Proceedings of the

American Association of Veterinary Laboratory Diagnosticians

50th Annual Conference

John Ascuaga's Nugget
Reno, Nevada
October 18-23, 2007

American Association of Veterinary Laboratory Diagnosticians

The American Association of Veterinary Laboratory Diagnosticians (AAVLD) is a not-for-profit professional organization which seeks to:

- Disseminate information relating to the diagnosis of animal diseases
- Coordinate diagnostic activities of regulatory, research and service laboratories
- Establish uniform diagnostic techniques
- Improve existing diagnostic techniques
- Develop new diagnostic techniques
- Establish accepted guidelines for the improvement of diagnostic laboratory organizations relative to personnel qualifications and facilities
- Act as a consultant to the United States Animal Health Association on uniform diagnostic criteria involved in regulatory animal disease programs

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Acknowledgments

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 everyone who attended the meeting. A special thank you also to all of our invited speakers for the AAVLD Plenary Session and the USAHA-AAVLD Scientific Session. And as a special feature of the 50th Annual Conference, thank you to all of the Pope Award winners and Life Members who were able to attend and make this meeting unique.

The Program Committee, listed below, deserves a special acknowledgement for all of their hard work, organization, review and editing of the abstracts, and moderation of sessions. Jay Kammerzell was again instrumental in computerizing and organizing the review process. Pat Blanchard, Allison Reitz, Vanessa Garrison, Jackie Cassarly, and Linda Ragland (USAHA) coordinated the meeting room arrangements, exhibitor booth arrangements, sponsor agreements, breaks, and all of the many other details that go into making a meeting a success.

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AAVLD Plenary Session

Saturday, Oct 20, 2007

Ponderosa B

"Past, present and future of veterinary laboratory medicine"

Moderator: Grant Maxie

Virology Scientific Session

Saturday, October 20, 2007

Bonanza A

Moderators: Kyoung-Jin Yoon, Kristy Lynn Pabilonia

Anatomic Pathology Scientific Session

Saturday, October 20, 2007

Bonanza BC

Moderator: Bill Layton

Molecular Diagnostics Scientific Session

Saturday, October 20, 2007

Ponderosa A

Moderator: Glenn Songer

* Graduate student presentation

Bovine viral diarrhea virus Scientific Session

Saturday, October 20, 2007

Ponderosa B

Moderator: Steven Bolin

* Graduate student presentation

Epidemiology/Molecular Diagnostics Scientific Session

Sunday, October 21, 2007

Bonanza A

Moderators: Francois Elvinger, Lindsay Oaks

* Graduate student presentation

Anatomic Pathology Scientific Session

Sunday, October 21, 2007

Bonanza BC

Moderators: David Steffen, John Adaska

* Graduate student presentation

Toxicology Scientific Session

Sunday, October 21, 2007

Genoa

Moderators: Catherine Barr, Patricia Talcott

* Graduate student presentation

Virology Scientific Session

Sunday, October 21, 2007

Ponderosa A

Moderator: Tim Baszler, Kyoung-Jin Yoon

***** Graduate student presentation

Bacteriology Scientific Session

Sunday, October 21, 2007

Ponderosa B

Moderator: Lorraine Hoffman

* Graduate student presentation

USAHA /AAVLD Scientific Session

Monday, October 22, 2007

Rose Ballroom

Co-chairs: Grant Maxie, President-elect, AAVLD; Jim Leafstedt, President-elect, USAHA

Examining the roles of AAVLD and USAHA concerning major diseases

07:30 AM **Welcome and announcements**
Jim Leafstedt, President-elect, USAHA
Grant Maxie, President-elect, AAVLD
Moderator - Will Hueston

07:45 AM **Control of *Viral hemorrhagic septicemia virus* (VHSV) of fish** - Gary Egrie..... 148

08:15 AM **Will we ever eliminate *Salmonella* from poultry?** - Stephen Roney 149

08:45 AM **Control and eradication of the scrapie prion from sheep** - Cindy Wolf 150

09:15 AM BREAK

09:30 AM **Comments on control of *Bovine viral diarrhea virus* in the United States** - Dale Grotelueschen..... 151

10:00 AM **Control and eradication of *Porcine reproductive and respiratory syndrome virus* (PRRSV)** - Scott Dee..... 152

10:30 AM **Control of equine infectious anemia should take new directions** - Charles Issel..... 153

11:00 AM **Building an infrastructure to address emerging diseases: the Johne's disease template** - John Adams..... 154

11:30 AM Summation

11:45 Adjournment

AAVLD Poster Session

October 19-22, 2007

Foyer

* Graduate student presentation

AAVLD Plenary Session

Saturday, Oct 20, 2007

Ponderosa B

"Past, present and future of veterinary laboratory medicine"

Moderator: Grant Maxie

The evolution of the AAVLD

Robert A. Crandell, Larry G. Morehouse, Vaughn A. Seaton

College Station, Texas 77845 (Crandell); Columbia, Missouri 65201 (Morehouse);
Ames, Iowa 50010 (Seaton)

Robert Crandell - I began my career with AAVLD in 1960. My first meeting was the 3rd Annual Meeting of the Conference of Veterinary Laboratory Diagnosticians in Charleston, WV. Dr. Ray Bankowski from the University of California was the president that year. There were 13 topics on the program. As Vice President in 1984 and program chairman, I initiated the poster session for the Fort Worth Annual Meeting. Today there are 120 scientific presentations, 30 some committee meetings, 30 plus posters, and many commercial exhibits.

Two of the purposes of the AAVLD are to "Establish uniform diagnostic techniques" and "Improve existing diagnostic techniques", and these purposes continue to evolve and to be fulfilled. Although AAVLD members didn't develop all procedures themselves, special committees did recommend minimum standards using these procedures which were approved by the USDA. For example, the FA test was approved as a standard test for hog cholera, and the serum neutralization test for pseudorabies was approved as an official test. Both of these procedures were instrumental in the eradication of these 2 diseases from the US.

New diseases were recognized between the early '50s and 1982, and another group was first recognized after 1982. Some of these diseases were eradicated while others are still of economic and public health importance. Diseases and disease patterns have changed over time, and the impact of wildlife-livestock interactions has been recognized.

Another purpose of the AAVLD is to "Disseminate information relating to the diagnosis of animal diseases". The proceedings of our Annual Meetings were first published in the USLSA's and in the USAHA's Proceedings book. In 1974, the AAVLD began to publish their Proceedings separately. Because universities didn't recognize articles in the Proceedings for promotion and tenure, it was decided in 1987 to create a referreed journal. The *Journal of Veterinary Diagnostic Investigation* was developed and the first issue dated 1 January 1989 was presented to the membership at the 1988 Annual Meeting. The telephone and the mail system were soon obsolete as computers, email and fax were introduced. More recently NAHLN, Worldwide Web, and other electronic communications systems have emerged.

Larry Morehouse - video presentation.

Vaughn Seaton - Anniversaries are happy occasions especially for organizations or associations because their continued existence implies success! Certainly that describes this AAVLD gathering. While it is often interesting and amusing to reflect on the past, thought should be given to a bit of analysis and instruction for the future. At the inception of the AAVLD many laboratories were parochial in scope. In the interim, administrative attitudes, newer technology, newer types of instrumentation, higher personnel standards, laboratory accreditation standards, broader diagnostic capabilities and responsibilities for animal and human health reporting have brought us to where we are today. Greatly expanded international interactions and responsibilities have been recognized with the formation of the WAVLD. This world organization was started by AAVLD personnel and is holding its 13th international meeting this year in Melbourne Australia.

Veterinary diagnostic toxicology: from spots to peaks to fragments and beyond (or why does diagnostic toxicology cause economic heartburn for laboratory directors?)

Robert Poppenga, Mike Filigenzi, Elizabeth Tor, Linda Aston, Larry Melton, Birgit Puschner

California Animal Health and Food Safety Laboratory System, School of Veterinary Medicine, University of California at Davis, Davis, CA 95616

The recent large-scale pet food recall as a result of contamination of imported wheat and corn gluten and rice protein powder illustrates the continuing need for responsive diagnostic and analytical toxicologic capabilities. Such capabilities do not come cheaply. However, when the economic and emotional impacts of such an event are considered, the high costs pale in comparison. Over the last 25 years, analytical systems have progressed from trying to identify ambiguous spots by thin layer chromatography (TLC) and peaks by high performance liquid chromatography (HPLC) to unambiguous molecular fingerprints by gas chromatography - mass spectrometry (GC/MS) and liquid chromatography - mass spectrometry (LC/MS). Using multi-stage mass spectrometry with HPLC (LC/MS/MS/MS) permits more rapid method development and sample preparation and increases the ability to confirm the presence of many toxicants in a variety of matrices at low parts per billion ranges. The diagnosis of intoxication by *Nerium oleander* provides an excellent example of how improvements in analytical systems have enhanced our diagnostic capabilities. Diagnostic confirmation of exposure to the plant initially relied on visual examination of gastrointestinal (GI) contents for leaf fragments. Subsequently, exposure confirmation relied on a relatively insensitive assay for the detection of the plant toxin, oleandrin, in GI contents. Currently, we are able to confirm exposure to oleandrin at 1 ppb or lower in a variety of antemortem and postmortem samples using LC/MS techniques. We have also been able to investigate the occurrence of meat and milk residues of oleandrin following exposure of dairy cows to the plant.

While the detection of metals does not receive the same attention as the detection of organic toxicants, analytical systems have progressed from detecting one metal at a time with atomic absorption spectrometry (AAS), to detecting dozens of metals with one analysis using inductively coupled plasma - atomic emission spectrometry (ICP-AES), to potentially pinpointing a source for metal exposure by measuring metal isotopes utilizing inductively coupled plasma - mass spectrometry (ICP-MS). The latter approach has been used by other investigators to measure Pb^{207}:Pb^{206}ratios in lead-intoxicated California condors and implicate lead ammunition as the source of exposure.

Future advances in analytical and diagnostic toxicology may come from proteomics and metabolomics, although much work needs to be done before such approaches can be used routinely. However, our ability to apply new analytical techniques to the detection of proteins as biomarkers of toxicologic and non-toxicologic diseases has the potential to improve diagnostics almost immediately. While it may be appealing in the near term to delay necessary expenditures for maintaining a cutting-edge analytical and diagnostic toxicologic capability, current trends such as increasing global trade and agroterrorist threats argue that such a delay would be short-sighted. **In an age of limited government resources, the AAVLD should give strong consideration to the establishment and support of regional centers of diagnostic and analytical toxicology centers of excellence.**

Microspheres and the evolution of testing platforms

Susan J. Wong

Wadsworth Center, New York State Department of Health, Albany, New York, USA 12201-2002

Serology and immunoassays for antibody detection or antigen detection have been prevalent diagnostic tools for over a century. Such ligand receptor binding techniques have evolved over the decades resulting in assays with improved analytical sensitivity and disease specificity. Some of the assays from the early 1900s, such as complement fixation, immunoprecipitation, and particle agglutination rely upon visual detection and remain in use today. Other assay formats such as indirect immunofluorescence and direct fluorescence assays have improved with the use of fluorochromes with higher extinction coefficients and use of better microscopes. Radioimmunoassays were a significant advance in the mid 20th century, but they were quickly replaced by immunoradiometric assays. Enzyme-linked immunosorbent assays (ELISA) became common in the 1970s. More recently electrochemiluminescence (ECL) and flow cytometric suspension phase microsphere immunoassay (MIA) have added power to the diagnostic armamentarium.

Contemporary testing platforms such as ECL and MIA have detection limits up to 5 logs lower than were possible a century ago. Advantages of the new platforms are: decreased amounts of reagents and specimens required, increased shelf-life of reagents providing better quality control, broader assay dynamic ranges, rapid turn-around-time, lower background (nonspecific binding), and ease of adaptation to multiple species. **Multiplex capacity allows for rapid evaluation of candidate antigens and peptides for new immunoassays.** Recombinant proteins, synthetic peptides, or highly purified antigens make these new assays possible. Our laboratory has developed and validated serological assays for bacterial, fungal and viral infections, all of which have been adapted to animal diagnostics or pathogenesis studies. West Nile virus immunoassays have been used for diagnostics, surveillance, and pathogenesis studies in horses, birds, and mice. Lyme borreliosis multiplex MIA has been applied to the rapidly progressive glomerulonephritis in retrievers.

The MIA platform is useful not only for serology but also for monitoring of therapeutic monoclonal antibodies. Oligonucleotide hybridizations can also be performed on the microsphere surface; so multiplex assays to identify PCR products can be used for syndromic disease surveillance. In similar fashion, the ECL platform can be used for multiplex testing and high-throughput analyses.

A production management (client's) perspective on diagnostics

Dale M. Grotelueschen

Beef Cattle Veterinary Operations, Pfizer Animal Health, Gering, NE 69341

Veterinary diagnostic laboratories in North America are important drivers of change for improvement of animal health in the food animal industry. Immediate impact focuses on the veterinarian and food animal production enterprise for typical case submissions, yet consumers, international markets, the public in general (protection of public health) and others also derive tangible benefits. Increasing focus on end users and markets has changed the relative importance of diagnostics to the livestock industry itself and many others.

Animal health is a science, and food animal production can be embraced as a scientific endeavor. Objective pursuit of the truth for all components is necessary, and increasing the levels of objective knowledge is not only critical but also the responsibility of all. Evidence-based medicine is an excellent example where all animal health disciplines are contributing. Quality control is being addressed by laboratories and is critical for continuous improvement, credibility, and increasing the economic value of diagnostics. Validation and standardization across laboratories addresses industry concerns.

Livestock production systems are in constant modes of change, initiated by economics, labor, efficiency, environment, feed supplies and other pressures. Priority to reasons for change varies greatly. For example, environmental stewardship and more recently the advent of alternative uses of feedstuffs has resulted in production shifts. Production goals and reasons for being in business greatly affect management and management decisions and therefore use of diagnostic services. Beef Quality Assurance, incorporating topics such as judicious use guidelines for antimicrobials, is an example of beef industry effort to educate producers and meet consumer expectations.

Food animal production is a competitive, economically driven business. Profit margins are narrow and range from negative to positive. Production and economic measures are increasingly important as business standards increase in the industry. Incorporation of these into diagnostic planning strategies focuses on common industry goals. **Proactive diagnostics that identify and embrace clinically relevant outcomes make laboratories invaluable partners for animal health improvement.**

The animal health component, relatively small in relation to the food animal industry as a whole, is composed of a diversity of disciplines and interests, ranging from production levels to basic sciences. Diagnostic laboratories play an integral role in this system. Epidemiologic applications, such as risk factor identification, rightfully increase the necessity of population-based approaches to diagnostics and problem solving. Sound applications of biosecurity and biocontainment principles to production medicine are improving production and meeting increased expectations. The diagnostic component cannot be underestimated to address needs at production levels as well as consumer criteria for food safety.

Gaps in communication and coordination across the food animal industry must continuously be filled. Creative efforts and priority are essential.

Future diagnostics, including use of new technologies must consider applicability to populations, clinical relevancy and scientific merit across production systems. Proactive identification and measurement of risk factors and agents significantly impacting productivity in changing production systems is critical.

AAVLD survey of pet food-induced nephrotoxicity in North America, April to June 2007

Wilson Rumbeiha, Dalen Agnew, Grant Maxie, Michael Scott, Brent Hoff, Barbara Powers

Diagnostic Center for Population and Animal Health, College of Veterinary Medicine, Michigan State University, Lansing, MI 48910-8104 (Rumbeiha, Agnew, Scott); Animal Health Laboratory, Laboratory Services Division, University of Guelph, Guelph, Ontario, Canada N1H 6R8 (Maxie, Hoff); Veterinary Diagnostic Laboratory, Colorado State University, Fort Collins, CO 80523 (Powers)

Following the outbreak of pet-food induced nephrotoxicity in cats and dogs around March 15, 2007, the AAVLD commissioned a voluntary survey of AAVLD-accredited laboratories that was eventually expanded to cover commercial laboratories and veterinary clinics across North America. Following this outbreak, there were numerous unsubstantiated reports of thousands of pets having been affected and/or died as result of having consumed melamine-contaminated food. There was also confusion surrounding the case definition of what truly counted as pet-food induced illness or death. This ongoing survey was designed to collect data from veterinary professionals across North America to determine: a) the numbers of confirmed cases of pets affected by melamine-contaminated food; b) the geographical distribution of cases across North America; c) common threads among affected animals; and d) to develop a case definition of what actually constituted a case of pet food-induced illness or death.

