This incurable infection typically occurs in the first year of life; however, the clinical symptoms (persistent, non-responsive diarrhea) may not appear for two years or more. Johne's disease causes severe economic losses worldwide due to reduced productivity, increased reproductive losses, and the eventual death or culling of infected animals.

The most common method to confirm Johne's disease is direct culture of MAP from feces, which can require 8 to 16 weeks. Recently, more rapid detection of MAP from fecal samples has been made possible by the use of PCR; however, current PCR assays have several weaknesses.
For example, fecal samples that are positive by culture methods and even samples with high numbers of bacteria are sometimes falsely identified as negative by PCR. PCR-based tests using fecal samples are often hampered by the presence of inhibitors such as phytic acid and other complex polysaccharides carried over during nucleic acid isolation. In addition to false negative results, PCR assays have also been criticized for targeting the *M. avium* subsp. *paratuberculosis* IS*900* element, a sequence found to share homology with other Mycobacterium species. These factors stress the importance of the development of improved nucleic acid isolation methods that remove PCR inhibitors and qPCR assays with internal controls that assess PCR efficiency and monitor PCR inhibition

For nucleic acid isolation, we developed a rapid high throughput isolation method based on Ambion's MagMAX™ technology. We use magnetic, nucleic acid binding beads to efficiently and effectively isolate nucleic acids (>50% recovery in model experiment) from various biological sources, such as blood and feces. By eliminating the culture step, pathogen detection time is reduced to 1 day. Also, the use of dispersible magnetic beads allows more effective washing compared to stationary glass-fiber filters; therefore, PCR inhibitors are more efficiently removed. Our method is performed in an automatable, 96-well format which allows rapid screening of a relatively high number of samples.

For the subsequent detection of purified MAP DNA, we developed a highly sensitive qPCR assay targeting ISMap*02*. The ISMap*02* genetic element (1,674 bp) is MAP-specific and is repeated 6 times in the MAP genome. Our qPCR assay includes a positive control (XenoDNA-01) to monitor qPCR efficiency and the presence of PCR inhibitors. This internal control decreases false negatives and provides highly reliable and confirmatory qPCR results. The analytical sensitivity of this assay is <10 copies of exogenous ISMap*02*.

By pairing of our robust nucleic acid isolation method with our highly sensitive and reliable qPCR assay, we were able to correctly identify MAP in fecal samples from naturally infected animals. These results demonstrate a highly effective, integrated process for rapid MAP detection by qPCR.

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AAVLD 49th Annual Conference
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Development and evaluation of a test for tuberculosis in live European badgers (*Meles meles*) based on measurement of IFNγ mRNA by real-time PCR

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A real-time PCR assay to measure IFNγ mRNA in European badger (*Meles meles*) blood cultures was developed. Levels of IFNγ mRNA in blood cultures stimulated with either bovine or avian tuberculin, or specific mycobacterial antigens, were compared relative to a non-stimulated control blood culture, as the basis of determining the tuberculosis (TB) status of live badgers. The assay was validated by testing 244 animals for which there were matching TB culture data. Relative changes in levels of IFNγ mRNA in response to bovine tuberculin and specific antigens were found to be greater among badgers with positive TB cultures. The test was at its most accurate (87% of test results correct) using cultures containing bovine tuberculin and when the response to avian tuberculin was taken into account. At a specificity of 90.7%, the test was 70.6% sensitive. At the same specificity, the current serological ELISA test for TB in badgers was only 53% sensitive. This work demonstrates that measurement of IFNγ mRNA by real-time PCR is a valid method for measurement of TB in live badgers and may provide an alternative to the current serological methods of diagnosis, the Brock Test. The testing procedure can be completed within 5 hours of receiving the blood culture samples. In addition, the use of a molecular based test offers the potential to fully automate the testing procedure through the use of robotics. To our knowledge, this is the first report utilising measurement of mRNA in cell-mediated assays for diagnostic purposes.

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Development of a Real-Time PCR for the specific detection of *Leptospira borgpetersenii* serovar hardjo

K.M. Harmon[1]

A real-time PCR test has been developed for the specific detection of *Leptospira borgpetersenii* serovar *hardjo* (type hardjo-bovis). The test is a modification of the procedure published by Woodward et al, 1991. Specificity of the real-time assay was determined by performing the PCR test on 30 different species/serovars of *Leptospira*, and 20 non-leptospiral bacteria and viruses. Initially, in addition to the hardjo-bovis strains, one other strain *(Leptospira borgpetersenii* serovar tarassovi) yielded a positive result in the assay. Increasing the stringency of the assay resulted in detection of only the hardjo-bovis strains. This test was subsequently used to evaluate specimens from infected animals from a challenge model study. The assay detected *Leptospira hardjo-bovis* in urine of animals (n=29) that were infected with the organism. Thirty days after infection animals were treated with one of two antimicrobial agents. A control group received saline instead of the antimicrobial treatment. Urine and kidney samples were collected 28 days after treatment, and tested with the PCR assay. *Leptospira hardjo-bovis* was detected by PCR in the urine specimens from all of the untreated (saline) animals (n=10) while urine from all the treated animals (n=19) was negative for the organism. The organism was not detected by PCR in the kidney samples from any of the animals which received either of the antimicrobial treatments. Kidneys from 5 of the 10 control animals which did not receive antimicrobial treatment tested positive by PCR. Culture results from the urine and kidney samples are pending. Additional studies are needed to determine the most appropriate samples for testing, as well as the optimal method for processing these samples for PCR. This assay should be useful in determining prevalence of this organism and its significance in disease status of cattle.

This project was funded by Pfizer Animal Health.

Reference:
Woodward, M. J., G.J. Sullivan, N.M.A. Palmer, J.C. Woolley, and J.S. Redstone. 1991. Development of a PCR test specific for Leptospira hardjo genotype bovis. Veterinary Record 128: 282-283.

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Evaluation of the HOOF-Print assay for genotyping *Brucella suis* strains isolated from the United States

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Epidemiological investigations of disease outbreaks often utilize information provided by molecular genetics to help determine the spread and possibly the source of infection in an outbreak. In this respect, genetic markers that are highly discriminatory can help in clarifying the genetic relationships among strains. Recently, a new method for genotyping *Brucella abortus* strains called HOOF-Print analysis was described. It is based on allelic variation at ten different tandem repeat loci within the *Brucella* genome. Because these loci are present in all *Brucella* species, this study was undertaken to determine if this methodology is appropriate for use in the analysis of *Brucella suis* outbreaks within the United States. For this, a total of 64 different field isolates of *B. suis* from outbreaks in domestic and feral swine (47 isolates total; 42 - *B. suis* bv 1, 5 - *B. suis* bv 3), as well as individual strains obtained from infected cattle (11 - *B. suis* bv 1), caribou (4 - *B. suis* bv 4), human (1 - *B. suis* bv 3) and horse (1 - *B. suis* bv 1) were analyzed. In addition, four laboratory reference strains (1 - *B. suis* bv 2; 2 - *B. suis* bv 3; 1 - *B. suis* bv 4) and two atypical isolates most closely resembling *B. suis* bv 3 from swine were included.

As expected, isolates obtained from specific outbreaks were consistently similar, varying from each other by only a single repeat unit at no more than three to four loci. In contrast, unrelated bacterial strains demonstrated allelic differences at five or more separate loci. Although there was no apparent genetic relationship between *B. suis* strains from cattle and swine that would suggest transfer from one species to the other, the lack of concurrent epidemiological data regarding source and geographical relationships among isolates in this study prevents definitive conclusions. Analysis of individual tandem repeat loci showed that Locus 2 was the least diverse, having only four different alleles present among all 63 diagnostic isolates. Furthermore, all 46 *B. suis* bv 1 strains in this study exhibited the same allele of two octal repeat units at this locus. Locus 4 was also very homogenous for *B. suis* bv 1 isolates, displaying only two different alleles. In contrast, Locus 9 and 10 demonstrated the highest diversity among all *B. suis* isolates, with twelve different alleles apiece. Furthermore, Locus 9 consistently displayed individual octal repeats of 10 or more in all biovars of *B. suis* analyzed. This, combined with the high diversity seen at this locus, suggests that it has a considerably higher mutation rate than the other loci, and thus may not contribute significant data for outbreak investigations. Overall, the results from this study indicate that HOOF-Print analysis can be applied to individual outbreaks of *B. suis* within the United States, and can provide useful data for concurrent epidemiological investigations.