To achieve the above objectives, a questionnaire consisting of 17 questions was designed. Collection of data started on April 5, 2007 and is still ongoing using a Web-based format via the on-line tool, Web Monkey™. For purposes of this abstract, analysis has been performed on data collected up to June 6, 2007; 486 cases had been entered in the survey database. Of those cases, 139 (29%) were excluded because the data did not meet the criteria for further analysis.

Of the 347 cases that met the criteria, 235 (68%) were cats and 112 (32%) were dogs. The sex of 8 cats was not identified, but of the remaining 227 cats, 48% were male and 52% were female. Fifty nine of the dogs (53%) were male and 47% were female. The age range for cats was 0.17-19 years and for dogs it was 0.33- 19 years. Of all the cases, 98% were reported from the US, with the remaining 2% reported from Canada. From North America as a whole, the majority of cases were reported from Texas, California, Ontario, Illinois and Michigan in descending order of frequency. It was interesting to note that of all the cat and dog cases, 61% and 74% respectively were dead and the rest were either ill or had recovered at the time of reporting. Among US cats, the top 2 foods responsible for illness were Iams and Special Kitty US in descending order of frequency. The top 2 food products most responsible for illness among dogs in the US were Alpo Prime foods followed by Ol' Roy US. In Canada, 20 of 27 cases reported were cats, and Special Kitty Canada and President's Choice were the 2 foods most frequently associated with illness. Another interesting finding was that of all the dog and cat cases, an equal proportion (18%) used 3 or more of the **8 diagnostic criteria used to confirm pet-food-induced nephrotoxicity**. These criteria were as follows: a history of having ingested recalled food; finding characteristic crystals in urine; gross findings of characteristic yellow crystals in the kidneys; histologic findings of distinct crystals in the kidneys; analytical confirmation of melamine in urine; analytical confirmation of melamine in tissues; analytical confirmation of melamine in food; and analytical confirmation of cyanuric acid, ammelide, or ammeline in tissues, urine, or food.

Only a minority of cases reported in the survey had pre-existing conditions that would be considered to make affected pets more vulnerable to pet food-induced nephrotoxicity. This was surprising considering that the vast majority of cases reviewed by authors of this abstract had preexisting conditions considered to render affected pets more vulnerable to pet food-induced nephrotoxicity, including glomerulonephropathy and tubulointerstitial nephritis.

Ecosystem health, agriculture, and diagnostic laboratories: challenges and opportunities

Thomas E. Besser

Washington Animal Disease Diagnostic Laboratory, Washington State University, Pullman, WA

Healthy ecosystems are those with intact and interacting physical, chemical and biological elements. They are resilient, have the ability to withstand changes and resist stressors, and are not experiencing drastic or abnormal changes in the abundance of native species, increasing concentration of contaminants or toxins, or other major landscape or ecological impacts. Healthy ecosystems provide services including food, water, shelter, economic livelihood, recreation and natural beauty. Agriculture is estimated to occupy 40% of the land surface of the earth and utilize 70% of the fresh water resources; as such it intimately affects ecosystem health. As the value that society places on a healthy ecosystem changes, agriculture must react in order to maintain the global food supply, which will require increasingly explicit attention to ecosystem health.

As our understanding of healthy ecosystems improves and as pressures on healthy ecosystems increase with population growth, climate change and global economic development, challenges are arising in areas as diverse as emergence and dissemination of zoonotic diseases, disease transmission between wild and domestic animals and humans, maintenance of healthy livestock as feed components rise in value and water shortages impact crop production and land use, beneficial utilization of manure wastes from intensive livestock operations, and many other areas.

Whether recognized as such or not, diagnostic laboratories have traditionally been involved in ecosystem health in roles such as the recognition of novel pathogens and diseases, development of appropriate diagnostic methods and management tools, surveillance for exotic disease agents, and detection of toxic substances. **In the future, it's likely that the interests of traditional diagnostic laboratory stakeholders will require new expertise and more complex interactions for laboratories and their staff, encompassing interactions and consultation with diverse partners including the public health community, wildlife managers, environmental health experts, land-use planners, and even ecologists.**

Virology Scientific Session

Saturday, October 20, 2007

Bonanza A

Moderators: Kyoung-Jin Yoon, Kristy Lynn Pabilonia

Further improvement and validation of MagMAX-96 AI/ND viral RNA isolation kit for efficient removal of RT-PCR inhibitors from cloacal swabs and tissues for rapid diagnosis of avian influenza virus by real-time RT-PCR

Amaresh Das, Erica Spackman, Mary J. Pantin-Jackwood, David E. Swayne, David Suarez

Foreign Animal Disease Center, APHIS, USDA, Plum Island, New York (Das); Southeast Poultry Research Laboratory, Athens, GA 30605 (Spackman, Pantin-Jackwood, Swayne, Suarez)

Real-time reverse transcription PCR (RRT-PCR) is a high-throughput molecular diagnostic test used for rapid detection of avian influenza virus (AIV) in clinical samples. However the performance of RRT-PCR can be adversely affected by RT-PCR inhibitors present in the sample. The tested commercial RNA extraction kits did not remove all RT-PCR inhibitors from clinical samples from cloacal swabs and tissues. In this study, we used a modified MagMAX-96 AI/ND viral RNA isolation kit (MagMAX, Ambion) for extraction of RNA from cloacal swabs and tissues. RRT-PCR was carried out in the presence of an internal positive control to detect inhibitors in the sample.

Cloacal swabs from 2668 wild birds sampled in Alaska and South America were analyzed by RRT-PCR after the RNA was extracted by the standard and modified MagMAX protocols. With the RNA extracted by the standard MagMAX protocol, 403 (15.1%) samples tested positive for AIV, 2167 (81.22%) samples tested negative for AIV, and 491 (18.40%) samples had evidence for PCR inhibitors (false negatives). Further analysis of 433 of the samples with inhibition with the modified MagMAX protocol indicated 36 (8.3%) of the samples tested were positive for AIV, 412 (95%) samples tested negative for AIV, and only 21 (4.8%) samples tested still had evidence of RRT-PCR inhibitors.

For tissues, the RNA was purified using a Trizol-MagMAX hybrid protocol in which the RNA was extracted by Trizol LS and purified using both standard and modified MagMAX protocols. Tissues of breast (n=28), thigh (n=28) and heart (n=28) from chickens infected with high pathogenicity (HP) Asian H5N1 AIV and those of brain (n=16), lung (n=18), spleen (n=11), heart, (n=9) muscle (n=11) and kidney (n=6) from HP H5N1 infected ducks were analyzed by RRT-PCR after the RNA extracted by the above protocols. The average C_ts corresponding to the virus in tissues from infected chickens and ducks were between 0.5 and 3.0 higher with the RNA extracted by the standard MagMAX protocol than the modified MagMAX protocol.

These results show more efficient removal of RT-PCR inhibitors from both cloacal swabs and tissues by the modified MagMAX RNA extraction protocol, which ensures high quality of the RNA for accurate detection of AIV by RRT-PCR.

Development of a high-throughput real-time PCR procedure including an internal control for detection of PCV-2 in various biological samples

Wonil Kim, Karen Harmon, Tanja Opriessnig, Wendy Stensland, Kathryn Behrens, Kyoung-Jin Yoon

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Iowa State University, Ames, Iowa

Porcine circovirus 2 (PCV-2) is implicated in porcine circovirus-associated disease (PCVAD), which is clinically manifested in various forms, and causes significant economic losses to the swine industry in North America. Since PCV-2 infection is widespread in swine populations and the demand for PCV-2 laboratory testing is growing rapidly, fast and accurate detection of PCV-2 from various matrices in a quantitative form has become critical in diagnostic service.

Fluorogenic PCR (a.k.a. real-time PCR) has the potential to be performed on a large number of samples with minimal labor. Furthermore, quantification of the target agent in a sample is an additional benefit of using a real-time PCR. Recently, our laboratory improved the real-time PCR procedure for PCV-2 by semi-automating the DNA extraction process and reducing the amplification time. In addition, an internal control plasmid was developed and incorporated into the PCR to ensure the accuracy of the test and PCV-2 quantification.

Two DNA extraction methods (Ambion® isolation kit in combination with Kingfisher® magnetic particle processor, and Qiagen® column-based isolation kit) were compared and evaluated for the efficiency of PCV-2 DNA recovery from serum and semen samples. The PCR procedure also was performed on various types of samples (i.e., tissues, serum, semen, and nasal swab) with and without addition of internal control to evaluate the internal control as an indicator of test validity. Furthermore, the internal control plasmid allowed for the quantification of PCV-2.

Ambion® isolation kit with Kingfisher® processor significantly reduced turnaround time without compromising sensitivity and specificity ($p>0.05$) of the PCR as compared to the previous procedure. The use of an internal control did not alter the test result and permitted the detection of putative PCR inhibitors that may interfere with the DNA extraction and amplification process.

Real-time RT-PCR testing for PRRSV:
does viral evolution outsmart molecular diagnostic capability?

Karen Harmon, Amy Chriswell, Kathryn Behrens, Raquel Hansell, Kyoung-Jin Yoon

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Real-time PCR has become a standard diagnostic tool in many veterinary diagnostic laboratories because of its sensitivity, specificity, rapid turn-around time, and capability for high throughput. The ability to detect various bacterial and viral agents directly from clinical specimens in a matter of hours has in many cases led to a dramatic decrease in the time required to make a diagnosis, thus allowing swift implementation of intervention strategies.

One of the purported benefits of real-time PCR is its high degree of specificity. Some types of probes are touted for their ability to distinguish single base pair changes. While in many cases this is an advantage, this high degree of specificity can be a drawback to real-time RT-PCR (rRT-PCR) testing for certain agents. In the case of RNA viruses, which undergo a high mutation rate, genetic areas that were chosen for the assay target because of their highly conserved nature can also undergo changes and fail to be recognized by the primer/probe set. **In our experience, a 1- or 2-base mismatch in the primer or probe binding sites can lead to lack of amplification or fluorescence signal production, resulting in a false negative outcome.** Several times since implementing rRT-PCR testing for PRRSV, our laboratory has needed to redesign the primer/probe set to compensate for the genetic changes we have observed in PRRSV field strains.

In this study, we evaluated the AgPath-ID® NA/EU PRRSV Multiplex Reagent kit (Applied Biosystems Ambion), which detects both North American and European PRRSV strains simultaneously. This kit contains 2 different primer/probe sets for each of the PRRSV genotypes. The use of multiple sets should increase the detection rate of the virus and decrease the incidence of false-negative results. Results from the AgPath-ID® kit were compared to those from our in-house PRRSV rRT-PCR test (ORF7 target) and a previously published gel-based PRRSV RT-PCR test (ORF7 target). Initially, all samples (n = 175) were tested with both real-time assays. Any samples yielding discrepant results were subjected to gel-based RT-PCR (n = 38). All samples that were positive with the ISU rRT-PCR were also positive by the AgPath-ID® kit (n = 66). However, ISU rRT-PCR was negative on some samples that were positive by the AgPath-ID® kit (n = 38). In all cases where the AgPath-ID® kit detected PRRS viral RNA but the ISU real-time assay did not (n = 12), PRRSV RNA was identified with the gel-based RT-PCR assay.

These results emphasize the benefit of using multiple primer/probe sets for real-time RT-PCR in order to decrease the number of false negatives that may arise from genetic variability of field strains.

Integrated high-throughput workflow for concurrent detection of North American and European strains of *Porcine reproductive and respiratory syndrome virus* by qRT-PCR

Angela M. Burrell, Weiwei Xu, Quoc Hoang, Roy C. Willis, Rohan Shah,
Mangkey Bounpheng, Xingwang Fang

Applied Biosystems, Austin, TX, 78744

Porcine reproductive and respiratory syndrome virus (PRRSV) is an RNA virus in the order Nidovirales of the Arteriviridae family. PRRSV is one of the most costly infectious diseases for US swine producers with an estimated annual cost of around $600 million. There are 2 main antigenic subtypes of PRRSV: the North American strain (ATCC VR-2332) and the European strain (Lelystad strain). Infected animals can suffer from both reproductive failure, characterized by abortions, stillbirths, and infertility, and from respiratory disease, often associated with secondary infections.

We have developed an integrated workflow process consisting of high-throughput sample preparation, nucleic acid purification, and qRT-PCR for concurrent detection of North American and European PRRSV. The performance of the workflow was evaluated using 129 field samples with PRRSV status known by RT-PCR. Using Ambion MagMAX™ technology, a rapid high-throughput magnetic bead-based viral RNA isolation method, RNA was isolated from swine serum, cell supernatant, PRRSV vaccine, tonsil, and lung tissues (68 from serum, 34 from tonsil tissue, 17 from lung tissue, 6 from cell supernatant, 4 from PRRSV vaccine), and the purified RNA was used for qRT-PCR using the AgPath-ID chemistry on the Applied Biosystems 7500 Real-Time PCR System. The AgPath-ID NA/EU PRRSV assay can concurrently detect both the American and European strains of PRRSV thus allowing PRRSV subtyping. As few as 50 copies of PRRSV RNA can be consistently detected using the assay. An internal control RNA, XenoRNA containing unique nucleotide sequences, is provided in the reagent kit to monitor nucleic acid purification efficiency, detect presence of reaction inhibitors, and assess functionality of reagents and reaction preparations. The XenoRNA ensures that false negative results are minimized, if not eliminated.

Results of this procedure were 98% concordant with secondary laboratory qRT-PCR results. Furthermore, subtyping of North American and European strains was 100% concordant, demonstrating that this method provides an economical and rapid solution for PRRSV detection and identification.

The kit described is for research use only, it is not for use in diagnostic procedures, and is not licensed by the USDA.

Development, optimization, and validation of a *Classical swine fever virus* real-time RT-PCR assay

Jill Bieker, August Eberling, Barbara Martin, Tammy Beckham

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Classical swine fever (CSF) is a highly contagious viral disease that affects both domestic and wild pigs. The etiological agent of CSF is a small (12.5 kb), enveloped, single-stranded RNA virus belonging to the genus *Pestivirus* of the Flaviviridae family. Other important animal pathogens within the genus *Pestivirus* are *Bovine viral diarrhea virus* (BVDV) of cattle and *Border disease virus* (BDV) of sheep, both of which can naturally infect pigs.

The highly infectious nature of CSF virus (CSFV), as well as the severe economic consequences that would result from a CSF outbreak, make early detection of this disease essential. Instrumental to the control of CSF is a well characterized assay that can deliver a rapid, accurate diagnosis prior to the onset of clinical signs. Implementation of an effective surveillance plan for CSF requires sensitive and specific diagnostic assays that are capable of detecting and identifying the virus early during the course of an outbreak.

The objective of this study was to develop, optimize and validate a real time reverse-transcriptase polymerase chain reaction (rRT-PCR) assay with increased sensitivity and specificity when compared to other CSF rRT-PCR assays currently in use. Primer/probe sets targeting the 5'UTR region of the CSF genome were designed and evaluated. A well characterized panel consisting of 21 CSFV, 5 BVDV, and 6 BDV isolates was developed for utilization in initial feasibility and optimization studies.

The newly developed CSFV assay was initially designed and validated for use on the ABI 7900HT using the Qiagen QuantiTect Probe RT-PCR chemistry. However, the versatility of this new assay was increased by demonstrating equivalency with multiple one-step RT-PCR chemistries and PCR platforms. One-step RT-PCR chemistries that were evaluated included the Qiagen QuantiTect Probe RT-PCR Kit, Qiagen QuantiTect Multiplex RT-PCR No ROX (NR) Kit, and the Invitrogen SuperScript III RT-PCR Kit. Equivalency of the assay was also demonstrated across two 96 well platforms (the Applied Biosystems ABI 7900HT and ABI 7500).

Analytical sensitivity and specificity of the newly designed assay were evaluated against a panel of 176 CSFV, BDV and BVDV isolates (obtained from the European Union Community Reference Laboratory (EU CRL), Hannover, Germany), samples from experimentally infected animals, and in limit of detection studies. Diagnostic sensitivity and specificity were evaluated using known positive (60) and negative (92) nasal swab samples collected from the Dominican Republic (DR) and Colombia, respectively.

Limit of detection experiments showed that the Invitrogen SuperScript III RT-PCR Kit was consistently the most sensitive one-step chemistry for use with the CSF PIADC primer/probe set. Analytical sensitivity of the CSF PIADC assay ranged from <1 to 2.95 $\log_{10}$ $TCID_{50}$/mL on the ABI platforms. Regardless of the chemistry utilized, the diagnostic sensitivity and specificity were 100% with the isolates tested.

The ability to perform this newly developed assay on multiple 96- well platforms provides an increased level of versatility for use in the CSF surveillance programs. In addition, the ability to perform this assay using multiple platform/chemistry combinations will enhance the nation's capabilities for responding to a CSF outbreak.

Comparison of two RNA extraction methods for *Classical swine fever virus* and *Foot-and-mouth disease virus*

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An introduction of classical swine fever (CSF) and/or foot-and-mouth disease (FMD) into the U.S. livestock population will require a rapid, precise and accurate diagnosis. In addition, early detection and high-throughput testing capabilities will be critical for responding to and controlling the spread of disease. Real time reverse-transcriptase polymerase chain reaction (rRT-PCR) assays for FMDV and CSFV have been validated for both single-tube and 96 well platforms. In addition, the versatility of each assay has been increased by demonstrating equivalency on multiple 96-well PCR platforms. The CSF assay is currently being used for surveillance purposes in the National Animal Health Laboratory Network (NAHLN). The FMDV assay will be deployed to the NAHLN when the vesicular disease surveillance plan is completed. Both assays utilize template RNA that is extracted via a single-tube extraction method (Qiagen RNeasy® Mini-Kit). In order to adapt these assays to a high-throughput testing capability, an efficient 96-well RNA extraction procedure that is readily adaptable to automation is needed.

The objectives of this study were to evaluate and optimize the performance of a 96-well RNA extraction procedure for CSFV and FMDV (Ambion MagMAX™-96 Total RNA Isolation Kit) and demonstrate equivalency between the currently accepted, single tube (Qiagen RNeasy® Mini-Kit) and 96-well extraction procedures.

Analytical and diagnostic performance characteristics of both extraction techniques were evaluated using tissue culture grown CSFV and FMDV. Diagnostic performance characteristics were evaluated utilizing samples collected from experimentally infected animals. The analytical sensitivity of the 96-well extraction method was equal to that of the single-tube extraction method. Diagnostic sensitivity was examined using samples collected from animals that were experimentally infected with CSFV (tonsils, tonsil scrapings and nasal swabs) and FMDV (epithelium and oral swabs). Statistical analysis indicated excellent correlation between the single tube and 96-well extraction methods with all sample types that were analyzed. In addition, when utilizing the 96-well method, a high correlation between operators was demonstrated.

The results obtained in this study indicate that there is no significant difference between the performance characteristics of the 96-well extraction method and those of the single tube extraction method. Acceptance of this rapid, automation-capable, high-throughput 96-well RNA extraction method will increase the nation's preparedness for response to a CSF and/or FMD outbreak. In addition, this method will enhance the efficiency and processing time for samples submitted as part of the CSF and the future vesicular disease surveillance programs.

A visual DNA chip for identification of different genotypes of *Foot-and-mouth disease virus*

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Gordon B. Ward, Brenda C. Donahue, Mary A. Kenny, Ming Y. Deng

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Foot-and-mouth disease (FMD) is a highly contagious viral disease that has a great potential for causing severe economic loss in susceptible cloven-hoofed animals. FMD virus (FMDV) is a member of the genus *Aphthovirus* in the family of Picornaviridae. There are 7 serotypes of FMDV: A, O, C, Asia 1, and South African Territories (SAT) 1, 2, and 3. Infection with any 1 serotype does not confer immunity against another. Within the serotypes, many subtypes can be identified. It is difficult to differentiate genotypes of FMDV in a routine diagnosis unless an analysis of nucleic acid sequences is conducted.

In this study, we developed a visual DNA chip for subtyping FMDV. Sixty-three synthesized FMDV genotype-specific oligonucleotide probes (20~30 mers) were spotted and immobilized on a single polyvinylchloride (PVC) chip to capture specific targets in the specimen. RNA of cell culture-grown virus was extracted with a QIAamp Viral RNA Mini Kit (Qiagen Inc., Valencia, CA) and subjected to a single-tube reverse transcription-polymerase chain reaction (RT-PCR) for FMDV. Both the forward and reverse PCR primers were biotinylated. A 5-µL portion of the product of the RT-PCR was used for a hybridization conducted on the oligonucleotide probe-immobilized PVC chip. A biotin-avidin alkaline phosphatase indicator system and NBT (nitro blue tetrazolium chloride)/BCIP (5-bromo-4-chloro-3-indolyl phosphate, toluidine salt) substrates were used in the colorimetric development. A perfectly matched probe-target hybrid, if present, forms a visible blue-purple precipitate on the PVC chip. Hybridization patterns of different genotypes of FMDV on the PVC chip were then observed and interpreted visually.

With this method, 63 different genotypes of FMDV could be identified including O/TAW/97, O/TAW/99, O_1/Campos, O_1/Manisa, A5, A12, A22, A24, A30, C1, C3, Asia 1, SAT 1, SAT 2 and SAT 3 subtypes. No cross-reaction was observed with other viral agents causing vesicular diseases such as *Vesicular stomatitis virus* or *Swine vesicular disease virus*.

This diagnostic tool is potentially useful for rapid detection and identification of different genotypes of FMDV. It is simple and rapid. No sophisticated equipment is needed.

Multiplexed *Foot-and-mouth disease virus* DIVA assay

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Liquid array technology allows simultaneous measurement of the relative responses of multiple signatures to a challenge sample. We have previously reported proof-of-principle of a multiplexed non-structural protein serological assay to differentiate *Foot-and-mouth disease virus* (FMDV) infected and vaccinated animals (differentiating infected from vaccinated animals; DIVA assay).

The current multiplexed assay consists of synthetically produced peptide signatures 3A, 3B and 3D and recombinant protein signatures 3ABC and 3D in combination with 4 controls. We have extensively evaluated the multiplexed assay against diagnostically relevant samples (n >4000) to determine the diagnostic specificity and sensitivity of each signature in the multiplex. The samples used for evaluation include FMDV naïve samples to determine specificity, vaccinated samples, samples from vaccine / challenge experiments, and sera panels to evaluate sensitivity. In each case, the samples were previously evaluated using a number of commercially available tests for comparison. The multiplexed assay shows >98 % diagnostic specificity when all signatures are evaluated as a whole. When evaluated against a serum panel (n = 36) to determine diagnostic sensitivity, the 3ABC signature in the multiplexed assay showed comparative sensitivity to a commercially available non-structural protein 3ABC ELISA. Moreover, the response of the 3A, 3B and 3D signatures give increased confidence in results, all in 1 assay. We have also carried out experiments to determine reagent stability (~ 6 months) and reagent reproducibility, along with optimization of assay conditions.

The encouraging performance of the multiplexed assay against diagnostically relevant samples promotes field evaluation to generate an assay for routine use in FMDV surveillance.

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Anatomic Pathology Scientific Session

Saturday, October 20, 2007

Bonanza BC

Moderator: Bill Layton

Coxiella-like infection in psittacines

Hulimangala L. Shivaprasad, Santiago S. Diab, Robert Nordhausen,
Maria Belen Cadenas, Edward Breitschwerdt

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Coxiella burnetii is an obligate intracellular bacterium that causes Q fever in people. These bacteria have been identified in many species of mammals, birds, reptiles and arthropods. In mammals, such as cattle, sheep and goats, *C. burnetii* causes abortions and neonatal deaths with placentitis and occasionally granulomatous hepatitis and interstitial pneumonia (particularly in goats). **Antibodies to *Coxiella* have been demonstrated in birds, but disease attributed to *Coxiella* has not been described before.**

Two psittacines, a 1-year-old female golden mantle rosella (*Platycercus eximius cecilliae*) and a 2-year-old female hawk-headed parrot (*Deroptyus accipitrinus accipitrinus*) were diagnosed with coxiellosis based on sequencing of 16S rRNA from the livers of the birds. The birds belonged to different owners, and both birds exhibited lethargy and weakness for several days before they died. Post mortem examination revealed that the birds were slightly emaciated and the livers and spleens were greatly enlarged. In addition, the livers were mottled pale. Microscopically there were multifocal necrosis of hepatocytes with infiltration of a mixed population of inflammatory cells including lymphocytes, heterophils, plasma cells and macrophages randomly scattered throughout. Within the macrophages, vacuoles compressed the nucleus and, when stained by Giemsa, were seen to contain basophilic small coccobacillary organisms measuring about 0.5 to 1 μm. The spleen had increased numbers of mononuclear phagocytic cells, some of which had vacuoles that contained organisms similar to those in the liver. There was similar inflammation in the epicardium, interstitium of the lungs, kidney, adrenal and bone marrow associated with similar organisms.

Transmission electron microscopy of the liver revealed some round to oval organisms and others elongated, measuring 0.2 μm in diameter and 0.7 μm in length. Most of these organisms contained electron-dense wispy material and were covered by a complex of tri-laminar membranes.

The birds were negative for *Chlamydophila* by fluorescent antibody technique, for aerobic bacteria including *Salmonella* by culture, and for *Avian paramyxovirus 1* and avian influenza virus by PCR.

Neuritis in a white leghorn chicken: Marek's disease or peripheral neuropathy?

Hulimangala L. Shivaprasad, Isabel Gimeno

California Animal Health and Food Safety Laboratory System, Fresno Branch, School of Veterinary Medicine, University of California, Davis (Shivaprasad); Departments of Population Health and Pathobiology, College of Veterinary Medicine, North Carolina State University, Raleigh, North Carolina (Gimeno)

Neuritis, a common condition in chickens, can be caused by infectious and non-infectious etiologies. Among infectious causes, Marek's disease virus (*Gallid herpesvirus*, an α-herpesvirus) induced neuritis is most common. *Reticuloendotheliosis virus* (REV), a retrovirus, can also cause neuritis in chickens. Among non-infectious causes, riboflavin (vitamin B2) deficiency is most common. Peripheral neuropathy characterized by edema and infiltration of a few lymphocytes and plasma cells has been described in certain lines of white leghorn (WLH) chickens and strongly influenced by major histocompatibility complex (B-complex).

An adult WLH rooster with a history of chronic lameness, such as ataxia, hopping on one leg, and inability to get up and eat and drink for the last 3 weeks before death, was presented for necropsy. There were no signs of central nervous disturbances in the chicken. The chicken was from a small backyard flock and had not been vaccinated for any common poultry diseases including for Marek's disease. Necropsy revealed the chicken was moderate to severely emaciated and weighed 1080 g. Both the right and left sciatic nerves were moderately enlarged and edematous. Histopathology of the nerves revealed moderate to severe interfibrillar edema, mild multifocal perivascular cuffing by lymphocytes and a sprinkling of lymphocytes and plasma cells scattered here and there. The brain, spinal cord, spinal ganglia and optic nerves were not affected, nor was there any evidence of lymphoma in any organs.

Frozen liver and nerves examined for Marek's disease virus were negative by real time polymerase chain reaction (PCR) specific for serotype 1 Marek's disease virus genes, *gB* and pp38 and by immunohistochemistry of the liver for identifying Marek's disease virus oncogene, *meq*. DNA extracted from the liver and examined by PCR was negative for REV and *Avian leukosis virus* subgroups, A, B, C, D, E, F and J. The bird was negative for *Mycoplasma* sp. and aerobic bacteria including *Salmonella* by culture, and for *Avian paramyxovirus-1* and avian influenza virus by PCR.

Based on histopathology of peripheral nerves in a WLH chicken and negative result for Marek's disease virus, REV and ALV–J, peripheral neuropathy was suspected in the chicken. The etiology of peripheral neuropathy is not known but an autoimmune cause has been suggested.

Peracute mass mortality of western grebes due to underwater primary blast trauma

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In the spring of 2006, 70 dead adult western grebes were discovered along a 1 mile stretch of beach at the high tide line in San Diego, CA. City, county and federal responders collected the dead birds and transported 10 to the County Animal Disease Diagnostic Laboratory and Sea World for testing.

All birds were in good nutritional and good post-mortem conditions. Virtually identical lesions were present in the birds, and consisted of abundant coagulated blood within the oral cavity, coelom, and thorax, Glisson's capsule rupture, and severe pulmonary hemorrhage. Cardiac lesions varied from bilateral rupture of the atria in one bird to transmural ventricular hemorrhage in another. Three of the 10 birds examined had hemopericardium. The esophagus and ventriculus of some birds contained recently ingested top smelt (*Antherinops affinis*). Radiographs of 4 birds did not reveal any broken bones, however, angiography of 2 birds indicated rupture of the renal veins. Microscopic examination demonstrated acute hemorrhage in multiple organs with no predisposing diseases. Viral cultures for paramyxoviruses and orthomyxoviruses were negative, as were PCR tests for WNV and *Chlamydophila psittaci*.

Primary blast trauma was determined to be the cause of death, although, the spectrum of lesions differed from typical mammalian primary blast injuries. This case illustrates the need for disparate agencies to work in a coordinated manner to safely and efficiently determine the cause of mass animal casualties that may be the first indication of emerging diseases, natural or man-made disasters, or terrorist attacks.

Severe, soft-tissue mineralization in bullfrog larvae from wastewater treatment wetlands

Kevin Keel, Aina Ruiz, Aaron Fisk, John Maerz

Southeastern Cooperative Wildlife Disease Study, College of Veterinary Medicine (Keel), and Warnell School of Forestry and Natural Resources (Ruiz, Maerz), University of Georgia, Athens, GA, USA; Great Lakes Institute for Environmental Research, University of Windsor, Windsor, Ontario, Canada (Fisk)

Constructed and naturally occurring wetlands are increasingly used for tertiary treatment of wastewater ultimately reclaimed for distribution through municipal water supplies. A perceived added benefit of such facilities is the production or preservation of wildlife habitat.

Wastewater treatment wetlands at one site in Georgia were surveyed to determine relative abundance and health of tadpoles. Effluent from this wastewater treatment facility is divided into 3 streams, each passing through a separate series of wetlands. Tadpoles were abundant, but many bullfrog larvae (*Rana catesbeiana*) had severe lesions consisting of large (up to 1 cm diameter), mineralized nodules protruding from the tail or gular region. The prevalence of lesions decreased with increased distance from the wastewater discharge site.

Sectioning of formalin-fixed specimens revealed more extensive mineralization involving the vertebrae or muscles of the head and tail. Nodules examined microscopically were not associated with parasitic or infectious agents, and individual connective-tissue fibers and muscle cells were also mineralized. The nodules consisted entirely of calcium phosphate.

Total serum calcium concentrations of tadpoles, and calcium concentrations in water samples did not differ significantly in wetlands with increasing distance from the discharge site. **The only significant finding to date is elevated vitamin D levels in tadpoles with mineralization**. At this time, the primary cause of elevated vitamin D levels is undetermined but it appears to be remediated by passage of water through the wetlands. However, based on reduced fitness of affected tadpoles, **the argument for wastewater treatment wetlands as habitat beneficial for wildlife may be inappropriate**.

An epizootic of stomatitis, rhinitis, tracheitis, and bronchopneumonia in captive white-tailed deer in Mississippi

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An epizootic of stomatitis and upper respiratory disease accompanied by high death losses occurred in juvenile offspring of captive white-tailed deer. The original breeding stock was assembled in 2005 from 3 different geographic regions of Mississippi, specified as Loess, Lower Coastal Plain, and Delta. Four juvenile animals were recognized with mouth lesions or swollen jaw during August and September, 2005. Selected necropsy examination revealed stomatitis with necrosis and mixed aerobic and anaerobic bacterial infection. During the 2006 fawn season, death losses increased markedly in juveniles in all 3 contiguous pens.

Excluding deaths due to capture and trauma, 43 juvenile deaths occurred in fawns ranging from 9-160 days old in the interval from August 28 to December 26, 2006. Lesions included mild to severe stomatitis (24 fawns); rhinitis, tracheitis, bronchitis (16), bronchopneumonia (38); 20 fawns had lesions in at least 2 of these preferential sites. Hemorrhagic disease was excluded in most cases (33/43 negative); epizootic hemorrhagic disease (EHDV-2) was diagnosed in only one juvenile and one adult. Histologic suggestion of viral rhinitis and tracheitis was evidenced by epithelial deciliation and desquamation or squamous metaplasia, with variable amphophilic intranuclear inclusion bodies suggestive of adenovirus.