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AAVLD 49th Annual Conference
October 12 – 18, 2006
Minneapolis Hilton – Minneapolis, MN

Salmonella enterica serovars: Differentiation by oligonucleotide spotted array

Yung-Fu Chang*, Raghavan U.M. Palaniappan, Ramanujam Subbupoongothai, David Chiu[1], Patrick McDonough

Salmonella is one of the most important zoonotic pathogens of major concern to public health. Genomes of *Salmonella* serovars are closely related impeding their efficient differentiation. We have developed an oligonucleotide spotted array targeting core genes of *Salmonella enterica*, subspecies I specific genes, fimbrial genes, genes from pathogenic islands, genes of phages Gifsy-1 and 2 and other variable genes which enable the differentiation of *Salmonella* serovars. Twelve reference strains of *Salmonella* from the *Salmonella* reference B (SARB) collection were used primarily to differentiate the serovars, and results were subsequently validated with 35 clinical isolates of *Salmonella*. Overall, the oligonuleotide array with 538 gene probes of *Salmonella* successfully differentiated serovars and even strains within the serovars of *Salmonella*.

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October 12 – 18, 2006
Minneapolis Hilton – Minneapolis, MN

Using a molecular approach to detect *Tritrichomonas foetus* in cattle

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Tritrichomonas foetus is a sexually transmitted protozoan parasite that causes abortion in cattle. It is asymptomatic in bulls and once infected, they become carriers. Due to reduced fertility and low calving rates, trichomoniasis causes significant financial loss. Recent state regulations require testing of bulls before being shipped or sold in NM. This has generated a need for a sensitive, rapid, and high throughput method of testing for *T. foetus* in NM. Microscopic examination of culture is time consuming, does not detect dead organisms that may be present if the sample was not transported appropriately, and does not differentiate between Trichomonad species. Here we report on the validation results of a PCR method that used published primer sequences specific to *T. foetus*. This assay was used for high throughput testing of all samples, not just culture positive samples. The initial assay was a conventional PCR, but the method was enhanced for higher throughput by transferring samples to a 96-well format for the extraction step and all downstream applications. New data will also be presented on a real-time PCR assay currently being validated. The probe was developed in-house and the assay includes an internal positive control to identify those samples that have significant inhibition to ensure that false negative results do not get reported.

To validate the conventional PCR assay, 266 split preputial samples were tested at NMDA-VDS and CSU using CSU as the gold standard. The results showed an accuracy rate of 99.26%.The analytical sensitivity of the assay was determined to be 12.5 cells per PCR reaction. The majority of the time, this assay can detect 1.25 cells per reaction. Some samples were also split between NMDA-VDS, CSU, and the NMDOH state lab so that they could be examined microscopically. In conclusion, 1) culture misses a significant number of positive samples, 2) laboratories using PCR only to confirm or speciate positive cultures may be missing positive samples, 3) the NMDA-VDS method is sensitive, rapid, and allows for high throughput.

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AAVLD 49th Annual Conference
October 12 – 18, 2006
Minneapolis Hilton – Minneapolis, MN

Development and validation of an ovine progressive pneumonia virus (OPPV) quantitative PCR

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Ovine progressive pneumonia virus infects at least one sheep in eight-one percent of U.S. sheep flocks and causes poor doer sheep and sheep with mastitis, arthritis, dypsnea, and cachexia. Diagnostic tests that quantify virus load are necessary to identify potential super spreader OPPV sheep. In this study, we developed primers and a Taqman™ probe to the highly conserved transmembrane region of the OPPV envelope gene and established a real-time quantitative (q) PCR assay. This new OPPV qPCR assay was validated against the caprine arthritis-encephalitis virus (CAEV) competitive inhibition enzyme-linked immunosorbent assay (cELISA) using 389 peripheral blood leukocyte samples and sera from 389 Dubois, Idaho sheep ranging in ages 3 to 6 years of the Columbia, Rambouillet and Polypay breeds. The new OPPV qPCR had a sensitivity of 96.1±2.3% and a specificity of 97.7±3.9% with a kappa value of 0.94. In addition, there was a significant correlation (Pearson two-tailed) between the mean copies of *envelope* and percent inhibition values using CAEV cELISA (P value=0.0003, R^2=0.033). Seroprevalence using the CAEV cELISA and mean copies of envelope using the OPPV qPCR positively correlated as sheep age increased. In addition, the Columbia breed maintained consistently higher envelope loads (>100 copies/ug DNA) in every age category than the Rambouillet or Polypay breeds. These results indicate that the OPPV qPCR may be used as another diagnostic tool for OPPV infection as well as identifying potential super spreader OPPV sheep.

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AAVLD 49th Annual Conference
October 12 – 18, 2006
Minneapolis Hilton – Minneapolis, MN

High Throughput Processing of FTA® Samples for Pathogen Detection by qRT-PCR

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Whatman FTA technology is a novel method designed to simplify the collection, shipment, archiving and purification of nucleic acids from a wide variety of biological sources.
Current methods for extracting nucleic acids from FTA samples are labor intensive, time consuming and not amendable for high throughput applications. To simplify the extraction process, we have developed a highly effective RNA Rapid Extraction Solution for RNA extraction from FTA Cards. This Solution is coupled with Ambion's MagMAX™ magnetic beads based RNA purification technology to provide a streamline process for a simple and rapid method for RNA isolation from FTA Cards. The purified RNA is of high quality and suitable for quantitative RT-PCR or other downstream RNA applications. This process enables high throughput RNA extraction and purification in a 96 well format manually or by automation and facilitates pathogen detection.

A direct comparison of our Extraction Solution with current FTA extraction methods demonstrates that our approach provides better RNA extraction efficiency resulting in better pathogen detection sensitivity. Our process is also less cumbersome and time-consuming. Only 5 minutes is required for the extraction process; this step is followed by a 30 minute RNA purification procedure. We have validated this process using diverse biological sample types including blood, tissue imprints, and cultured cells and bacterial cultures.
Validation studies were performed using blood, tissue imprints and culture cells for the detection of ALV (Avian Leukosis Virus), BVDV (Bovine Viral Diarrhea Virus), WNV (West Nile Virus), AIV (Avian Influenzy Virus), *Mycobacterium smegmatis*, and *Mycobacterium murale*. Our results demonstrate concordance with secondary ELISA and RT-PCR data.

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AAVLD 49th Annual Conference
October 12 – 18, 2006
Minneapolis Hilton – Minneapolis, MN

High-Throughput System for Diagnostic Surge Capacity

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We have developed an integrated high throughput laboratory system that could be used to provide diagnostic laboratory support during a foreign animal disease outbreak response. This semi-automated system is comprised of two robotic workstations that together can process a minimum of 1000 individual clinical samples in a 10 hour shift with two laboratory technicians. Automation encompasses the transfer of liquid samples from collection vials to a 96-well plate, addition of an internal control, magnetic bead-based nucleic acid purification, multiplexed reverse transcriptase polymerase chain reaction (RT-PCR) amplification, microsphere array hybridization, Bio-Plex detection and data analysis.

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Minneapolis Hilton – Minneapolis, MN

Initial Results Using a Real Time RT-PCR Assay for Canine Distemper Virus

K. S. Bowles, R. P. Poston and A. F. Roy

Canine Distemper Virus (CDV) is a highly infectious disease of Canids, Procyons and other carnivores. CDV is a single stranded enveloped RNA virus with a helical capsid from the Morbilivirus group in the family Paromyxoviridae. Clinical onset often involves the respiratory, intestinal and central nervous systems and has a case fatality rate in unvaccinated animals second only to rabies virus. Recently, there has been an increase in the reporting of CDV cases, often in dogs with a limited or incomplete vaccination history. Because the initial clinical picture in dogs resembles a great variety of other infections fast clinical diagnosis is critical in providing proper supportive care and segregation of infected animals. Serolologic diagnosis has limited usefulness given the vaccination rate in the population and the ability of CDV to cause immunosuppression. Virus isolation can take days and field isolates are often difficult to culture. Molecular diagnostic assays can be fast and sensitive. While numerous molecular diagnostic assays have been published for CDV, to date most involve nested-PCR protocols, or other gel based assays. Due to the labor involved in gel-based PCR assays we were interested in developing a real-time based CDV assay.

Primer and probe design was performed using Primer Express software using sequences aligned from three vaccine strains and six wild type CDV strains (Applied Biosystems, Foster City, CA). The P gene was chosen for primer and probe design as this is reported to be the most highly conserved of the CDV sequences. Specificity testing was done using a plasmid containing the P gene of phocine distemper virus, and against a vaccine strain of Measles Virus (Attenuvax, Merck).

Preliminary testing was performed using a cryolysate from an Onderstepoort based vaccine strain (Galaxy-D, Schering-Plough Animal Health, Kenilworth, NJ). Vaccine was reconstituted as per manufacturer's instructions and extracted using a commercial RNA extraction kit (RNeasy; Qiagen, Valencia, CA). Based on initial virus titer in the vaccine, we were able to reliably detect as little as 0.0125 $TCID_{50}$ equivalent per PCR reaction. Test of this PCR on archival samples has demonstrated a sensitivity near 70% as compared to fluorescent antibody exam. While this RT-PCR is unable to distinguish between vaccine strains and field isolates it is known that vaccine strains can be pathologic in species other than dog, and in rare instances vaccine induced pathology has been suggested in dogs. Caution must be used in interpreting results from this assay as a recent report suggests that vaccine shedding in healthy dogs is detectable by RT-PCR at times up to 3 weeks post vaccination.

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AAVLD 49th Annual Conference
October 12 – 18, 2006
Minneapolis Hilton – Minneapolis, MN

Amblyomma variegatum in the Caribbean: Current status in selected islands

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The original model that was adopted for the eradication of the tropical Bont tick (TBT) in the Caribbean was technically faulty as it was based on 24 months of treatment rather than 48 months. There were several factors that contributed to lack of overall successes including the following:

1. Livestock management practices, particularly lack of enclosures, were not conducive to pest eradication.
2. The program was inconsistently and inadequately funded.
3. Compliance with treatment schedules and the legislation were not enforced.
4. Management procedures were inconsistent and too complex with ten Government Administrations and six International Agencies involved.
5. National staff and farmers suffered from fatigue after about five to six years.

St Kitts showed excellent progress during the first five years (1995 to 2000) but, thereafter, for various reasons the status declined markedly with prevalence rates of TBT infestation increasing from less than 1% in 2001 to between 10 and 20% in 2005 – 2006, and the number of infested areas from one to five. Similarly, prevalence rates of TBT infestation in St Lucia have also increased since 2003 through 2006. Nevis took 10 years to reduce the prevalence to less than 1%. Marie Galante (French West Indies) has the highest prevalence of TBT infestation in the region with 74% of herds infested. This island is believed to be a source of persistent re-infestation in Dominica.

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AAVLD 49th Annual Conference
October 12 – 18, 2006
Minneapolis Hilton – Minneapolis, MN

Bovine Viral Diarrhea Virus and *Mycoplasma* Coinfection in Cattle with Respiratory Disease in Alabama

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Bovine respiratory diseases (BRD) are historically the most significant cause of mortality of feedlot calves. According to Grooms and Coe (Cattle Call, http://beef.ans.msu.edu) the most common bacterial pathogen from feedlot cattle with BRD in Michigan was *Mycoplasma bovis,* and the most common virus isolated was bovine viral diarrhea virus (BVDV). Cattle in Alabama are typically in pasture environments. From 2001 to 2005, 433 bovine samples (3 respiratory swabs, 21 buffy coats, 47 lungs, and 362 lung and spleen pooled) were collected and tested for BVDV and bovine mycoplasmas by PCR at the Alabama State Diagnostic Laboratories. Twenty-four samples were from live animals, the rest were from dead calves. One hundred twenty four samples (28.9%) were BVDV positive; 151 (35.29%) samples were mycoplasma positive. In mycoplasma-positive cases, mycoplasma species included 100 (66%) *M. bovis,* 25 (16%)*M. bovirhinis* , 17 (11) *M. dispar,* 7 (4.6%) *M. arginini (%)*, and 2 (1%) *M. alkalescence*. Out of 124 BVDV positive samples, 67 (54%) BVDV positive samples had mycoplasma coinfection. Out of these 67 BVDV positive samples, there were 55 (41%) positive for *M. bovis;* ten (8%) for *M. bovirhinis,* and six (4.84%) for *M. dispar*. Other mycoplasma positive samples did not show BVDV co-infection by PCR tests. More than 90% of these coinfected animals had pneumonia. These data show that BVDV and *M. bovis* coinfection are common in cattle with pneumonia in Alabama. Most of these cattle were from Alabama farms and were on pasture and not in a feedlot situation. *Mycoplasma bovis* and BVDV coinfection appears to be common in cattle in feedlot and pasture environments.

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AAVLD 49th Annual Conference
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Minneapolis Hilton – Minneapolis, MN

Bovine Viral Diarrhea Virus Persistently Infected and Acutely Infected Calves: Assays for Viral Infectivity, Polymerase Chain Reaction Analysis, and Antigen Detection

Robert W. Fulton[1*], Nathan Elam[2], Bill E.Hessman[3], Bill J. Johnson,[1,5] Sanjay Kapil,[1,5] Julia Ridpath [4], Lurinda J. Burge,[1] Barbara Braziel,[1] Kira Kautz[1], Amy Reck[1]

There are numerous assays for bovine viral diarrhea virus (BVDV) detecting infectious virus, nucleic material, and antigen. Persistently infected (PI) and acutely/transiently infected calves with BVDV represent two different manifestations. Diagnostic test results impact on differentiation of PI or acutely infected cattle, particularly relating to management of the two forms.

The objectives of the initial study included: (1) assay for infectious virus and polymerase chain reaction (RT-PCR) in serums and nasal swab materials of acutely infected and PI calves. The second study's objectives used PI calves held over several months. (1) determine if PI calves remain positive over time by the antigen capture ELISA (ACE) test on notches in PBS and immunohistochemistry (IHC) on fixed notches; (2) quantitate virus in nasal swab materials and serums of PI calves; and (3) assay for BVDV using RT-PCR in nasal swab materials and fresh notches. Samples included in these studies were from acutely infected and PI calves. Cell culture assays for BVDV used two methods (1) qualitative cell culture assay (QCCA) based on positive antigen in bovine monolayer MDBK cultures; and (2) quantitative assay using viral titration (VT) in 96-well plates using staining for BVDV in MDBK cells. The ACE test and IHC were used on notches, and the RT-PCR on serums, ear notches, and nasal swab materials.The detection limit on dilution factors for the VT were: $10^{1.6}$ per ml for serum and $10^{2.9}$ per ml for nasal swabs. Nasal swab materials were obtained by placing commercial viral culturettes into 2 ml cell culture media. Acutely infected calves with positive QCCA from nasal swabs were either negative by the VT ($<10^{2.9}$) or undiluted samples were toxic under cell culture conditions used in VT. The VT for three PI calves' nasal swab materials had titers of $10^{3.9}$ to $10^{4.4}$ per ml. There were 14 acutely infected calves positive by the QCCA in PBL (7 with positive QCCA in serum), and 13/14 were negative by the VT ($<10^{1.6}$). Only one calf had measurable titer in the serum, $10^{1.6}$ per ml. The PI calves' serums had titers of $10^{3.6}$ to $10^{3.8}$ per ml. However, 6 acutely infected calves negative by the QCCA in nasal swabs were positive by RT-PCR in those same nasal swabs. There were 3 PI calves with negative PCR in ear notches.In the second study there were two groups of PI calves. (1) Trial 1. 18 PI calves ,sampled over a 215 day period. One calf was added on day 73. Of the 19 calves 16 were infected with BVDV1b and 3 with BVDV1a. During the study 3 calves died; and (2)Trial 2. 12 calves sampled over a 155-day period.In this second study, all samples collected monthly were ACE and IHC positive. There were instances where there was a weak positive staining on IHC. All 31 calves had infectious virus quantitated by the VT in the serum ranging from $10^{1.6}$ to $10^{5.6}$ per ml.The VT for the nasal swab fluids ranged from $10^{2.9}$ to $10^{7.85}$ per ml. There were instances where the nasal swab materials were toxic to VT cultures. Nasal swabs were PCR positive in the nasal swab fluids in most cases; however there were calves with VT positive titers that were negative by the RT-PCR. Some fluids with VT positive PCR negative fluids inhibited the RT PCR of VT positive samples.