Eight suspect cases were evaluated by IHC for adenoviral hemorrhagic disease (AHD) by UC Davis (Dr. Leslie Woods) and were negative. A 144-day-old juvenile that died early in the course of disease had focal stomatitis with necrosis, nephritis, rumenitis, enterocolitis and widespread lymphocyte depletion. A portion of the genome of a unique adenovirus was PCR amplified from spleen and sequenced by Dr. Larry Hanson. The sequence was clearly distinct from AHD published sequence. Kidney was strongly positive by IHC using a polyclonal anti-adenoviral human antibody, but negative with anti-AHD antibody. **Adenoviral virions were confirmed by electron microscopy in renal tubular epithelial cells.** Stomatitis lesions are pathogenetically reminiscent of those seen in AHD outbreaks in western states and experimental studies. No deaths occurred past 160 days of age suggesting cessation of viral infection following maturation of the immune system. **Conclusive proof of the contribution of adenovirus is lacking and further studies are in progress.**

Detection of PrP^{CWD} in rectal lymphoid tissues in postmortem and live Rocky Mountain elk (*Cervus elaphus nelsoni*), a possible preclinical test for CWD

Terry R. Spraker, Thomas Gidlewski, Kurt VerCauteren, Aru Balachandran, Randy Munger, Lynn Creekmore, Katherine O'Rourke

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Introduction. 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. These biopsies are then stained with specific antibodies for the abnormal prion protein associated with CWD. A live animal test has not been described for Rocky Mountain elk (*Cervus elaphus nelsoni*).

Material and methods. The utility of examination of the rectal mucosal lymphoid tissues for detecting chronic wasting disease (CWD) was investigated in Rocky Mountain elk. Postmortem rectal mucosal sections were examined from 308 elk from two privately-owned herds that were depopulated. The results of the postmortem rectal mucosal sections were compared to immunohistochemical (IHC) staining of the brain stem, retropharyngeal lymph nodes and palatine tonsil. Approximately 450 captive elk from 3 ranches that had previous cases of CWD were examined by rectal biopsy in 2006 and 2007. All elk were biopsied 2-3 times.

Results. Seven elk were found positive using the brain stem (dorsal motor nucleus of the vagus nerve), retropharyngeal lymph nodes and palatine tonsil. Six of these elk were positive in postmortem rectal mucosal sections. 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 postmortem rectal mucosal sections. Approximately 450 live elk from 3 CWD infected farms were biopsied over a 2-year period. Most elk were biopsied twice over this 2-year period. Seven infected elk with no or minimal clinical signs were found. The lymphoid follicle count decreased with age, starting about 5-6 years of age.

Discussion/Conclusion. The use of rectal mucosal lymphoid tissues may be suitable for a live animal diagnostic test as part of an integrated management strategy to limit CWD in elk. There are several limitations to this test. The exact time at which the rectal lymphoid tissues become positive post exposure is not known. The apparent decrease in follicles after the age of 6 years does cause some problems, however most cases of CWD in captive elk are detected before an animal is 6 years old.

Detection of PrP^{CWD} in retinal tissues in white-tailed deer (Odocoileus virginianus) and Rocky Mountain elk (*Cervus elaphus nelsoni*) with CWD

Terry Spraker, Thomas Gidlewski, Justin Greenlee, Delwyn Keane,
Amir Hamir, Katherine O'Rourke

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Introduction. Chronic wasting disease (CWD), a transmissible spongiform encephalopathy, has been reported in captive and free-ranging mule deer (*Odocoileus hemionus hemionus*), white-tailed deer (*Odocoileus virginianus*) and Rocky Mountain elk (*Cervus elaphus nelsoni*). An abnormal isoform of a prion protein (PrP^{CWD}) that has been associated with CWD has been reported in numerous internal organs other than the brain and lymphoid tissues, including the retina of mule deer. The objective of this project was to investigate the possibility of PrP^{CWD} in the retina of white-tailed deer and Rocky Mountain elk with CWD.

Materials and methods. Eyes from 80 captive white-tailed deer that were killed during a depopulation program were collected and placed in 10% neutral buffered formalin. None of these 80 white-tailed deer showed any clinical signs suggestive of CWD prior to euthanasia (gun shot); however 79% were positive for CWD by using immunohistochemical staining of the brain stem and head lymphoid tissues. Eyes from 7 captive and 2 research elk were collected and placed in Davison's fixative. Clinical signs typical of CWD were observed in 5 elk (3 with M/M genotype, 1 M/L and 1 L/L). The other 4 were non-clinical (3 with genotype M/M and 1 L/L), but did have a previous positive rectal biopsy. The globe from each animal was trimmed and embedded in a single paraffin block and sectioned at 5 μm. Tissue sections were stained with H&E and immunostained with Anti-Prion 99 and P4 for the detection of PrP^{CWD}.

Results. Prion was only found in the retina, all other regions of the eye (cornea, lens, ciliary body, iris, choroid, sclera) were free of PrP^{CWD} in the deer. Examination of the eyes from the white-tailed deer revealed 4 to have detectable PrP^{CWD} within the retina. PrP^{CWD} was restricted to the inner and outer plexiform layers of these deer. Sections from all 9 elk had PrP^{CWD} in 8 of the 10 retinal layers and in the optic nerve. All other regions of the eye were free of PrP^{CWD}. The most prominent features in the elk were heavy PrP^{CWD} staining in the inner and outer plexiform layers with minimal intracytoplasmic staining in ganglion cells in the M/M and M/L elk. The 2 L/L elk had minimal PrP^{CWD} staining in the plexiform layers, but relatively heavy staining in the cytoplasm of ganglion cells and an unusual accumulation of PrP^{CWD} just inside outer limiting membrane layer. An occasional ganglion cell within the ganglion cell layer contained an intracytoplasmic vacuole in the M/M elk.

Discussion/Conclusion. Deer and elk do have an abundance of PrP^{CWD} in retinal tissues and optic nerve (elk). This accumulation of PrP^{CWD} may affect vision especially in elk. Genotypes did result in different patterns of PrP^{CWD} accumulation in elk. The LL genotype at codon 132, which has a prolonged incubation period, had much less PrP^{CWD} in the retina, especially within the inner and outer plexiform layers. In addition, the LL elk seemed to have more intracytoplasmic staining within ganglion cells as compared to the MM and ML elk.

Plague in mountain lions (*Puma concolor*) from the greater Yellowstone area of Wyoming

Todd Cornish, Cynthia Tate, Rosemary Jaffe, Toni Ruth, Howard Quigley, Brian Parrie, Terry Kreeger, Amy Boerger-Fields, Ken Mills

Wyoming State Veterinary Laboratory, University of Wyoming, Laramie, WY (Cornish, Parrie, Boerger-Fields, Mills); Wyoming Game and Fish Department, Laramie, WY (Tate); Montana Fish, Wildlife, and Parks, Bozeman, MT (Jaffe); Wildlife Conservation Society, Gardiner, MT (Ruth); Beringia South, Kelly, WY (Quigley); Wyoming Game and Fish Department, Wheatland, WY (Kreeger)

Plague (*Yersinia pestis* infection) was diagnosed in 4 radio-collared mountain lions that died in the summer and fall of 2005 and 2006 in the greater Yellowstone area of Wyoming. The 4 cases, submitted to the Wyoming State Veterinary Diagnostic Laboratory, included a female lion and one of her cubs. A second cub was found alive and never developed clinical signs of disease, although it had a plague titer of 1:512 via fluorescent antibody testing. The other 2 cases were a 10-year-old female pregnant with near-term triplets, and a young adult male.

The 3 adult lions were in good nutritional condition, suggesting that the course of disease was rapid. Gross lesions included pneumonia in all cases with lung available, and cervical lymphadenitis in all cases with lymph nodes available. Microscopically, affected mountain lions had corresponding suppurative to necrotizing bronchointerstitial pneumonia and/or suppurative and necrotizing lymphadenitis with abundant intralesional coccobacilli.

The diagnosis was confirmed in all animals by bacterial culture and identification and either by fluorescent antibody test or immunohistochemistry for *Y. pestis*. These 4 cases contradict the general impression that mountain lions are relatively resistant to plague, and suggest that **plague may be a more common cause of mortality in this species than previously recognized**. These cases also highlight the need for appropriate personal protective measures for wildlife professionals and hunters when handling mountain lion carcasses in the laboratory or the field.

Molecular Diagnostics Scientific Session

Saturday, October 20, 2007

Ponderosa A

Moderator: Glenn Songer

* Graduate student presentation

Real-time PCR testing of pooled (1:5) fecal samples for MAP compared to HEYM culture

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Introduction. Increased sample submission for laboratory testing for the detection of *Mycobacterium avium* subsp *paratuberculosis* (MAP) in bovine fecal samples has necessitated utilization of techniques to enhance efficiency of MAP detection. Pooling of fecal samples offers a method to enhance diagnostic efficiency when coupled with real-time PCR (RT-PCR) with minimal loss of test sensitivity. Most importantly, the action cut-point for the identification of individually infected cattle can be adjusted to account for herd prevalence and for owner management decision making.

Materials and methods. Individual fecal samples from 736 cows in 4 dairy herds were processed by standard techniques for the detection of MAP and 1:5 pools were created concurrently for both culture and for RT-PCR with the Tetracore Vet Alert™ Johne's Real-Time PCR assay. All individual and pooled fecal samples were cultured using the standard 3-day culture protocol with 4 tubes of HEYM. The 1:5 pools were created by transfer of 5 mL of each standard fecal water tube-step to a 50 mL conical tube. From the 25 mL of pooled fecal water tube, 5 mL were transferred to 25 mL of BHI and incubated overnight; centrifuged at 900*g* for 30 min and the pellet was re-suspended in 1 mL of antibiotic brew on day 2 and incubated overnight. On day 3, the sample was vortexed and approximately 200 µL inoculated on the surface of each of 4 tubes of HEYM with mycobactin J. The remaining 20 mL of the pooled fecal water tube were centrifuged at 900*g* for 30 minutes, decanted and the pellet re-suspended in water and processed according to manufacturer's recommendations for RT-PCR. Samples tested by RT-PCR were assayed in duplicate wells.

Results. Of the 148 pooled fecal samples representing 736 individual cows, 34 pools were RT-PCR **positive on both wells**; all individual samples within 19 of the 34 positive pools were tested, and 18/19 (95%) contained at least 1 RT-PCR positive individual sample. Culture identified MAP in 14/34 (41%) of the RT-PCR positive pools, and in 26/170 (15.2%) of the individual samples from those 34 pools. Only 1 pool was RT-PCR positive where all individual samples were both RT-PCR negative and culture negative. Of the 31 culture-positive fecal samples among the 736 tested, only 1 individual fecal sample with a colony count of 1, 0, 0, 0 was not detected by either RT-PCR or by culture in the 1:5 pool.

An additional 24 pools had **1 of 2 wells positive** on RT-PCR. These represented lower concentrations of MAP with Ct values between 37 and 42, the cut-off value for a negative sample. Of 9 positive pools with 1 positive well and all individual samples tested by RT-PCR, 8/9 (89%) had at least 1 positive individual sample. Only 4 (3.5%) individual fecal samples within the 115 fecal samples represented by the 24 pooled samples were culture positive, all were low shedders and 3 of 4 were in 1 pooled sample.

Conclusion. Testing pooled fecal samples with a commercially available RT-PCR offers an economical, flexible, rapid and exquisitely sensitive method to identify those MAP-infected cattle at the greatest risk to spread MAP infection to herdmates. Only individual samples in pools with the highest concentration of MAP (lowest Ct values) need to be tested for MAP, thus significantly reducing the testing cost for the herd.

Acknowledgements. Financial support for this work was provided by the USDA Agricultural Research Service Cooperative Grant (58-1265-3-115) and by USDA-APHIS-VS field studies funding.

A streamlined workflow for rapid and sensitive detection of *Mycobacterium avium* subsp. *paratuberculosis* (MAP) in bovine fecal samples by real-time PCR

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Johne's disease is a chronic wasting disease of ruminants, caused by an incurable infection of the intestinal tract by the bacterium *Mycobacterium avium* subsp. *paratuberculosis* (MAP). Currently, the laboratory standard for diagnosing Johne's disease is fecal culture. Cultures of samples taken from a herd's environment are also used to identify a herd's overall risk for Johne's disease. Unfortunately, due to the slow growth of the MAP organism, cultures typically take between 6 and 16 weeks to determine MAP presence. This allows more time for infected animals to shed MAP into the environment, increasing the risk of infection to healthy animals in the same herd.

Quantitative real-time PCR (qPCR) provides a much faster solution for the detection of MAP from animal feces with results available within hours. Applied Biosystems has developed an integrated workflow, consisting of streamlined sample preparation, high throughput nucleic acid isolation and purification, and qPCR for detection of the bovine strain of MAP in bovine fecal samples as well as environmental samples from cow alleyways and manure pits. The MAP DNA isolation protocol combines the use of chemical and mechanical cell lysis with the Ambion® MagMAX™ technology - a rapid, high-throughput magnetic bead-based nucleic acid isolation and purification method - to purify MAP DNA with minimal reaction inhibitors commonly found in animal feces. The purified nucleic acid is analyzed using our AgPath-ID™ MAP reagent kit on the Applied Biosystems 7500 Real-Time PCR system. To minimize false negatives, our MAP assay contains XenoDNA, a unique nucleotide sequence that serves as an internal process control to monitor the presence of qPCR inhibitors and reagent functionality.

Using 80 field samples with known MAP culture status, our workflow method resulted in 100% sensitivity and specificity, out-performing the currently available commercial fecal DNA isolation methods and MAP qPCR. In addition, we identified all samples in the 2007 NVSL Johne's Check Test with 100% accuracy using our MAP detection method. We also tested 87 bovine fecal samples that grew ≤1 colony of MAP per culture tube on Herrold's egg yolk medium with mycobactin J and antibiotics (HEY) resulting in 78 samples detected as MAP positive samples by our method. With our method 17 of 19 fecal samples with growth of ≥100 MAP colonies on HEY culture were easily distinguishable from all fecal samples we tested that grew ≤30 MAP colonies on HEY culture (n=145). This allows samples with high concentrations of MAP to be distinguished consistently from medium to low MAP-positive samples, providing reliable data for determining the risk that an animal presents to the rest of a herd.

Performance of our MAP detection protocol was evaluated using 409 field samples of unknown status that were later tested by HEY culture at an independent NVSL certified laboratory. For high and medium-low MAP shedders (≥3 colonies MAP/tube on HEY culture, n = 47), this evaluation resulted in 97.9% sensitivity and 100% specificity for our MAP detection method when using HEY culture as the "gold standard." HEY culture results for low MAP shedders (<3 colonies MAP/tube on HEY culture, n = 80) and cultures with no MAP growth (n = 282), show that our MAP detection method for these samples has a sensitivity of 87.5% and a specificity of 55.7%. We believe that the low specificity is due to the higher sensitivity of our method compared to HEY culture. In conclusion, **our workflow provides a reliable method to identify animals shedding MAP within 4 hours of sample collection.**

Evaluation of the AnDiaTec ParaTub® Immunomagnetic Separation-PCR for the high-throughput detection of *Mycobacterium avium* subsp. *paratuberculosis* in bovine milk

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Mycobacterium avium subsp. *paratuberculosis* (MAP) is the etiologic agent of paratuberculosis, also known as Johne's disease, a chronic and incurable enteric disease affecting cattle and other ruminants. The disease occurs throughout the world and the economic losses to dairy and beef cattle producers are severe. Animals most commonly become infected in the first weeks of life via colostrum, contaminated milk or feces. After a prolonged incubation period, typical diarrhea, progressive emaciation and decreased milk production develop. During the incubation period, MAP is shed intermittently in lower numbers in feces and milk; in contrast, clinically infected animals can shed as many as 5×10^{12} MAP organisms per day. Clinically affected animals maintain a regular appetite and normal body temperature at the onset of disease, and may go undetected. Consequently, infected animals can introduce MAP into the environment via feces and into the food chain via milk and meat.

Molecular techniques such as PCR could potentially provide a rapid means of detecting MAP. However, due to inhibitory substances in feces (the most commonly used sample matrix), the PCR reaction is often not able to proceed under optimal conditions to maximize sensitivity. Further, all of the so far described PCR methods are labor- and time-intensive and thus not suitable as rapid routine diagnostic systems for large number of samples.

The AnDiaTec *ParaTub-SL*® IMS-real time PCR is an assay for automated high-throughput detection of MAP in raw milk of naturally infected dairy herds. The screening method is capable of analyzing the status of a herd for paratuberculosis (analysis of bulk raw milk samples) and also detecting single animals that are Johne's disease positive (symptomatic and asymptomatic). In this test, MAP are isolated within 45 min from milk by high-throughput immunomagnetic bead separation using an automated magnetic particle pipetting robot (modified Hamilton liquid handling station, commercially available), and released subsequently for analysis directly into PCR amplification mixtures for real time PCR (microtiter plate real time PCR machines). The threshold detection level of the test as determined with artificially contaminated samples is 2-5 bacteria/mL raw milk. The specificity was proven with 19 MAP strains from cattle, deer and moufflon and 34 non-MAP organisms, all of which were non-reactive in the test.