In summary, serums and nasal swabs of BVDV acutely infected calves had no detectable or greatly reduced viral infectivity compared to PI calves. Also, positive BVDV PI calves remained ACE and IHC positive on ear notches in all cases. Also, some ear notches and nasal swab fluids were inhibitory in the RT –PCR test.

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AAVLD 49th Annual Conference
October 12 – 18, 2006
Minneapolis Hilton – Minneapolis, MN

Evaluating stability, size requirements, viral load and pooling of ear notch samples in BVDV testing

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Infections with bovine viral diarrhea viruses (BVDV) are a major source of economic loss for the U.S. cattle industry. BVDV control efforts in the U.S. are geared towards identifying and eliminating persistently infected (PI) animals. Several tests based on detection of either antigen or viral RNA in blood, serum, bulk milk or skin biopsies are currently in use for detecting PI's.

While ear notches have become one of the samples of choice, there is little information available regarding sample size requirements and stability. Further, while pooling of ear notch samples has been proposed for reducing the cost of surveillance programs, the viral load available for detection from ear notch samples is largely undetermined. The purpose of this study was to establish working parameters for sample size, viral detection limit and sample storage conditions for real time PCR and antigen capture ELISA and to compare reproducibility of tests between three laboratories based on a blinded panel of pooled and unpooled samples.

For these experiments, notch samples were extracted by soaking for a minimum of 60 min in 2 mls PBS. There was no difference in the amount of virus detected in ear notch extractions using ear notch samples weighing between 0.75 and 0.05 gms. However, extracts generated using ear notches weighing 0.03 gms or less containing at least a log lower virus concentration. There were no significant differences between storage at -20, 4 and 25 C for 7 days. In contrast, detection was reduced in lyophilized samples and samples stored at 37 C for 7 days.

The concentration of virus in standard size ear notch (avg. weight 0.75 gms) extractions averaged 452.3 virions/ml. Of the 153 samples evaluated for virus concentration, 16 (10.5%) had between 10 and 100 virions/ml, 86 (56.2%) had between 100 and 1000 virions/ml, 50 (32.6%) had between 1000 and 10,000 virions/ml and 1 (0.7%) had more than 10,000 virions per ml. The detection limit of the real time PCR test was determined to be 10 virions/ml.

The most consistent results between laboratories was observed using the ACE tests on non pooled tissues followed by ACE tests on samples pooled 1 to 5 and ACE tests on samples pooled 1:10. The least consistency was observed with PCR based tests based on sample pools of 1:100 or greater.

These results suggest that ear notch samples are relatively stable for at least 7 days if stored between -20 and 25 C. However, both exposure to higher temperature and drying reduced detection. Similarly the amount of virus extracted was not significantly affected by sample size over a wide weight range.

The concentration range of virus in ear notch extractions and the detection limits of real time PCR suggest that pooling of samples in surveillance programs must be approached cautiously. Pooling of 10 samples, where a sample pool includes 1 positive and 9 negative samples, could result in the failure to detect 10% of the samples used in this study. Pooling of 100 samples, where the sample pool includes 1 positive and 99 negative samples, could result in failure to detect over 50% of the samples used in this study. The comparison of the blind panel results were consistent with these assumptions.

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Pooled PCR on Skin Extract as a strategy for BVDV screening

Steffen DJ, Brodersen BW, Galeota JA, Smith, DR, Rupp GA, Kelling CL

Introduction: Reverse transcriptase polymerase chain reactions RT PCR) technology has been proposed as useful using pooled PBS extracts of skin as a substrate. The supernatant can then be used for ELISA and skin for IHC to determine the test status of each individual. We tested this approach under field conditions.

Methods: Fresh field-origin ear notches were extracted with PBS for 10 minutes and briefly vortexed. Pools of the PBS extract were made by combining 100 ul from each of 100 consecutive samples in order of receipt. RNA was extracted with Trizol. Nested RT-PCR was performed as described by Ridpath. A universal set of primers was used for the first round of amplification and two sets of nested primers were used to specifically amplify type 1 BVD and type 2 BVD. The test status was determined by commercial ELISA and IHC by indirect procedure. IHC was performed room temperature. TBS (pH 7.6) and Tween 20 used as wash buffer. Pretreatment was in 20% acetic acid (4C). Antigen retrieval with 0.05% protease XIV, followed by Powerblock (BioGenex). Incubation (TBS, pH 7.6) primary antibody (15C5, Syracuse Bioanalytical, Inc.). Biotinylated secondary antibody (LSAB 2 kit Dako Corporation) is applied. Alkaline phosphatase-conjugated streptavidin (Dako, LSAB 2 kit) is used followed by BioRed - Fast red chromagen (Biopath Laboratories, Inc.). Test outcomes from individual samples by ELISA and IHC were analyzed for agreement. Ten-fold serial dilutions of individual positive ear notch extracts were used for RT nested PCR analysis to estimate RNA load in positive samples. Fifty-one pools were analyzed. True-positive pools were defined as having at least one sample within the pool positive by at two of three test methods (ELISA, IHC or PCR).

Results: 5,100 individual samples were analyzed. Twenty-three individual samples were positive by IHC and 19 were positive by ELISA. All ELISA-positives were IHC-positive. Agreement of the two tests beyond chance was high (Kappa=0.90). The four samples with discordant results (classified IHC-positive/ELISA-negatives) were PCR-positive. Thirteen pools were true-positive as defined. Ten pools were positive by PCR analysis of pooled supernatant. Twelve pools were positive by individual sample IHC and nine by individual ELISA. Sensitivity of PCR to detect BVDV RNA in the supernatant pools was determined to be 0.769 (10/13) and specificity was 1.00 (38/38). Two PCR negative pools which contained positive IHC and ELISA samples were sent to 3 independent labs for PCR and confirmed negative. False negative pools were tested for inhibitors by spiking with serial dilutions of cell culture virus, ranging from 10^5 to 2 viral copies. Inhibition was found in 1 sample. In the second pool the individual sample was PCR negative and no inhibition detected. In the third case the individual sample was PCR positive with no inhibition.

Discussion: Agreement between IHC and ELISA on individual samples was high although ELISA failed to identify a few BVDV-infected individuals. It is important for herd testing strategies that the presence of BVDV-infected cattle be detected. The failure to identify the presence of BVDV within pools by ELISA, IHC or PCR appears to be sample dependent.