The test kit was evaluated in several experimental series at high-throughput with bulk milk samples that were tested in parallel with culture (feces and milk) and serology (using commercial serologic ELISAs). The evaluation studies demonstrated that the sensitivity of the PCR test is superior to fecal and milk culture by 21% and 22.5%, respectively, and sufficiently sensitive to detect single shedders in pooled milk samples from up to 30 animals (as judged by single-animal milk screening). Further it could be shown for 14 animals that MAP organisms were detected in milk prior to detection of seroconversion by any of the 3 commercial antibody ELISA kits. **The experiments proved that the newly developed test is sensitive, specific and fast and thus, for the first time allows the standardized large-scale routine MAP screening of bulk milk samples at acceptable cost.**

Development of a real-time PCR (rt-PCR) assay to detect *Tritrichomonas foetus* in cattle

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Tritrichomonas foetus is a sexually transmitted protozoan parasite of cattle. Infection is spread by infected bulls that become chronic carriers. Cows recover but can become reinfected by breeding to carrier bulls. *T. foetus* infection can lead to lower fertility and decreased calving rates causing significant production loss. The slaughter of carrier bulls also causes substantial economic loss to ranchers.

Identification of infected bulls has been challenging due to limited methods available for the detection of *T. foetus*. Direct microscopic examination of preputial scrapings grown in selective enrichment media (TF Inpouch, Biomed Diagnostics) for the presence of *T. foetus* has been the standard method of detection. With the development of a molecular assay to detect *T. foetus*, confirmation and speciation of positive cultures using PCR has become the diagnostic tool of choice in most veterinary diagnostic laboratories.

We undertook a validation study to enable the New Mexico Department of Agriculture, Veterinary Diagnostic Services (NMDA-VDS), to bring on line a novel real-time PCR (rt-PCR) assay with an internal positive control (IPC). The validation study compared results for the same preputial samples tested by enrichment culture followed by microscopic examination, the standard PCR method currently in use, and the new rt-PCR assay developed at NMDA-VDS. The IPC included in the new method ensures the differentiation between a true negative test and a failed PCR reaction, further reducing the rate of false negative results due to inhibition. Although both assays are specific to *T. foetus*, the rt-PCR assay is more sensitive (1.5 copies of the *T. foetus* genome). Due to the large number of samples submitted seasonally in New Mexico, a 96-well high-throughput method was optimized for DNA extractions, decreasing turnaround time. Real-time PCR detected 0.125 organisms compared with 1.25 organisms detected by standard PCR. Of 100 preputial samples tested, 7 positive samples were detected by microscopic examination compared with 20 positive samples detected by rt-PCR; as well, microscopic examination alone cannot speciate trichomonads. The increased sensitivity of the rt-PCR over standard PCR, and the greater specificity of rt-PCR over microscopic examination, reduced the false negative rate and increased the positive rate.

Results of our validation comparing standard methods of detecting *T. foetus* with rt-PCR demonstrate that rt-PCR is a superior method of testing for *T. foetus* in cattle. Implementing real-time PCR as the standard testing method will lead to better control of trichomoniasis in cattle.

Pooled PCR to detect *Tritrichomonas foetus* in beef bulls

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Preputial scraping samples from 305 mixed-breed beef bulls were examined for the detection of *Tritrichomonas foetus* infection. All samples were collected by veterinarians and transported in commercial media to an accredited lab.

Upon arrival, samples underwent microscopic examination for the presence of *Tritrichomonas foetus* and were then incubated for 3 additional days at 37°C before final microscopic examination. *Tritrichomonas foetus* infection was detected in 14 bulls by culture. After final microscopic examination, the 305 samples were randomly grouped into pools of 5, technicians were blinded as to culture results of the individual samples constituting each pool. From each sample within a group of 5, 250 µL of fluid sediment were removed and pooled to form 61 pools; 200 µL were then removed from each pool for PCR. PCR detected 16 positive pools; 2 positive samples were identified by individual PCR on samples previously diagnosed by culture as negative. When referenced to culture, the 95% confidence interval for sensitivity PCR pools to detect *Tritrichomonas foetus* was found to be 76.8% to 100% (mean value 100%) and the CI of specificity was 85.5% to 99.5% (mean value 93.4%). If referenced to individual PCR, all of the pools that were positive by individual PCR were also positive in the pool of 5, while none of the pools falsely detected evidence of the organism.

PCR testing of *Tritrichomonas foetus* samples in pools of 5 showed no loss of sensitivity compared to PCR testing of individual samples. Single PCR showed a greater sensitivity to detect *Tritrichomonas foetus* than a single culture, and this study indicates that no loss in sensitivity of PCR occurred when samples were pooled in groups of 5. **PCR testing of pools of 5 samples for the detection of *Tritrichomonas foetus* appears to be a viable alternative to individual culture or individual PCR.**

Development and comparison of a fluorescent microsphere immunoassay with the virus neutralization test for the detection of antibodies to *Equine arteritis virus*

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Equine arteritis virus (EAV) is the causative agent of equine viral arteritis (EVA), a respiratory and reproductive disease of horses. Serological and clinical studies indicate that EAV is widely distributed in equine populations around the world. In more recent years, EAV infection has increasingly been recognized in a number of countries and in multiple states in the United States.

Currently, diagnosis of EAV infection is based on detection of the virus either in cell culture or by serologic testing of paired (acute and convalescent) sera. The virus neutralization test (VNT) is the OIE prescribed test for international trade for detection of antibodies to EAV. The assay is used for diagnosis, surveillance, trade, and pre-vaccination monitoring purposes. Although highly sensitive and specific, the assay is expensive, labor intensive and time consuming. Accordingly, our primary objective was to develop a rapid, sensitive and reliable immunological assay to detect antibodies to EAV in equine sera.

A microsphere immunoassay (MIA) was developed to detect equine antibodies to the major structural proteins of EAV. We cloned and expressed full-length individual major structural proteins [GP5 (aa 1-255), M (1-162) and N (1-110)] as well as the partial length of each structural protein [GP5 (1-116), GP5 (75-112), GP5 (55-98), M (88-162) and N (1-69)] comprising putative antigenic regions of the virus. Purified recombinant proteins were covalently bound to fluorescent polystyrene microspheres. After incubation with diluted equine serum, antibodies bound to recombinant antigens were detected with fluorescent labeled anti-equine IgG and analyzed using the Luminex 100 instrument. The assay was validated by testing 2,500 equine serum samples previously characterized in the VNT. These included 1,500 serum samples received at the OIE EVA Reference Laboratory at the Maxwell H. Gluck Equine Research Center as well as 1,000 equine sera received at the Livestock Disease Diagnostic Center, University of Kentucky, Lexington.

Among 8 recombinant proteins, highest concordance with the VNT results on the selected equine sera was obtained with the partial GP5 (55-98) protein**.** Using an optimal cutoff value of 992 median fluorescence intensity (MFI), the sensitivity and specificity of the test using GP5 (55-98) protein was 92.6% and 92.9%, respectively. The GP5 (55-98) MIA and the VNT outcomes were significantly correlated ($r=0.84$; p-value<0.0001). **Although GP5 (55-98) MIA is less sensitive than the standard VNT, it has the potential to provide an accurate, rapid, convenient and more economical test for screening equine sera for the presence of antibodies to EAV while using the VNT as a confirmatory assay.**

* Graduate student presentation

Comparison of 2 real-time RT-PCR assays for the detection of *Equine arteritis virus* nucleic acid in equine semen and tissue culture fluid

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Equine arteritis virus (EAV) is the causative agent of equine viral arteritis (EVA) in horses and other equids. Up to 10-70% of stallions infected with EAV may become carriers, and these persistently infected stallions transmit EAV solely by the venereal route to susceptible mares during breeding and can be responsible for new outbreaks of EVA. Persistently infected stallions function as natural virus reservoirs that harbor the virus between breeding seasons. Therefore, identification of the carrier stallion is critical to the control of EAV infection. The presence of EAV in the semen of carrier stallions is frequently detected by virus isolation (VI) in cell culture or by test breeding of stallions to susceptible mares, both of which can be time consuming, expensive and cumbersome. Compared to VI, which is the current gold standard for the detection of EAV in clinical specimens, nucleic acid based assays are frequently more sensitive, less expensive and less time-consuming.

In this study, we compared and validated two previously developed TaqMan® fluorogenic probe-based one-tube real-time RT-PCR (rRT-PCR) assays [Balasuriya et al. J Virol Methods 2002;101:21-28 (North American rRT-PCR; NA rRT-PCR) and Westcott et al. Vet Res 2003;34:165-176 (European rRT-PCR; EU rRT-PCR)] for the detection of EAV nucleic acid in equine semen and tissue culture fluid (TCF). The specificity and sensitivity of these two assays were compared to the traditional VI in cell culture.

Three hundred semen samples received between April 2006 and May 2007 at the OIE EVA Reference Laboratory, Maxwell H. Gluck Equine Research Center, University of Kentucky, were tested for the presence of EAV by VI and 2 previously described rRT-PCR assays. In addition to this, 155 TCF samples were also evaluated in the study. The latter included TCF containing modified live virus vaccine strain of EAV (ARVAC®, Fort Dodge Animal Health, Iowa), 64 North American and European field strains of EAV, 74 EAV negative TCF samples, and 16 TCF samples containing other equine viral pathogens (*Equid herpesvirus* 1-5, *Equine rhinitis virus* A and B, *Equine adenovirus* 1 and 2, equine influenza virus type A1 and A2, and Salem virus). Attempted isolation of EAV from semen samples was performed in 2 rabbit kidney cell lines, RK-13 (KY) and RK-13 (ATCC-CCL37), according to the OIE-approved protocol. The primers and probes used in each TaqMan® RT-PCR assay were identical to those described in the 2 previous publications and targeted the ORF7 of EAV. Inter-assay variability and analytical sensitivity of these 2 assays were compared by using *in vitro* transcribed EAV ORF7 RNA.

The NA rRT-PCR had a higher sensitivity than the EU rRT-PCR in detecting EAV nucleic acid in semen samples (93.4% and 42.6%) and TCF (96.9% and 89.2% respectively). However, the NA rRT-PCR was less sensitive (93.4%) than the OIE prescribed VI test (gold standard) for the detection of EAV in semen. In light of these findings, **semen that is negative for EAV nucleic acid by rRT-PCR should be confirmed virus negative by VI**. In summary, **the results of this study illustrate the importance of comparative evaluation and validation of rRT-PCR assays prior to their recommended use in a diagnostic laboratory for the detection and identification of infectious agents.**

* Graduate student presentation

A real-time PCR assay to detect single nucleotide polymorphisms at codon 171 in the prion gene for the genotyping of scrapie susceptibility in sheep

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The objective of this study was to assess and review a reliable real-time polymerase chain reaction (rt-PCR) assay compatible with the Roche LightCycler® 2.0 capable of genotyping sheep for scrapie susceptibility at codon 171. The single nucleotide polymorphisms (SNPs) in the prion protein gene in sheep that may govern resistance to scrapie at codon 171 encode for lysine [K], histidine [H], glutamine [Q], and arginine [R].

A modified proteinase K method for leukocytes or whole blood was used to isolate genomic DNA from sheep blood. Fluoresentric developed and optimized primers and probes for the codon 171 SNP assay. The assay was initially validated using 218 determinations from whole blood of known genotypes with a 100.0% correct identification rate. The assay was further validated through a whole blood check test provided annually by the National Veterinary Services Laboratory (NVSL) with a correct identification rate of 100.0%. As of December 2006, 3,672 samples from blood were genotyped at codon 171. The genotypes were QR_{171} (n = 1,838, 50.05%), RR_{171} (n = 1,423, 38.75%), QQ_{171} (n = 407, 11.08%), HR_{171} (n = 2, 0.05%), and HQ_{171} (n = 2, 0.05%).

The combination of this simple extraction method and the novel Fluoresentric assay is exceedingly accurate, capable of identifying all 4 SNPs at codon 171 in 1 reaction, and has proven useful to producers in their selective breeding programs.

Bovine viral diarrhea virus Scientific Session

Saturday, October 20, 2007

Ponderosa B

Moderator: Steven Bolin

* Graduate student presentation

Persistent *Bovine viral diarrhea virus* infection in cervids in Colorado

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Bovine viral diarrhea virus (BVDV) is an economically important disease of cattle worldwide. Attempts to eradicate the virus from populations are complicated by the unique pathogenesis of the disease, which includes vertical and horizontal transmission and variable outcomes. While there are many published recommendations for testing and managing BVDV within cattle populations, limited progress has been made in the control of this disease within North America. One potential source that has been largely overlooked is the presence of other virus reservoirs, such as wild animals. Seroprevalence studies of numerous North American wild cervid populations have identified populations with exposure to BVDV, and experimental infections suggest cervids can become infected with and shed the virus. Persistent infection has also been demonstrated in wild and experimentally infected cervids suggesting fetal pathogenesis in cervids similar to that in cattle. Persistently infected individuals shed abundant virus and are recognized to be one of the most significant variables influencing the epidemiology of the virus in a herd. A better understanding of the disease in wild species and the risk they pose to domestic is required before control measures can be developed. The objective of this study was to determine the prevalence and distribution of persistent BVDV infection in wild cervids in Colorado, and to evaluate the risk of BVDV infection in cattle posed by wildlife in Colorado.

In this cross-sectional study, samples were collected through a voluntary hunter submission surveillance program for chronic wasting disease (CWD) in Colorado. During the 2005-2006 hunting season, for every head processed as a part of the CWD surveillance, a full thickness ear notch (dorsal aspect of ear) was collected, formalin-fixed, paraffin-embedded and stained for BVDV antigen by immunohistochemistry. Other information collected on individual animals included geographic location where the animal was harvested, species and age.

Immunohistochemistry was conducted on 5,951 samples and a single positive animal was identified. The positive animal was an adult male mule deer, negative for chronic wasting disease. Appropriate geospatial references were available for 5,895 samples, and the distribution of samples was mapped relative to cervid populations, cattle populations and information on BVDV infection in cattle obtained from the Colorado State University Veterinary Diagnostic Lab. The prevalence of BVDV infection in cervids of Colorado is very low (<0.02%), however the risk of interspecies transmission is presumably highest in geographic areas with high cervid and bovid densities and BVDV in cattle populations. Unfortunately the lack of systematically collected data on BVDV infection and difficulty obtaining good population estimates of wild cervids negates the application of conventional, quantitative risk assessment techniques; however control programs should focus on minimizing infection spillover from cattle into wild animals in any areas with high population densities.

* Graduate student presentation

A summary of test results from large-scale BVDV antigen ELISA testing performed in a private laboratory setting

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From November 2005 through April 2007, ear notch samples representing over 500,000 animals were tested using the IDEXX HerdChek® BVDV Antigen Test Kit. Ear notch samples were obtained from various types of cattle and included samples from 34 states (n=375,360) as well as Mexico (n=22,222) and Canada (n=522). Animal origin could not be determined for 177,046 samples. Testing was performed at 4 different laboratory locations in Texas (3) and Kansas (1). **The prevalence of BVDV positives in the sample set was 0.40% (2229/552,406) and ranged from 0.00% to 1.59% on an individual state or origin basis (a single submission of 2 samples from one state yielded 2 positive results = 100%).**

Samples from sale barn cattle accounted for the majority of samples tested (59.21%) with 1302/327,094 (0.40%) of samples yielding positive test results. Ear notch samples were also tested from other classes of cattle including Mexican (4.66% of total, 0.50% positive), dairy (1.37% of total, 0.41% positive), pre-conditioning (8.90% of total, 0.29% positive), ranch (1.94% of total, 0.32% positive), natural and show (0.10% of total, no positive results). There were 130,977 samples tested from animals from undetermined class (23.71% of total, 0.45% positive).

The weight classes for cattle tested ranged from <300 lbs class (4.59% of total, 0.76% positive) to ≥900 lbs (1.18% of total, 0.08% positive). The largest number of cattle tested where weights were known was with cattle in the 400-499 lbs class (22.75% of total). In this weight class, 387/125,671 samples yielded positive results (0.31%). There were 205,026 (37.11%) samples tested from cattle of unknown weight class; for this set of samples, 872 (0.43%) samples yielded a positive test result.

BVDV ELISA testing utilizing ear notch samples continues to be a valuable method for identifying BVDV infected cattle and understanding the prevalence of BVDV infection within various segments of the cattle industry. The results in this report are similar to previous reports regarding BVDV prevalence (1,2).

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BVDV antigen ELISA false-positives associated with Brahman cattle

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During March and April of 2007, customer calls regarding high levels of presumed serum false positives on the HerdChek® BVDV Antigen Test Kit were received by the IDEXX technical services group. **Investigations revealed that the high level of reactivity was associated exclusively with female Brahman cattle from 3 accessions. The observed reactivity within each of the 3 accessions was 72.7%, 94.7% and 100% (48/66, 18/19 and 25/25 reactive/total, respectively).** This level of reactivity had never been documented by IDEXX technical services nor had it been observed in testing hundreds of bovine serum samples during the kit licensing process.

The remaining samples were shipped to IDEXX technical services where initial BVDV positive results were confirmed. Clinical chemistry values including total protein and albumin levels were within the normal range for all animals (age range 3 months to 10 years). Follow up with herd veterinarians indicated that animals had received typical vaccinations (IBRV, BPIV-3, BRSV, etc.) and there was no history of health issues associated with BVDV infection.

Upon testing with the modified assay protocol (without goat anti-BVDV detector), all but 5 of the samples yielded negative results (similar optical densities with and without detector). The 5 serum samples yielding suspect or positive S/P values using the modified detector protocol were then tested on the European version of the IDEXX BVDV antigen kit (HerdChek® BVDV Antigen Serum Plus), yielding negative results. All available paired ear notch samples were negative by BVDV IHC.

Additional serum samples (n=66) were collected from one herd (2 physical locations) and were tested under a number of scenarios. Freeze-thaw cycles, heat inactivation, microtiter plate blocking and microtiter plate type had no effect in reducing the reactivity. The reactivity was also not associated with BVDV antigen kit lot nor was it temporal in nature. Fifteen serum samples were selected from this sample set and were sent for testing by PCR; all 15 samples yielded negative results.

Given that the reactivity was apparent with and without kit detector antibody (goat anti-BVDV) and was most likely associated with the anti-goat conjugate, 8 different anti-goat IgG conjugate preparations were evaluated in order to find one that would eliminate or reduce the reactivity. Samples were also tested with 7 other conjugates (including 3 anti-bovine IgG) in order to determine the extent of non-specific sample binding during the assay incubation steps.

Testing with various conjugates revealed that sample IgG was binding to the microtiter plates and was subsequently recognized by the current anti-goat conjugate. **One anti-goat conjugate, specifically manufactured to remove reactivity with serum proteins from other ruminant species, reduced the reactivity in each of the 3 accessions by 98.5%, 100% and 100%, respectively. The use of a more specific conjugate in the BVDV antigen ELISA will reduce the incidence of false positives associated with Brahman cattle and may improve overall test specificity.**

Interlaboratory comparison of diagnostic testing methods for *Bovine viral diarrhea virus*

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The NVSL, in collaboration with ARS, developed a proficiency panel for detection of *Bovine viral diarrhea virus* (BVDV). Twenty-eight participants used the panel for BVDV detection by antigen ELISA, virus isolation, or PCR. The panel consisted of 15 or 16 diluted serum and buffy coat samples from known BVDV-negative and BVDV-persistently infected cattle. Four of the samples were deemed negative, and 12 were positive by PCR (expected results).

Proficiency panel participants applied their tests to the panel as follows: 28 for the antigen ELISA, 21 for virus isolation, and 25 for PCR; 10 of the labs that conducted PCR testing also included virus typing. For the antigen ELISA, 10 of 28 participants identified all samples correctly. For virus isolation, 5 of 21 participants identified all samples correctly. For PCR, 10 of 25 participants identified all samples correctly.

Approximately 50% of our samples were identified as expected by all participants across all tests.

Laboratories consistently identified negative samples, with 1 false positive by antigen ELISA, 2 false positives from 1 participant by virus isolation, and no false positives by PCR.

Improved BVDV-1b challenge model for evaluating efficacy of protection against clinical signs following acute infection

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Introduction. Efficacy of *Bovine viral diarrhea virus* (BVDV) vaccines in preventing acute infections is evaluated based on reduction of clinical disease. While high virulence BVDV-2 strains are used in U.S. vaccine efficacy studies, the BVDV-1 strain, NY-1, made available by the USDA as a challenge strain, produces very little in the way of clinical disease. Further, NY-1 is also present in at least one commercial vaccine. In order to identify a BVDV-1 strain that generates a more pronounced clinical presentation, clinical presentations following inoculation with 3 field strains were compared to clinical presentation following inoculation with NY-1.

Materials and methods. The strain NY-1 was isolated, by researchers at Cornell University, from dairy cattle residing in the United States. Strains 5013 and 2360 were isolated at the University of Bern, Institute of Veterinary Virology from animals suffering from bloody diarrhea and pyrexia. Strain CA0401186a was isolated from tissues from a PI calf submitted to the National Animal Disease Center (NADC) by diagnosticians from the Tulare Laboratory of the California Animal Health and Food Safety Laboratory. All viruses were characterized as noncytopathic based on growth characteristics in MDBK cells. Based on phylogenetic analysis, strains NY-1 and CA0401186a were segregated to the BVDV-1b subgenotype, strain R5013 to the BVDV-1k subgenotype and R2360 to the BVDV-1e subgenotype.

Three-to-nine-month-old Holstein calves were tested free of BVDV in buffy coat (BC) samples by virus isolation, free of BVDV antibodies in serum, and free of BVDV antigen in ear notch samples by immunohistochemistry. Animals were inoculated via the nasal route with 5 mL of the appropriate viral dose in cell culture medium. Inoculated animals were individually penned in a climate-controlled barn for the duration of the experiment and observed a minimum of twice daily for cough or loose stool. Basal temperatures were taken daily from day -2 to day 14 post inoculation. Blood samples for virus isolation, determination of circulating lymphocytes and platelet counts and determination of presence of serum neutralizing antibodies were collected on days -2, 3, 6, 9, 11 and 13 post inoculation. These time points correlated with the typical first observation of clinical signs (day 3), midpoint of clinical disease (days 6 and 9), and recovery (day 13) based on previous studies.

Results. All virus-inoculated animals developed serum titers greater than 1:4 by day 13. Virus was isolated from the buffy coat of all virus-inoculated animals at least once between days 3 and 11 post inoculation. There were not significant differences between number of days virus was isolated or serum titers between inoculated groups. Neither serum antibodies nor virus were detected in any of the control animals.

Infection with any of 3 of the BVDV-1 field strains resulted in a clinical presentation that was more severe than that observed with NY-1. Lymphocytopenia and the development of loose stools were significantly greater following infection with R5013 compared to NY-1. The number of animals developing temperatures greater than 41.1°C (106°F) was greater following infection with either BVD 2360 or CA0401186A. Further, there was a significantly greater decrease in circulating platelets following infection with CA0401186A.

Conclusions. Based on these results, strain CA0401186A was selected as a potential BVDV-1 challenge strain. The clinical presentation following inoculation with this virus was reproducible and was not dependent on dose over 2 logs of virus.

Utilization of multiple diagnostic tests to identify cattle with *Bovine viral diarrhea virus* infections and persistence of positive tests in persistently infected cattle

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Bovine viral diarrhea virus (BVDV) infections have a significant impact on the cattle population and production. Persistently infected (PI) cattle represent the principal reservoir of infection. Identification and removal of PI animals are critical to the control of BVDV. There are numerous assays for BVDV including viral isolation, antigen capture ELISA (ACE), immunohistochemistry (IHC), and various polymerase chain reaction (PCR) assays.

The objectives of this study included using different methods to identify PI cattle, and assessing the duration and consistency of the positive results in PI cattle. These assays included viral isolation, ACE, IHC, and PCR using samples collected from 12 PI cattle held from day 0 to day 342 at approximately 1 month intervals. The PI cattle were penned together allowing commingling of the calves that were infected with BVDV-1a, BVDV-1b, or BVDV-2a (four calves per subtype, naturally occurring PI). The calves had received BVDV vaccine prior to the start of the study as part of the normal processing regimen. None of the viruses from PI calves were of vaccine origin based on biotype (noncytopathic) and sequencing of a 5'-UTR using known sequences for vaccinal strains. Sera collected throughout the study were assayed for antibodies to the 3 BVDV subtypes. 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. Viral isolation and titrations were performed on sera and nasal swab materials. The ACE test was used on sera, nasal swabs, and ear notches, and IHC was performed on notches. The reverse transcriptase (RT)-PCR was performed on sera, ear notches, and nasal swab materials. The detection limits based 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 medium.

During the study, 3 calves died with mucosal disease lesions. The remaining calves were IHC and ACE positive in all ear notch collections from day 0 to day 342. The cattle were positive for virus in the nasal swabs by viral isolation for all collections with titers ranging from $10^{3.4}$ to $10^{7.85}$. In some instances, the nasal swab materials were toxic to VT cultures at the lowest dilution. All of the calves had virus in the serum ranging from $10^{1.6}$ to $10^{5.35}$. One calf did not have quantifiable virus ($<10^{1.6}$) in the serum on 3 collections. All of the PI calves were ACE positive on sera and nasal swabs in collections throughout the study. PCR results were positive on sera, nasal swabs, and notches(except for 1 collection of 1 animal); however there were instances in which calves were initially test negative by PCR on ear notches, but positive on subsequent testing. Serotesting of the PI calves using day 0 and day 342 samples indicated that some, but not all, of the PI calves seroconverted to heterologous subtypes (likely from the other PI calves).

In summary, the PI cattle remained IHC and ACE positive on ear notch samples for the duration of the study (342) days without going negative at any time. The infectivity of the serum and nasal swabs indicates that the PI cattle remain as continual shedders of the virus throughout their lives. **The ACE and PCR tests on sera and nasal swabs may be used as screening tests, however repeated testing or confirmation with other tests are required to confirm PI status**. Plus PI calves may respond to BVDV vaccinations or exposure to heterologous strains.

BVDV ear notch survey to reveal potential false-negatives associated with a mutation in the E^{rns} glycoprotein

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In September of 2004, a Holstein calf was born in Alabama that was persistently infected with a type 2 strain of BVDV exhibiting a mutation in the E^{rns} glycoprotein. **The E^{rns} mutation was associated with failure of immunohistochemistry and the 15C5-based IDEXX HerdChek® BVDV Antigen Test Kit (Westbrook, Maine) to detect the persistent infection.** Further testing on a different IDEXX BVDV Antigen Test Kit (HerdChek® BVDV Antigen Serum Plus, Bern, Switzerland) yielded a positive result on both serum and ear notch tissues (1).

In order to determine the prevalence of BVDV-PI animals that may escape detection with a single capture antibody format, test-negative ear notch samples were obtained from 4 IDEXX customers currently performing BVDV antigen testing with the 15C5-based kit. The samples were then retested using the HerdChek® BVDV Antigen Serum Plus kit (manufactured with a blend of 3 BVDV monoclonal antibodies for antigen capture). Additionally, the Alabama sample was tested with individual BVDV antibody coated plates in order to determine the antibody responsible for detection in the HerdChek® BVDV Antigen Serum Plus kit.

A total of 10,096 ear notch samples previously testing negative with the 15C5-based kit were obtained and tested on the HerdChek® BVDV Antigen Serum Plus kit. The samples were taken from routine laboratory submissions or local collections and were of diverse geographic origin. Of the ear notch samples tested, 16/10,096 (0.16%) yielded suspect or positive results. All samples yielded relatively weak test values at or about the negative/suspect (S/N value = 0.20) or suspect/positive (S/N value = 0.30) cutoffs and were much weaker than that seen for the Alabama sample (S/N value = 3.50). One of the 16 samples yielded a weak positive result when retested on the 15C5-based BVDV kit (S/P value = 0.28), all others tested negative. Reactive samples were submitted for testing by PCR; 11/16 samples were PCR negative with the balance of results still pending. It was determined that a BVDV type 2-preferential monoclonal antibody was responsible for Alabama sample detection in the HerdChek® BVDV Antigen Serum Plus kit.

At this point in the study, the BVDV ear notch survey has failed to uncover any samples yielding false-negative results similar to that of the Alabama sample. Modifying the current HerdChek® BVDV Antigen Test Kit to include additional capture antibodies for broadened (Alabama sample) detection would require both sensitivity and specificity assessments as well as USDA approval. Further studies are planned to assess individual antibody detection with accessible BVDV strains.

Reference

1. Gripshover EM, et al. Variation in E(rns) viral glycoprotein associated with failure of immunohistochemistry and commercial antigen capture ELISA to detect a field strain of bovine viral diarrhea virus. Vet Microbiol 2007;May (in press).

Evaluation of the AnDiaTec BoVir® real-time RT-PCR kit for the detection of BVDV in pooled bovine ear notch and blood samples

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Bovine viral diarrhea (BVD) occurs as a worldwide disease of cattle, and it continues to cause severe economic losses to the dairy and beef cattle industries. There is a considerable interest in strategies for BVDV monitoring, and indeed, several European countries have already started control and eradication programs.

The genetic variability of BVDV strains is remarkable. BVD viruses have been subdivided into genotypes 1 and 2 based on genomic sequence and antigenic variation. However, disease caused by BVDV of either genotype can appear similar, and therefore, strains of both genotypes must be detected by a screening assay. In the past, blood samples had been the sample of choice, however, recently, ear notches are becoming the predominant sample type for routine diagnosis. The advantage of ear biopsies is the ease of sampling, which can be performed by producers themselves. Recently, several real-time RT-PCR assays for the detection of BVDV have been established. Although ear notch sampling is preferred, difficulties arise in testing ear biopsies by RT-PCR due to the need for labor- and time-intensive, complicated homogenization and RNA extraction. Thus, it is not surprising that disappointing results have been reported in some evaluation studies comparing RT-PCR to antigen-capture ELISA (ACE), immunohistochemistry (IHC), or virus isolation (VI).

The AnDiaTec BoVir-SL BVDV RT-PCR is a completely automatable diagnostic test using a proprietary 2-step lysis buffer to extract viral RNA from ear notch samples into the supernatant that is then used directly (in pools of 10 samples) with the real-time PCR process. The test was evaluated independently in government reference laboratories from 6 European countries. The specificity and broad reactivity of the test was confirmed with 68 well-characterized reference strains, including atypical European and North American strains, such as HoBi and H138. The detection limit of the kit was determined to be 50 to 100 viral particles in 1 mL whole blood, which surpassed the sensitivity of other RT-PCR systems by a factor of 50 to 500.

Blood samples (n = 24,169) (EDTA-blood, plasma, serum) were tested in an evaluation study in 9 laboratories. The study comprised pre-tested samples (VI and commercial ACE; VI and in-house RT-PCR or VI, ACE and in-house RT-PCR) but also parallel testing of untested samples. **The AnDiaTec test diagnostic sensitivity was 99.8% and diagnostic specificity was 100%.** Only 1 hemolyzed sample, previously tested positive, could not be confirmed by the AnDiaTec test due to PCR inhibition.

Ear biopsies (n = 1,423), obtained with approved sampling devices from Allflex, Caisley and Typifix, were tested in a second study. Again, the study comprised mainly samples from pre-tested animals (IHC and commercial ACE or IHC, commercial ACE and in house RT-PCR) but also parallel testing of untested samples. All (n = 833) pre-tested positive ear notch samples (100%) were confirmed positive, while 13 additional samples were tested positive only with the BoVir® system. Additional screening of blood samples proved these animals to be BVDV-positive. The AnDiaTec test is approved by European reference laboratories and used in eradication programs in several countries.

Epidemiology/Molecular Diagnostics Scientific Session

Sunday, October 21, 2007

Bonanza A

Moderators: Francois Elvinger, Lindsay Oaks

* Graduate student presentation

Laboratory-based early animal disease detection utilizing a prospective space-time permutation scan statistic

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The veterinary diagnostic laboratory is in a unique position to analyze data from a large number of clinical cases. We developed a system for early detection of clusters of health events in animal populations using diagnostic laboratory data. This system provides early cluster alarms/alerts and near-real-time medical situational awareness. This aids the decision-making process regarding conducting field investigations and/or mounting a medical response. In addition, the system could possibly detect a cluster of animal health events that might also put the human population at risk, e.g., bioterrorist attack.

Kulldorff's prospective space-time permutation scan statistic has been utilized. The method uses a cylindrical window of variable size to scan for potential clusters in space and time. The statistic only requires case health events (e.g., confirmed diagnoses, deaths), thereby eliminating the need for population-at-risk data. We developed an engine that performs the necessary statistical analyses at the close of business each day. A likelihood ratio test is used to test statistical significance of potential clusters. Identified clusters are automatically mapped for study each morning by the laboratory epidemiologist.