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Minneapolis Hilton – Minneapolis, MN

Development of an Avian Influenza Antibody ELISA with Multi-Species Detection Capability

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Avian influenza is an important zoonotic disease with serious economic consequences. Available serological diagnostic tests include hemagglutination inhibition, agar gel precipitation and ELISA. ELISA tests have the advantage of high throughput and ease of use. However, the available commercial ELISA test kits are only validated and approved for use with chicken and sometimes turkey serum samples. In the present study, IDEXX Laboratories has developed an avian influenza antibody ELISA which would have broad application for detection of avian influenza exposure in multiple species. Performance characteristics evaluated include specificity, reproducibility, analytical sensitivity and diagnostic sensitivity in chickens, turkeys and ducks. The test detected antibody in chicken sera following exposure to different avian influenza subtypes and, in temporal series, was able to detect seroconversion between day 8 and 13 post experimental exposure. The specificity of the test on normal chicken (broiler breeders), turkey and duck populations tested to date is >99.9%. Performance of the test indicates it will be an important tool for monitoring avian influenza serologic status in different species.

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AAVLD 49th Annual Conference
October 12 – 18, 2006
Minneapolis Hilton – Minneapolis, MN

Evaluation of serological assays for the diagnosis of Bovine Herpesvirus 1

J.A. Godhardt[1], J.A. Zoromski[1], K.L. Toohey-Kurth[1,2]

Bovine herpesvirus 1 (BHV-1) can infect the respiratory and genital tract of cattle. Although high mortality rates are not an issue with BHV-1, the virus can cause serious economic loss. BHV-1 is of consequence to commercial exporters of bovine semen because infected semen can lead to reproductive problems such as abortion. The virus is endemic in the U.S. but has been eradicated in many countries in the European Union (EU). Thus export of semen to the EU requires accurate assessment of the BHV-1 status of a bull which is usually accomplished by measuring antibody level to the virus. The gold standard test approved by the Office of International Epizootics (OIE) is a serum neutralization (SN) using an overnight incubation of test sera with virus. The competitive ELISA is also an approved test for international trade and results are more quickly obtained than with an SN test. In the U.S. there is one commercial provider for a BHV-1 ELISA. However, this assay had a sensitivity and specificity which was unacceptable. To improve upon the sensitivity and specificity we developed our own competitive ELISA.

We first evaluated four monoclonal antibodies available through VMRD (Pullman, WA) and found the G2 antibody against glycoprotein 82 to be the most reliable in a competitive ELISA format. Results of our competitive ELISA were compared to the OIE approved serum neutralization assay and all positive samples were further confirmed at another institution using a kinetic ELISA. We tested 117 well characterized positives and 198 well characterized negative serum samples. The competitive ELISA showed 100% sensitivity and 99% specificity compared to the OIE SN. These results indicate that it is a reliable test for detection of BHV-1 antibody.

We also evaluated the effect of a 1 hour incubation period of virus with test sera in the serum neutralization assay. As expected, the hour incubation led to a lower sensitivity at 89% and a specificity of 98%. Although the shorter incubation period is commonly used for BHV-1 serum neutralizations, these results underscore the need for use of the OIE SN procedure for screening animals prior to introduction of a bull into a seronegative population of animals.

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October 12 – 18, 2006
Minneapolis Hilton – Minneapolis, MN

Genetic Analysis of PCV2 Sequences from an Outbreak of Severe PCVAD in Kansas

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Porcine dermatitis and nephropathy syndrome (PDNS) and postweaning multisystemic wasting syndrome (PMWS) are categorized as porcine circovirus associated diseases (PCVAD). Historically, PDNS has a low incidence (usually < 0.1%) but a high fatality rate, and is characterized by necrotizing, exudative glomerulonephritis, interstitial nephritis, and necrotizing vasculitis in the kidneys, dermis, and, in many cases, other tissues. PMWS is more common and characterized by failure to thrive and microscopic lesions of lymphoid depletion and granulomatous inflammation in lymphoid and other tissues, especially the liver and lungs.

Beginning in December 2005, numerous swine producers in central and eastern Kansas noted a dramatic increase in mortality beginning at 4 to 5 weeks after placing pigs in the finisher buildings with mortality reaching 10% to 20%. Many pigs had clinical signs of "severe" PMWS and PDNS. In addition, affected pigs often exhibited neurological signs. Histologic examination of PMWS pigs at the microscopic level showed that inguinal lymph nodes were depleted of lymphocytes with an influx of macrophages and granulocytes and occasional multi-nucleated giant cells. Germinal centers were absent. Immunohistochemistry (IHC) identified PCV2 antigen-positive cells in lungs, lymph nodes, kidneys, and Peyer's patches. Inflammatory lesions in brain and spinal cord were observed in the neurologic pigs. However, PCV2 antigen was not associated with the neurological lesions.

The initial investigation featured PCV2 sequences of 14 affected pigs from four different farms in the same geographic region of northeast Kansas. PCV2 DNA sequences were obtained by PCR amplification from tissues using a modification of the approach described in Fenaux et al. (J. Clin. Micro. 38:2494-2503, 2000). Phylogenetic trees were constructed using whole genome sequences. For the purpose of comparison, we included PCV2 sequences from North America, Europe and Asia, which were obtained from GenBank. All 14 sequences formed a single group, which was well separated, and only about 95% similar to a large body of "historical" North American isolates. The northeast Kansas sequences showed a 99.5% similarity to a 1998 French isolate, GenBank No. AF055393 (J. Gen. Virol. 79:2171-2179,1998). We also sequenced PCV2 on a farm in the same geographic location that was not experiencing severe PCVAD. These sequences were distinct from the AF055393 group and fell within the main body of historical North American isolates. Translation of ORF2 showed four non-conserved amino acid changes, located between amino acids 151-206 of the capsid protein.

Based on the results from this study we can make the following conclusions, which are important for the diagnosis and for understanding the pathogenesis of PCVAD.
1. Severe PMWS in northeast Kansas is associated with a single PCV2 isolate that forms a close association with a group of European PCV2 isolates.
2. The presence of neurological lesions suggests that other co-factors (viruses) may contribute to the severe PCVAD syndrome.
3. Significant amino acid changes in the capsid protein of the Kansas PCV2 isolates have important implications in pathogenesis and vaccine efficacy.

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Minneapolis Hilton – Minneapolis, MN

Bayesian Estimation of Rabies Ab ELISA performances in absence of a gold standard

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Background: Today, international rabies control is based on antibody measurement after vaccination and relies partially on diagnostic tests interpretation. Unfortunately, it is well admitted that there is no perfect reference test.

Objectives: Bayesian approach for latent class analysis has been one of several statistical methods proposed to estimate tests parameters such as sensitivity and specificity.

Material and Methods: Two European populations of vaccinated dogs and cats were used to determine the performances of two tests FAVN and ELISA. Estimation of prevalence, sensitivities and specificities of the two tests are obtained by combining observed data from populations to information from previous studies named informative priors.

Results: Estimates of the sensitivities and prevalence were high (prevalence: 96.1%; FAVN sensitivity: 98.9%; ELISA sensitivity: 93.8%) and are in agreement with previously published data. Estimations of the specificities were close to prior information and did not differ significantly between the two tests.

Discussion: The results obtained underline the need for adequate informative priors in Bayesian estimations as well as the need for adequate sample size. Data suggest also that FAVN and ELISA specificities are often imperfectly determined.

Conclusions: This innovative approach is useful in designing the right tools in the Pet Travel Scheme or any move of vaccinated dogs and cats to rabies free countries.

Keywords: Diagnostic, Rabies, Neutralizing antibody, International movement, ELISA, FAVN

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AAVLD 49th Annual Conference
October 12 – 18, 2006
Minneapolis Hilton – Minneapolis, MN

Measurement of the immune response to rabies vaccination using a novel and traditional assays

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Detection of neutralizing antibodies to rabies virus is the basis for determination of adequate immunity while specific lymphocyte responses to rabies vaccination has not been fully investigated The objective of this study was to develop a nonradioactive multi-parameter flow cytometry assay to identify antigen specific memory lymphocytes in individuals previously vaccinated against rabies virus and compare it against the tritiated thymidine method. A cell tracking dye (carboxyfluorescein succinimidyl ester) was used in combination with surface staining for CD4 and CD8 molecules to determine the response of memory lymphocytes to killed rabies virus in an antigen recall assay. Rabies virus-specific lymphocyte responses were compared to antibody responses in the same individuals. Lymphocytes and serum samples from 10 healthy subjects previously vaccinated with a cell culture rabies vaccine and from five non-vaccinated subjects were analyzed. Memory T lymphocyte responses to rabies virus were identified in all vaccinated subjects; some noted as early as 3 days after stimulation and others not until 7 days of stimulation. The proliferation index of the CFSE assay correlated well with the simulation index of the [^{3}H] thymidine assay (kappa statistic = 0.73). An inverse relationship between antibody and lymphocyte responses to rabies virus was detected in the vaccinated subjects. Two vaccinated individuals with high lymphocyte and low antibody responses produced Th1 type cytokines (IFN- gamma and TNF-alpha) to rabies virus stimulation, whereas two vaccinated individuals with low lymphocyte and high antibody responses did not produce these cytokines. Currently this CFSE assay is being adapted to veterinary species for the detection of cellular immunity memory response to various recall vaccine antigens.

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AAVLD 49th Annual Conference
October 12 – 18, 2006
Minneapolis Hilton – Minneapolis, MN

Genetic drift in Infectious Salmon Anemia Virus Detected in US Farmed Salmon

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Infectious salmon anemia (ISA) has been a disease issue for Maine marine salmon aquaculture sites since 2000. Initial industry efforts to eradicate the disease from farmed Atlantic salmon were unsuccessful leading to the development of a voluntary industry wide ISA prevention and control plan. A major component of the ISA management plan includes aggressive surveillance for detection of ISA virus (ISAV) and early removal of cages with infected fish. Surveillance includes state-mandated, veterinary onsite fish health inspection and collection of diagnostic samples for ISAV detection. The frequency (monthly, biweekly, or weekly) of site visits is determined by the risk associated with farm site location, season, previous ISAV diagnostic testing results, and current disease status.

Sequence analysis of ISAV cDNA fragments of gene segments 6 and 8 was utilized to evaluate potential epidemiologic relationships between ISAV positive aquaculture marine sites and to track changes in the virus on a site over time. Both North American and European-like sequences were detected in Maine farmed Atlantic salmon; but only North American sequences were associated with clinical disease, and a virus was isolated in cell culture. Analysis of the highly polymorphic region (HPR) of the haemagglutinin-esterase (HE) gene in isolates showing the North American sequences revealed two separate, stable, single nucleotide changes during five years of surveillance. These single nucleotide changes resulted in amino acid substitutions. A single European-like sequence was detected. Analysis of the HPR associated with this European-like sequence revealed the putative full length HPR of the HE gene. Genetic drift in ISAV isolates associated with clinical disease was limited to single point mutations in the region of the HE protein analyzed.

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Minneapolis Hilton – Minneapolis, MN

Update on the APHIS, USDA Spring Viremia Carp Surveillance Program

J.V.Warg[1], A.O. Joe[1], and A.E. Goodwin[2]

Spring viremia of carp (SVC) was first diagnosed in a US commercial koi carp and goldfish operation in the spring of 2002. The Animal and Plant Health Inspection Service funded efforts to eradicate the disease and initiated a voluntary surveillance program to determine by virus isolation if SVCV was present in major koi carp and goldfish breeding farms and other cyprinid farms in the US. From November 2003 through May of 2006 a total of 8079 fish from 23 states were evaluated by virus isolation. Cyprinid fish sampled included koi carp, grass carp, bighead carp, Israeli carp, goldfish, rosy red minnows, fathead minnows, and shiners. Fish were collected when water temperatures where between 41°F and 68°F for a minimum of ten days. No spring viremia of carp virus was isolated from any of the fish sampled. Aquareoviruses were isolated from four different lots of minnows received from three different premises during the study period. A ranavirus was isolated from a single lot of minnows.

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Minneapolis Hilton – Minneapolis, MN

An investigation of selenium and vitamin E values in goats in the Northeast.

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Vitamin E (Vit E) and selenium (Se) deficiency produce similar lesions and clinical signs in many species. A feed ration that is low in Vit E or Se can result in illness in livestock of various ages, making these values an important component to herd health in areas defined by Selenium deficient soils. There is scant data on reference values for Vitamin E and Selenium for goats in the literature. In many instances, the values for trace minerals and vitamins are borrowed or extrapolated from other species and much of the work being done with Se and Vit E in small ruminants seems to focus on sheep.

This investigation was conducted with 220 goats from four different herds in New York State. These animals were placed into three subcategories based on age and reproductive status (pre-kidding/late gestation, peak lactation, kid). The purpose of this investigation is to establish a relative reference range for Se and Vit E in goats of varying age, production purpose, and dietary nutrient composition in the Northeast United States. The resulting mean values for blood Se were higher than published results from other countries. Vit E levels were similar to those reported in plasma by Kessler et. al. in supplemented goats and their kids, but these values are much lower than those considered acceptable for other classes of livestock. All groups in the study population produced mean PCV and TP values within the expected reference range and there were no reports of illness during the study period. Therefore, the study population is considered to be healthy at the time of collection.

Tentative reference ranges are determined using two standard deviations above and below the observed mean values for each category. The exception to this determination will be the kid population, where two standard deviations below the mean for Se would be below the critical limit of 5µg/dL as defined by the literature. Similarly with this group, two standard deviations below the mean for Vit E would result in a negative number. For this group, the lower end of the observed range of values is used.

Table I Blood Se Ranges (µg/dL) and Vit E Ranges (µg/dL) for goats in the Northeast tentatively accepted for normal reference values

Pre-Kidding		Lactating		Neonate	
Se	Vit E	Se	Vit E	Se	Vit E
14.0 – 42.2	10.49 – 278.25	5.55 – 43.83	20.27 – 310.28	7.6 – 36.19	11 – 375.66

Conclusions

Assuming no cross protection from Se status, Vit E levels in goats are much lower than those accepted for other species. Adequate blood Se and serum Vit E values can be attained by goat herds in the Northeast without injectable supplementation. Because of the ongoing nature of the study, the ranges found in the total population are tentatively accepted as the reference range for goats in the Northeast not receiving parenteral Se or Vit E supplementation.

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Minneapolis Hilton – Minneapolis, MN

Determination of Gentamicin in Feline Kidney: A Case of Apparent Nephrotoxicity

P.M. Imerman[1] and M.J. Yaeger[1]

Aminoglycosides are broad spectrum antimicrobials used intravenously to treat serious infections. The margin of safety between effective and toxic doses is small, and care should be taken when choosing to administer this class of compound. Gentamicin, an aminoglycoside effective against gram-negative bacteria, is known to ionically bind to tissue protein. The compound tends to concentrate in the renal cortex, where it causes necrosis that can result in kidney failure.

A case of feline bladder infection with dehydration and anemia was initially treated with gentamicin. Fluid therapy and a blood transfusion were administered later. The cat retained fluids with no production of urine and died 48 hours later. The animal was submitted to the ISU Veterinary Diagnostic Lab for evaluation, with a clinical diagnosis of suspected gentamicin intoxication. Necropsy findings included increased peritoneal fluid and very pale (white) kidneys. Histopathology indicated severe acute diffuse necrosis of the proximal tubular epithelium with preservation of the basement membrane.

Fresh kidney tissue was extracted and cleaned up by solid-phase extraction using acetonitrile and an ion pairing reagent. A pre-column fluorescent derivative (FMOC) was made from the gentamicin, and the compound was detected and quantified by reverse-phase HPLC using standard addition. Gentamicin was detected in the kidney at 1.7 ± 0.2 ppm.

The clinical history, clinical signs, necropsy findings, histopathology and analytical chemistry results in this case are suggestive of gentamicin toxicity. Reference data on renal gentamicin concentrations in cases of feline poisoning was lacking, although information was available for feline serum gentamicin concentrations. A serum sample in this case may have provided corroborating data to complete the diagnosis.