We used this methodology to successfully detect significant clusters of bovine blackleg and equine leptospirosis that occurred in 2006-2007. This prompted laboratory epidemiology staff to conduct detailed investigations of the outbreaks and to release situational awareness bulletins to the Kentucky Office of the State Veterinarian, laboratory clients (via email listserv), and announcements at local veterinary meetings. **The appropriate analysis of animal health events can be successfully used to generate medical alerts, but sensitivity and specificity of generated alerts need to be further characterized.**

Epidemiologic characteristics of outbreaks associated with changes in the *Foot-and-mouth disease virus* genome in the 2001 epidemic in Argentina

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Because *Foot-and-mouth disease virus* (FMDV) replication has a high error rate, most infections are expected to produce a large number of strains that represent a mutant distribution referred to as quasispecies. Knowledge of epidemiological factors that influence, or are influenced by, changes in the genetic distribution of prevailing quasispecies would improve our understanding of the epidemiology of FMDV evolution and of our ability to prevent and mitigate disease. We present here work that quantifies the relationship between changes in the VP1 gene of FMDV strains isolated during the epidemic in Argentina in 2001 and epidemiological features of the disease in affected herds.

The number of different nucleotides (dn) in the VP1 gene was computed for each pair (i,j) of 23 FMDV serotype A isolates obtained from 23 different herds experiencing outbreaks during 2001 in Argentina. The association between $dn_{i,j}$ and 6 hypothesized factors epidemiologically characterizing each outbreak was assessed using a Bayesian binomial regression model. The factors were difference in the duration and onset of the outbreaks (days), arithmetic difference in the attack rates, spatial distance between premises, and arithmetic difference of the easterly and northerly direction of movement of the outbreaks. Regression coefficients were modeled using non-informative prior distributions. The deviance inference criterion (DIC) was used for model selection. The model that best fit the data was used to predict the expected genetic distance (EGD) between each isolate obtained in 2001 and an isolate obtained in January 2002. The accuracy of the model was evaluated by estimating the correlation (R) between the values of EGD and the corresponding observed genetic distance.

The model that best fit the data was the model that included the difference in time of onset (OR: 1.14, CI95%: 1.02-1.26), the spatial distance between premises (OR: 1.19, CI95%: 1.08-1.32), the interaction between the difference in the attack rates and difference in durations of the outbreaks (OR: 1.07, CI95%: 1.00-1.14), and the northerly (OR: 1.08, CI95%: 1.00-1.16) and westerly movements of the epidemic (OR: 1.10, CI95%: 1.03-1.20). The genetic distance observed between each isolate obtained in 2001 and an isolate obtained in 2002 was significantly associated ($R=0.6$; $P<0.01$) with the genetic distance predicted by the model, indicating the model was able to predict genetic variability of an unknown strain.

Spread of FMDV into neighboring herds was expected to result in outbreaks caused by closely related strains, whereas more distant spread, in both time and geographic distance, was expected to allow more time for strains to mutate and thus outbreaks would be caused by strains that were less closely related. The association between $dn_{i,j}$ and northerly and westerly directions indicates that genomic change increased more as one moved in those directions. The interaction between a high attack rate and a long duration of an outbreak suggests that viruses infecting herds with both a large difference in outbreak durations and in the attack rates were expected to have a large number of nucleotides that differ in their VP1 gene.

Further development and application of the methods presented here may be useful in the investigation of factors influencing genome changes during FMD epidemics and in forensic analysis to identify the origin of FMDV reintroductions during the course of an epidemic.

FMD BioPortal: a system for global surveillance of foot-and-mouth disease

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Countries and agencies need to have a global situational awareness for foot-and-mouth disease (FMD) and to be able to estimate in real time or near real time elevated risks of FMD so that appropriate measures can be taken to prevent or mitigate disease and its impact. One of the strategies for early detection of and response to FMD is that of global surveillance, which would aim to seek out specific information about new FMD cases, changing risks of FMD, and genomic changes in the FMD virus as necessary in planning and preparing for an FMD incursion. Although there has been considerable discussion about the needs and prospects for a global surveillance system for FMD, little in the way of formal action has taken place to create such a system. In this report we describe a system referred to as the FMD BioPortal that is aimed at providing real time information, analysis, and visualization of FMD surveillance and monitoring data.

The FMD BioPortal was developed initially as a collaborative effort of the FMD World Reference Laboratory at Pirbright, U.K., the Artificial Intelligence Laboratory at the University of Arizona, and the FMD Laboratory at the University of California, Davis. Version 1.0 was made operational in January, 2007 (http://fmd.ucdavis.edu/bioportal/). At the time of this abstract submission, the FMD BioPortal has had 122 users from 26 countries. An initial goal was to create a Web-based system that would make all FMD-related data presently banked at the Pirbright laboratory available to the public. A primary objective was to be able to apply basic search and analytic tools to the data, including graphic and tabular presentation and spatial-temporal clustering analysis, and to be able to download selected records. Since its first release, additional databases have been captured by the FMD BioPortal, including FMD virus genomic data from GenBank and weekly in-country incident data from OIE (version 2.0). Major systems components of the FMD BioPortal include secure, real-time data transfer, data analysis modules, and interactive visualization tools that allow for integrated analysis and display of epidemiological and genomic sequence data, including linkages with Google Earth.

Another version of the FMD BioPortal, which is planned for July 2007, will have additional functionality to access models for real-time development and comparison of phylogenetic trees of virus isolates using FMDV sequence data. One analysis module allows for user adjustment of a threshold genetic distance between any 2 isolates to assess genetic relatedness among FMDV strains, using a phylogenetic tree display.

The presentation will illustrate data display, analyses, and models currently available for FMD surveillance and monitoring through the FMD BioPortal.

Development of the FMD BioPortal represents an important step forward in realizing a goal of global infectious disease surveillance and in recognizing that global surveillance will not be possible without a system for international real-time information sharing and analysis.

The use and limitations of expert opinion data for foot-and-mouth disease surveillance

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Surveys of subject area experts are commonly used by national and international groups, such as USDA-APHIS and the European Commission for the Control of Foot-and-Mouth Disease (EUFMD), to offer an assessment of disease status of countries for trade purposes. The relative ease of obtaining expert opinion makes expert opinion data attractive in other aspects of disease surveillance and risk assessment when data representing the preferred measurements cannot be obtained. Expert opinion can be used appropriately to address the critical lack of data necessary for prevalence and incidence estimates for many diseases, including foot-and-mouth disease (FMD). However, when a conclusion based on expert opinion applies to a topic as sensitive as FMD, which has tremendous economic, political, and social implications, care should be taken to understand the accuracy and variation of the accuracy of the opinion data.

Using Bayesian methods for diagnostic test evaluation, we estimated the accuracy of expert opinion for "diagnosing" country-level FMD presence for the years 1997 to 2003, compared with results of a statistical model and using OIE-assigned FMD status (reporting FMD cases, "positive," and listed as free of FMD, "negative") as a "gold standard" test. In addition, we evaluated the responses of 8 international FMD experts and identified differences in both the answers they gave and the possible logic behind their answers.

Based on our survey, we found that individual experts provided different values for the probability of finding FMD in a country, but the weighted average of the experts' responses was relatively accurate (sensitivity of 91% and specificity of 85%) at identifying the FMD status of a country, compared with OIE-assigned FMD status and our statistical model. The most disagreements between individual experts and OIE were found for Indonesia, South Korea, and South American countries, and, in general, the experts seemed to believe that countries in South Asia were more likely to be positive than other countries that were specified as FMD-positive by OIE.

Though our study found that a group of experts can be accurate at diagnosing country-level FMD status, we identified problems with this type of data that should be addressed when expert opinion data are used to assess the disease status of a country.

* Graduate student presentation

Risk factors associated with spatial distribution of foot-and-mouth disease in Nepal

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Foot-and-mouth disease (FMD) is considered one of the most important diseases of livestock in Nepal, where it has been reported to occur in all of the 75 districts of the country. Since 1965, 4 of the 7 FMD virus serotypes (O, A, C, and Asia 1) have been identified in several regions of Nepal. It has been estimated that FMD is responsible for 26% of the economic losses in livestock production in Nepal. Despite the social and economic impact of the disease on the country, the nature and extent to which epidemiological factors contribute to the spatial distribution of FMD risk has not been reported. Here we offer preliminary results of a study aimed at understanding host, demographic, and environmental factors associated with the spatial distribution of FMD in Nepal.

The association between the proportion of village development committees (VDCs) reporting clinical cases of FMD in 2004 and 20 factors hypothesized to be associated with FMD risk at the district level was assessed, using information obtained from responses to a questionnaire completed by each of the 75 district veterinary officers and a Bayesian Poisson regression model with spatially structured and unstructured random effects and non-informative priors for distribution parameters. The model with the lowest deviance information criterion (DIC) value was considered the combination of covariates that best predicted the distribution of FMD cases.

Of the 75 districts and 3,913 VDCs, 29 districts (38.7%) and 214 VDCs (5.5%) reported at least one FMD outbreak in 2004. The number of FMD-positive VDCs per district ranged from 1 to 27. The model providing the best fit indicated the number of FMD-positive VDCs increased with both increasing district level human and buffalo population and increasing number of animal health technicians. For each standardized unit increase in the values of number of human, number of buffalo, and number of technicians, the overall risk of FMD in the district increased by factors of 1.1, 1.8, and 1.3, respectively. Additionally, high risk districts identified by the model were similar to clusters identified by a spatial scan statistic and the human population was found to affect FMD risk most in districts bordering India. In addition, results of the Cuzick Edwards test suggested that the risk of FMD in a given district was influenced only by the number of FMD-positive VDCs in the bordering districts, and not in more distant districts.

Results suggest that FMD risk in Nepal was associated with increasing human population, which was considered here as a proxy for animal movement to abattoirs or markets as expected to meet the high demand for meat and draft power for agriculture. Further, increased risk in districts bordering India warrants our attention to the possible role of widespread legal and illegal animal and human movement across the unregulated border. Increased number of buffalo was also associated with increased risk, suggesting that buffaloes or buffalo management might be worthy of further investigation to assess the reasons for this association. The finding that FMD risk increased with increasing technicians could indicate improved detection with more technicians and/or increased indirect transmission by technicians who might function as a vehicle to spread the virus.

As control of FMD by test-and-slaughter is impossible given the religious norms in Nepal, the model predictions will be useful to formulate programs to control and eradicate FMD by making more efficient use of scarce resources to identify and target high risk areas and factors for intensive follow up and risk assessment.

* Graduate student presentation

A high-resolution global hierarchical Bayesian model to predict foot-and-mouth disease presence

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An objective of disease surveillance programs is to obtain confident estimates of the occurrence or risk of the disease of interest and of predictions for when and where the disease might be found in the future. However, assessing the presence of foot-and-mouth disease (FMD) on a global scale is difficult due to limitations of disease reporting in remote or underdeveloped areas. Voluntary reports from remote or underdeveloped areas typically provide annual information at the country level, but specific temporal and spatial data exist only in certain areas.

The purpose of this study was to create a high-resolution statistical model to predict FMD presence worldwide. FMD data and hypothesized predictors for 187 countries from 1996 to 2006 were used to construct a Bayesian hierarchical logistic regression model to predict FMD presence at a resolution of 2500 km^2 cells. Cells in which FMD reports had a town or city of the outbreak recorded, which was considered to be a resolution of 2500 km^2 or better, were considered cases (n = 2272). Controls were all cells in areas specified as "free" or "free with vaccination" by the World Organization for Animal Health (OIE) (m = between 17,206 and 18,660 cells per month). Hypothesized risk factors for FMD in a given 2500 km^2 cell were distance from FMD-positive cells; prior FMD presence; bovine, small ruminant, buffalo, pig and human population densities; and bovine, small ruminant, and pig meat deficits. Annual country-specific variables were gross domestic product (GDP) per capita, governance, and historical FMD presence. The model was validated with subsets of data and was used to predict FMD in areas with unknown FMD information through the year 2007.

Preliminary data maps match the existing low-granularity OIE maps, but show finer distinctions in FMD risk within countries. As expected, preliminary data suggest that cells in the USA and Canada are unlikely to have FMD (with probabilities ranging from $5x10^{-8}$ to $2x10^{-6}$ for the USA and $2x10^{-8}$ to $1x10^{-7}$ for Canada), whereas cells in Pakistan have relatively high probabilities of having FMD (0.93 to 1.0). An unexpected preliminary finding was that cells in South Africa appeared to have a lower risk of FMD (probabilities range from $1x10^{-15}$ to $1x10^{-14}$) than cells with similar risk factor characteristics in the USA and Canada.

Though the findings are preliminary, South Africa's low predicted probability of FMD appears to be due to its GDP and its region (Africa). In the preliminary model, countries in the 'middle GDP' category had lower risk for FMD than high- or low-GDP countries. Also, once we controlled for 'GDP', 'governance', etc., countries in Africa had lower risk for FMD than countries with similar risk factor characteristics in the Americas. The preliminary model suggests that the strongest force in creating a low risk of FMD in the USA and Canada is governance. It should also be noted that we have modeled 'reported cases of FMD', so some of the effects of region or GDP might truly be effects on reporting of FMD rather than FMD itself.

With further validation, the model and maps created here could be used to help concentrate surveillance and control efforts in local high risk areas and could provide data-based estimates for risk analysis and border surveillance.

* Graduate student presentation

Spatial and temporal characterization of animal disease risk in two provinces of Spain using network analysis

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Zoning is an Office International des Epizooties (OIE) ruled-procedure implemented by individual countries with the objective of delimiting geographical regions with different animal health status or at different risk for a given disease. Zoning is a critical tool to limit market losses to countries that are experiencing animal disease epidemics, to identify regions at high risk of suffering an epidemic, and to increase cost-effectiveness of resources allocated to prevent and control animal disease spread. Prerequisite for the determination of high-risk zones is the description and analysis of patterns for animal movement, which is considered the main risk for the introduction of infectious diseases into free areas and regions. A technique referred to as social network analysis (SNA) has been used in veterinary medicine to describe animal movement networks and to identify groups of premises more related to each other in terms of the number or frequency of animal movements among them. The objective of this paper is to illustrate the use of SNA to quantify temporal and spatial variation of risk for animal disease introduction and spread in Salamanca province of Castilla y Leon, Spain. Methods discussed on this contribution may also be applied for the assessment of risk associated with livestock movement elsewhere.

We used SNA, in combination with techniques for the detection of spatial and temporospatial clusters, to identify premises at high risk of receiving ('support') or shipping ('influence') cattle and pigs from January through December, 2005, in the Spanish province of Salamanca. An animal shipment was defined as a group of animals shipped simultaneously from a given premises to another. Six social networks were created to describe cattle (17,215 premises), pig (8,369 premises), and cattle and pig (25,222 premises) movements within Salamanca, and between Salamanca and other regions of Spain. Geographical areas and times of the year at higher risk ($P<0.05$) of receiving or shipping livestock were identified on each of the networks using a time-space scan permutation model.

Significant ($P<0.05$) clusters of influential and supportive premises were located at different geographical locations and times of the year on each of the networks analyzed. Differences in the location of temporospatial clusters suggest that temporal variation in the pattern of animal movements influences estimates of risk for animal disease. For this reason, SNA that consider the temporal dimension of the data are likely to produce more accurate estimates of the level of risk due to animal movements in a region, compared with results obtained ignoring the temporal information. Results presented here will be useful for the implementation of differential surveillance, prevention, and control strategies at specific times and selected regions of Salamanca.

Considerable attention has been given during recent years to the potential consequences of establishing a system for recording and tracing-back movements of livestock in the United States. An approach similar to the described on this paper may be used in the United States to characterize risk emerging from the pattern of animal movements in the country.

* Graduate student presentation

Development and application of serological screening for bovine digital dermatitis: application of Bayesian modeling to no gold standard data

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No 'gold standard' diagnostic test exists for bovine digital dermatitis (BDD). The disease is defined by the presence of typical epidermal lesions; however, routine foot inspection is labor-intensive to carry out, and small lesions may be overlooked. As incidence of clinical lesions and serology are correlated, serology could prove to be a more sensitive indicator of infection status.