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AAVLD 49th Annual Conference
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Minneapolis Hilton – Minneapolis, MN

Normal Manganese and Molybdenum in whole blood, Plasma and Serum

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Abstract: Manganese (Mn) is an essential element. It is required for normal growth and reproduction. Manganese deficiency is more of a concern than toxicosis. Manganese deficiency is characterized by improper formation of bone, growth retardation, inflammation of the skin, and reproductive failure. Molybdenum (Mo) is a cofactor of several enzyme systems and is therefore an essential element. Molybdenum deficiency impairs growth and reproductive efficiency in livestock. However, because the requirement for molybdenum is low, clinical deficiency in livestock is rare. Ruminants are however more susceptible to molybdenum toxicosis. There is very little information in the literature on normal reference values of manganese and molybdenum in whole blood, blood plasma or serum. This makes interpretation of test results for these elements in blood and other blood derived matrices very problematic. This study was designed with 3 objectives in mind: 1) to determine the ideal blood matrix to use for molybdenum and manganese analysis; 2) to obtain preliminary reference values; and 3) to determine if any changes exist under normal sample handling conditions. Whole blood, blood plasma, and serum samples were obtained from ten of bovine, ovine, and equines species. The samples were analyzed by ICP/MS. In all three species, there were statistically significant differences ($p<0.01$) among serum, plasma and whole blood manganese concentrations, with highest concentrations of manganese present in whole blood. For molybdenum, in all three species serum and plasma molybdenum concentrations were similar, but there was a statistically significant differences ($p< 0.01$) between whole blood and serum or plasma. Whole blood had the lowest molybdenum concentration in all 3 species. Except for sheep where statistically significant differences were observed between plasma, serum and whole blood manganese and molybdenum concentrations between samples analyzed same collection day and replicates analyzed 5 days later, no such differences were observed for other species ($p< 0.05$). These results suggest that whole blood is the best sample for analysis of manganese and either serum or plasma is suitable for molybdenum analysis. Overall, it is not necessary to analyze samples immediately as no significant differences were present between samples analyzed on the same day and those analyzed 5 days later.

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Validated method for quantification of toxic elements in whole blood

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An ICP/MS method has been developed and validated to measure heavy metals such as chromium (Cr), cadmium (Cd), antimony (Sb), mercury (Hg), thallium (Tl), lead (Pb), as well as arsenic (As) in whole blood. To analyzed a blood sample, 200 µL of the whole blood was diluted by adding 5.0 mL of pre-prepared diluent, which contained 0.05% EDTA, 1% ammonium hydroxide, 0.05% Triton-X 100, 2% butanol and 10 ppb scandium, 15 ppb germanium, 15 ppb rhodium, 15 ppb indium and 15 ppb bismuth as internal standards. The toxic metals were monitored at the following m/z (metal, m/z): Cr, 53; As, 75, Cd, 111; Sb, 121; Hg, 202; Tl, 205; Pb, 208. The standard curve for the quantification of Cr was constructed at 0, 1, 10, and 100 ppb and was constructed at 0, 5, 50, and 500 ppb for each of the other elements. Standard additions of the above mentioned elements at 1.0, 5.0, 10, 20, 40, 2000 ppb levels were performed on the control whole blood samples. The analyses were run on six different days with three sets of samples being analyzed on each day. Standard addition curves were constructed and the average recovery rates were obtained for each spiking level. Method precision was expressed as inter-day and intra-day %RSD at different spiking levels. The quantification limits for Cr and Hg were 5.0 ppb. The quantification limits for other elements were 1.0 ppb. Statistical data at other concentrations were also obtained. Method accuracy was assessed by running an NIST reference material, SRM 966, Bovine Blood, level 2, which contains Cd, Hg, and Pb, and by running spiked bovine whole blood samples at 5.0 and 40 ppb.

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Minneapolis Hilton – Minneapolis, MN

Exposure-to-treatment interval and clinical severity in canine poisoning: A retrospective analysis at a Portland Veterinary Emergency Center.

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The amount of published information regarding the exposure-to-treatment interval (ETI) and the severity of canine poisoning cases is limited. The ETI is a critical factor in the effectiveness and risk:benefit ratio of decontamination of the upper digestive tract by induction of emesis, gastric lavage and single-dose activated charcoal. For agents that do not affect gastric emptying, the efficiency and risk:benefit ratio of upper gastrointestinal (UGI) decontamination following poisoning declines rapidly as the ETI increases, particularly when this interval exceeds 1–2 hours. The objectives of this study were to test the hypothesis that the minimum estimated ETI in canine poisoning requiring emergency treatment is usually too long for UGI decontamination to be effective, and to test the hypothesis that the majority of canine poisoning cases are mild and have a high probability of a positive outcome, to determine the most common types of canine poisoning in canine emergency practice, and to document the cost of treatment. All canine clinical case records between July 2003 and July 2004 with a presumptive clinical diagnosis of poisoning (a total of 416 cases) were included in the analyses. Parameters extracted from the clinical case records included: age, breed, sex, likely toxin/toxicant involved, method of presumptive clinical diagnosis, estimated minimum ETI, clinical severity score at the time of first examination and/or admission (0 - 4), case outcome (death, partial recovery, full recovery), and the total cost of all treatment.

The average age of dogs requiring emergency treatment following a presumptive diagnosis of poisoning was 3.97 ± 3.66 years (mean ± SD) with a range of 0.15–13 years. Sex and breed had no significant effect on the frequency of poisoning ($p > 0.05$). The overall average estimated ETI was 7.44 ± 12.64 h (range 0.5–72). Neither the toxin/toxicant involved nor the clinical severity score significantly ($p > 0.05$) effected the estimated minimum ETI. The majority of cases presented with no to mild clinical signs median 0, range 0–4). The type of toxicant involved had no significant effect ($P > 0.05$) on the clinical severity score. Although a wide range of agents were involved in canine poisoning cases requiring emergency treatment, the most common were the rodenticides (25.7% of cases), chocolate (25.5% of cases), metaldehyde (10.6% of cases), and non-steroidal anti-inflammatory drugs (10.4% of cases). Poisoning with human pharmaceutical products constituted 22.8% of cases. The main method of presumptive clinical diagnosis (99.7% of cases) was the case history (often owner witnessed ingestion) combined with the clinical signs at presentation (if any). Only a single case in the series was diagnosed using toxicological testing (rapid ethylene glycol test). The overall average cost of treatment per patient was US$385.94 ± 17.84 (range US$73.00–2072.38). The type of agent involved did not significantly ($P > 0.05$) affect the average cost of treatment.

Given that the average minimum ETI in canine poisoning was about 7–8 h and that UGI decontamination techniques have limited or no effectiveness against agents that do not form gastric concretions or delay gastric emptying beyond approximately 2–3 h post-ingestion, it is unlikely that emergency UGI decontamination would have been of any benefit in the majority of the cases examined. Effective UGI decontamination performed with due clinical prudence is a critical part of the treatment of oral poisoning. However, it should not be used indiscriminately: its use should be restricted to those patients who present within 2–3 h (preferably within 1 hour) following oral ingestion of a poison and who have a good risk–benefit profile in relation to these procedures. It is noteworthy that although the majority of canine poisonings were relatively mild, these episodes represented a significant financial cost to owners given that the average cost of treatment approached US$400.00 with upper range of cost extending beyond US$2000.

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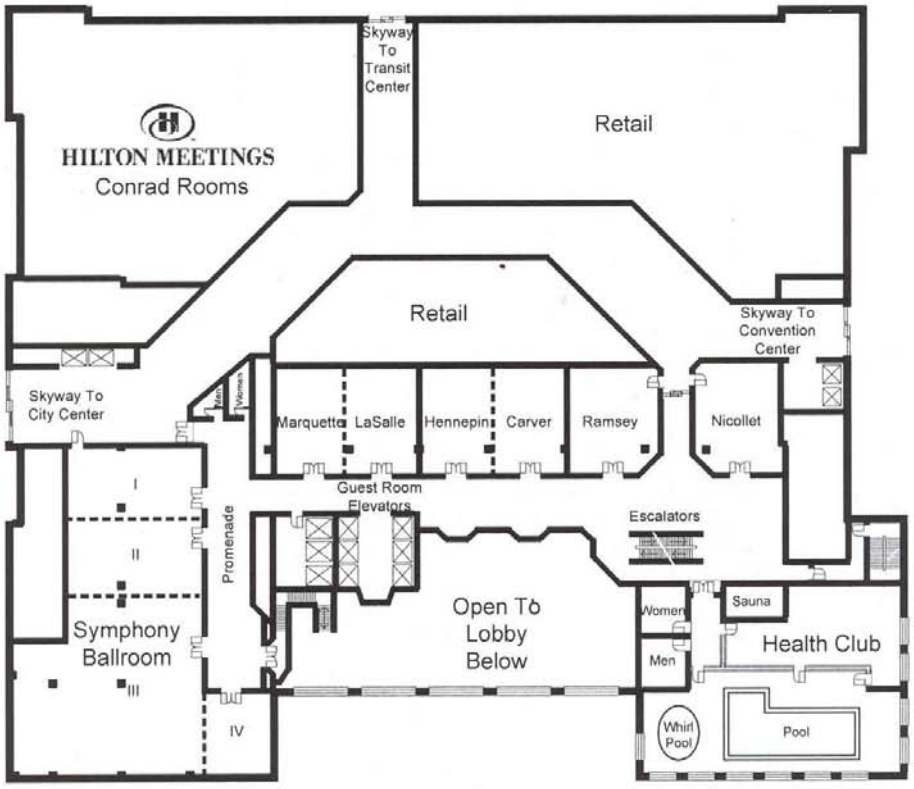
MEETING FACILITIES - SECOND FLOOR



Hilton Minneapolis

ROOM NUMBER	ROOM DIMENSIONS Square Feet	Ceiling Height	ROOM CAPACITIES Theater	Classroom	Banquet	Reception
Marquette & LaSalle	2,120	11'	208	132	150	240
Marquette	1,000	11'	110	48	70	115
LaSalle	1,120	11'	124	68	80	130
Hennepin & Carver	2,120	11'	208	132	150	240
Hennepin	1,120	11'	124	68	80	130
Carver	1,000	11'	110	48	70	115
Nicollet	1,312	11'	130	68	90	140
Ramsey	1,312	11'	130	68	90	140
Symphony Ballroom	7,399	10' 6"	615	245	500	815
I	1,543	10' 6"	140	75	110	160
II	1,413	10' 6"	115	48	80	150
III	3,749	10' 6"	390	162	260	425
IV	694	10' 6"	60	38	60	80
I & II	2,956	10' 6"	255	123	190	310
III & IV	4,443	10' 6"	400	168	310	505
II, III & IV	5,856	10' 6"	470	194	390	655
I, II & III	6,705	10' 6"	615	245	450	735
II & III	5,162	10' 6"	470	194	340	575
Symphony Promenade	1,750	10' 6"				

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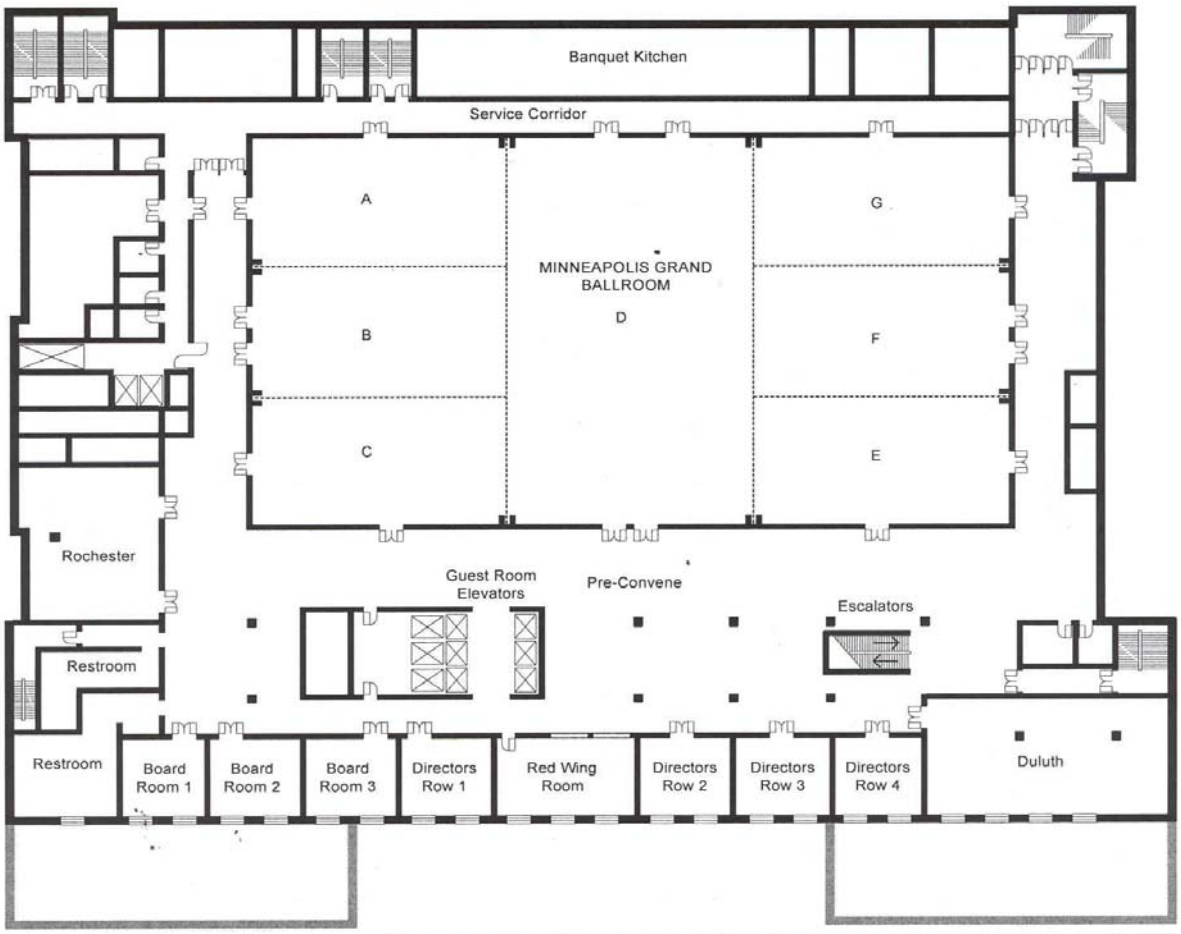

Hilton Minneapolis

ROOM NUMBER	ROOM DIMENSIONS Square Feet	Ceiling Height	ROOM CAPACITIES Theater	Classroom	Banquet	Reception
Grand Ballroom	24,780	20'/14'5"	2,576	1,440	1,950	2,850
Salon A, B, C Each	2,769	20'/14'5"	290	172	220	320
Salon E, F, G Each	2,652	20'/14'5"	270	154	210	305
A, B, C Combined	8,378	20'/14'5"	930	524	660	960
Salon D	8,378	20'/14'5"	1,010	524	700	960
E, F, G Combined	8,024	20'/14'5"	880	486	640	920
Rochester	1,785	11'	132	48	150	205
Board Room 3	676	10'	Permanent Seating for 18 People			
Board Room 1 & 2	624	10'	60	32	50	70
Directors Row 1	676	10'	65	32	50	80
Directors Row 2, 3, 4	624	10'	60	32	50	70
Duluth	2,145	10'	150	96	150	250
Red Wing Room	1,040	10'	96	48	80	120

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