Utilizing outputs of microbiological research, we developed and investigated an indirect ELISA for serological screening for *Treponema* spp. Data from a cross-sectional study were used to validate this ELISA in the absence of a 'gold standard'. We assumed the existence of a latent state of infection, and did *not* dichotomize the test outcome to prevent loss of information. Using Bayesian statistical methodology, a model was constructed that enabled the probability of true infection of an individual to be estimated on the basis of the serological test outcome, supported by observations of foot inspection for a subset of animals.

Probability of infection was modeled as a function of serologic outcome, foot hygiene score, age, and lesion status. This was accomplished by first modeling serology score to depend on the latent infection status. Then lesion status, which was observed for only a subset of animals in the study, was modeled through a logistic regression to depend on foot hygiene score and age. And finally, the unknown infection status was modeled to depend on lesion status by virtue of its implicit imputation through the Bayesian procedure for the majority of cases for which it was not observed. A key feature of the analysis is plots of infection probabilities versus serologic outcome for lesion status known and unknown.

The model more effectively classifies BDD infection status of an individual animal than serology or foot inspection alone, while simultaneously being more informative than lesion inspection, thus facilitating the investigation of farm-level distribution and prevalence of infection.

Data performance of assays for the detection of antibodies to PRRSV in muscle transudate samples ("meat juice")

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"Meat juice," i.e., transudate recovered from frozen meat as it thaws, has been used extensively in Europe on post-slaughter *Salmonella* monitoring and surveillance programs (Nielsen, 1998). This sampling method was also successfully used to conduct pseudorabies virus surveillance (ELISA) (Le Potier, 1998), and is now being used in *Porcine reproductive and respiratory syndrome virus* (PRRSV) surveillance. However, performance data (sensitivity, specificity) is lacking for PRRSV "meat juice" diagnostic assays and, therefore, interpretation of surveillance results may be uncertain. The aim of this study was to compare the performance parameters of ELISA, SN and IFA assays for "meat juice" samples and serum samples of known PRRSV infection status.

Meat samples were collected in a large longitudinal study wherein 165 pigs (109 infected; 56 negative controls) were followed for up to 202 days post inoculation (DPI). Animals were euthanized at ~2-week intervals, serum and meat samples collected, and stored frozen (-20°C). Meat juice and serum samples were run on the HerdChek® PRRSV 2XR ELISA (IDEXX Laboratories, Inc.). Serum samples were assayed by ELISA as recommended by manufacturer. Meat juice samples were assayed by the ELISA at 5 dilutions (1:2 to 1:40) and by IFA at 4 dilutions (1:2 to 1:16). Serum and meat juice samples were assayed by FFN at 2-fold dilutions (1:2 to 1:512).

Diagnostic sensitivity and diagnostic specificity was estimated using a ROC analysis in which PRRSV inoculation (Y/N) was used as the basis for determining infection status. Correlation coefficients of comparison between ELISA results on serum and different dilutions of meat juice indicated a correlation between 0.75 and 0.87. ROC analyses of the ELISA data indicated good sensitivity (>95%) and specificity (~100%) at any of the dilutions tested. For an IFA cut-off of 1:4, in the infected group 68% of animals were positive, specificity was 100%, and sensitivity was 63.3%. The AUC curve indicated that IFA was a good test to add to PRRSV diagnostics.

In terms of meat juice and test performance, the ROC analyses of the 2XR ELISA data indicated that meat juice samples can be run at any of the dilutions tested (1:2, 1:5, 1:10, 1:20, 1:40) if the cut-off appropriate for the dilution tested is used for interpretation. Under the conditions of this study, a dilution of 1:5 and an S/P cut-off of 0.1 provided the best performance.

These data justify the use and implementation of PRRSV surveillance based on meat samples collected at slaughter.

* Graduate student presentation

Feasibility of testing pooled serum samples by ELISA to detect antibody to PRRSV

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ELISA is commonly used for surveillance for *Porcine reproductive and respiratory syndrome virus* (PRRSV) in negative sow farms. The objective of this study was to evaluate the feasibility of pooling serum samples for detection of antibody to PRRSV by ELISA.

The effect of pooling on sensitivity and specificity of the antibody ELISA was evaluated by testing 113 true-positive samples and 100 false-positive samples respectively. True-positive samples were obtained from 29 experimentally infected swine at 10 to 28 days post-infection. False-positive samples were obtained from routine submissions from PRRSV-negative sow farms to the University of Minnesota Veterinary Diagnostic Laboratory, and were confirmed negative by IFA. All samples were tested undiluted and diluted 1:2, 1:4, 1:6, 1:8 and 1:10 in negative sera to simulate the effect of pooling in pool sizes from 2 to 10. The results were evaluated at 3 different sample/positive ratio (S/P) cut-off values of 0.4, 0.3 and 0.2.

Simulated pooling of serum samples resulted in a decrease in sensitivity and an increase in specificity, compared to testing individual samples, whereas the reduction of the S/P cut-off value recommended by the manufacturer had the opposite effect. Sensitivity estimates ranged from 0.92 (undiluted sample, cut-off 0.2) to 0.42 (pools of 10, cut-off 0.4). Specificity estimates ranged from 0.952 (undiluted sample, cut-off 0.2) to 0.999 (pools of 10, cut-off 0.4). Based on these estimates, different surveillance protocols were compared in terms of herd sensitivity and herd specificity. Most of the protocols evaluated were superior to the standard protocol (undiluted samples, cut-off 0.4).

Results reported in this study show that the conventional monitoring protocols based on ELISA on individual samples can be improved by using pooled-sample testing.

Diagnostic performance of PRRSV PCR and ELISA assays using porcine oral fluid samples

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Oral fluid samples may contain pathogens and antibody at diagnostically useful levels. Archibald et al. (1) suggested the use of oral fluids for HIV diagnostics in 1986. Subsequent oral fluid research resulted in the development of rapid (20 min) point-of-care diagnostic kits for HIV and other infectious agents (2). Oral fluids from non-human species contain similar diagnostic characteristics. In swine, specific antibodies in oral fluids were detected following inoculation with group E *Streptococcus* (3), *A. pleuropneumoniae* (4), and cholera toxin B subunit (5).

We have explored the use of porcine oral fluids for PRRSV detection using PCR and ELISA (IDEXX Laboratories, Inc) under experimental and field conditions:

- Under experimental conditions, the mean diagnostic sensitivity of qPCR based on 209 pen-based oral fluid samples for the first 4 weeks post-inoculation was 88% versus a mean diagnostic sensitivity of 89% in individual pig serum samples. Diagnostic specificity of both oral fluid and serum was 100%.
- Oral fluid samples (6 pens) and serum samples (5 pigs per pen) were collected from placement to close-out at approximately 4-week intervals in three 1,100 head finisher units. PCR results suggested that oral fluid samples could be used in place of serum samples to survey for PRRSV infection by testing at 2 - 4 week intervals.
- Antibody assays originally developed for analyzing serum samples can frequently be adapted to oral fluid samples by modifying incubation time, sample dilution, detection threshold (cut-off), etc (6). Our research indicates that the commercial PRRSV ELISA can be adapted to oral fluids using these modifications with a resulting diagnostic sensitivity of ≥85% and specificity approaching 100%.

Although research on the use of oral fluids in veterinary diagnostics has been limited, our research has shown that porcine oral fluids are easily collected, compatible with available diagnostic assays, and present a viable alternative to serum samples. Although research has focused on PRRSV, preliminary data suggest that oral fluid samples may also serve for the detection of other infectious pathogens of swine (*Porcine circovirus 2*, *Mycoplasma hyopneumoniae*, influenza virus), using PCR- and antibody-based assays. Especially for antibody detection assays, achieving this objective will require further development.

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* Graduate student presentation

Improved estimate of persistence of *Porcine reproductive and respiratory syndrome virus* (PRRSV) in a population of pigs

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Management strategies directed at prevention and/or control of PRRS requires a qualitative and quantitative understanding of virus circulation at the population level. Inherent in this issue is the length of time PRRSV persists in individual pigs. In previous studies, infectious virus had been recovered for up to 154 days after infection. However, these studies also indicated that persistent infection is eventually terminated. The purpose of this study was to provide an improved estimate of the percentage of persistently infected pigs within a population over time.

The experiment was designed as a longitudinal study in which pigs were followed for up to 202 days post inoculation (DPI). On day 0, 109 3-week-old pigs were intramuscularly inoculated with PRRSV strain VR-2332. Negative control pigs (n = 56) were sham inoculated with MEM by the intramuscular route. Thereafter, at approximately 2-week intervals, serum samples were collected from all animals and a subset of randomly selected animals was euthanized and tissues collected. The presence and amount of virus in tissue and serum was assessed using RT-qPCR, standard virus isolation, and bioassay.

Detection of PRRSV in serum by qPCR showed that most pigs cleared the viremia by 42 DPI, but some pigs continued to test positive up to 154 DPI. Lymphoid tissue was qPCR positive through 202 DPI in one or more pigs at each sampling point. Infectious virus was recovered from serum and lymphoid tissue by virus isolation on MARC-145 cell culture in a few pigs up to 28 DPI. Bioassays based on lymphoid tissue homogenate showed that infectious virus was present in these tissues up to 175 DPI.

These results suggest that infectious virus is able to persist in populations for a longer period of time than previously thought. RT-PCR was the most sensitive assay for detecting PRRSV, but the discrepancy between PCR and bioassay results indicated that PCR is detecting non-infectious virus.

* Graduate student presentation

PrioCHECK® Trichinella Ab, a new highly sensitive and specific ELISA for the detection of antibodies against *Trichinella* spp. in serum and meat juice of pigs

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Trichinellosis is a zoonotic disease that occurs worldwide and is caused by the nematode *Trichinella*. The roundworm *Trichinella* spp., infects many carnivorous and omnivorous animal species, including domestic pigs. Humans can be infected by eating raw or insufficiently cooked meat. During 1997-2001, an average of 12 human cases per year was reported in the US. In the European Union, trichinellosis is common in areas where pigs are reared in small holdings with insufficient control measures, but occasional cases also occur in holdings with good farm management practices in place.

Under the current EU regulation, all pigs slaughtered for human consumption have to be tested for trichinellosis by artificial digestion. According to regulation EC 2075/2005, testing of all pigs can be reduced for holdings that are certified as *Trichinella*-free. A pilot program for certifying herds as *Trichinella*-free was evaluated in the US and the voluntary trichinae certification program for US pork proposed by APHIS has recently been published in the Federal Register (9 CFR Parts 149, 160, and 161). Under this program, serological methods, such as enzyme-linked immunosorbent assay (ELISA) are used to determine and monitor the *Trichinella* infection status in herds.

The PrioCHECK® Trichinella Ab is a diagnostic kit based on ELISA technology for the detection of antibodies directed against *Trichinella* spp. in serum and meat juice samples of pigs and was developed for surveillance, monitoring and certification purposes. The PrioCHECK® Trichinella Ab follows a 4-step protocol, consisting of sample preparation, sample incubation, conjugate incubation, and detection. The excretory/secretory antigen (E/S antigen) as the major antigenic protein complex is coated on the ELISA plate. Serum or meat juice samples are incubated on the plate. A peroxidase (POD) labelled anti-pig antibody is used for detection of antibodies bound to the E/S antigen. Color development using TMB substrate measured optically at a wavelength of 450 nm shows the presence of antibodies directed against *Trichinella* spp.

We have performed an in-house test equivalence study using 222 *Trichinella* negative and 59 *Trichinella* positive serum samples from pigs. The test results were referenced to the diagnostic status of the samples as determined by an E/S ELISA from an EU national reference laboratory. Based on these serologically confirmed samples, the PrioCHECK® Trichinella Ab showed a diagnostic sensitivity and a diagnostic specificity of 100%. There was no cross reactivity observed with commonly found swine parasites such as *Ascaris*, *Trichuris*, *Hyostrongylus* and *Strongyloides*.

We conclude that the PrioCHECK® Trichinella Ab ELISA represents a valuable tool for monitoring, surveillance and certification purposes as suggested in regulation EC 2075/2005 and according to the US Trichinae certification program.

Comparison of diagnostic tests to detect Johne's disease positive animals in western farm goats and range flock sheep

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Johne's disease is caused by *Mycobacterium avium* subspecies *paratuberculosis* (MAP), which infects the mesenteric lymph nodes and intestines of ruminant species. Adults transmit these bacteria to their fetus in utero or young via colostrum, milk and fecal contamination. This disease does not respond to treatment, so control depends on eradication. Since many large western sheep flocks graze on open range, there is further concern about the livestock-wildlife-human interface.

This diagnostic testing was initiated to accurately identify positive animals and their offspring for elimination from the flocks/herds. Because an individual sheep or goat has little economic value, we are searching for the most accurate but cost-effective means to identify a Johne's-positive animal. Range sheep are rarely confined, thus there are few opportunities for sampling during the year. Therefore, we attempted to set up our sampling protocol to find the best samples, the most convenient time, and the most cost effective method for Johne's disease flock/herd-level prevalence testing.

Three antemortem tests for detecting MAP-positive animals are being compared:

- the bovine serology **ELISA** (IDEXX HerdChek) using 0.250 S/P cutoff on sheep and goat serum, plasma and milk samples;
- **culture** of feces, tissues, and buffy coats from EDTA blood tubes, milk and colostrum; and
- the **johnin intradermal skin test** for status of cell-mediated immune response to MAP infection.

An increased sediment inoculum is being used on two culture media: BACTEC™ MGIT™ para TB liquid medium with the fluorometric manual read method; and Herrold's egg yolk agar (HEYA) with or without mycobactin J (to determine if the isolate is MAP or the MAP bovine strain).

Our samples are coming from two cooperator producers. One is a 4,000 ewe range flock, and one is a 20 doe farm herd. We have ELISA tested 170 samples from adults and young. At this stage of testing, we have found that the sera/plasma (130) or milk (40) from adults with S/P results greater than 0.300 for milk and 0.400 for sera are all culture positive. Also, the serum sample S/P results from positive dams' lambs or kids range from 0.400 to 1.500 until colostral antibodies wane at 4 months of age. We have cultured 100 samples from adults from tissues and feces of both the farm herd and range flock animals. We see growth in 2 weeks from positive tissues (11) and multibacillary feces (2); and within 10 months from fecals (10) from very low shedders. None of the MAP isolates grow on the initial HEYA medium. The isolates are confirmed as MAP by subculture from MGIT acid-fast positive tubes to HEYA. Two paucibacillary does with serum S/Ps of 2.0 to 3.0 have been identified. These clinical animals were fecal culture positive but with few acid-fast bacteria in their tissues. The johnin intradermal skin test agrees (12/13) with the serology results early in infection, but this test becomes negative as the animal becomes clinical (5/11). The johnin test is more easily used on a farm goat herd than a large range sheep flock. At certain times, milk is a more easily collected sample than serum, plus the milk pellet can be cultured. **The ELISA is the most expedient test for both the serum and milk samples from adults and for serum from offspring less than 3 months of age.**

As we identify individual positive animals, owners donate these animals. The animal is eventually euthanized, necropsied, and sampled for culture and histopathology. Johne's disease is only one cause of wasting in small ruminants; we also test for other diseases. The sheep flock is positive for OPP virus; the goat herd is negative for CAE virus. Fecal samples are also tested for intestinal parasites. Generally, MAP-positive animals tend to have high parasite loads that never clear after repeated anthelmintic treatments.

Characteristics of an outbreak of equine leptospiral abortion in the Bluegrass region of Kentucky, 2006-2007

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Leptospirosis was diagnosed by the Livestock Disease Diagnostic Center, Lexington, Kentucky on 37 late-term fetuses and 1 stillborn foal from October 2006 through March, 2007. The index case was received on October 5, 2006 and the last case for the season was received on March 9, 2007. These confirmed cases were distributed across 5 Kentucky counties and involved 34 Thoroughbred and 5 Standardbred foals.

Diagnoses were confirmed by fluorescent antibody (FA) testing on placenta, liver, and kidney specimens from the foals that were necropsied, gross and histological lesions, and positive mare and fetal antibody titers. During the same foaling season in 2005-2006, the laboratory diagnosed only 1 equine leptospiral abortion. Total rainfall was 14" greater in 2006 as compared to 2005, and may have been a factor in the increased number of cases.

Regarding the last 14 years of equine leptospirosis data in Kentucky, high leptospiral titers (> 1:3200) are predominantly serovars pomona, icterohaemorrhagiae, and grippotyphosa. However, the icterohemorrhagiae titers are considered to be cross-reactions.

The 2006-2007 abortions resulted in well over $1 million in losses to the equine industry in the Bluegrass region of Kentucky.

Anatomic Pathology Scientific Session

Sunday, October 21, 2007

Bonanza BC

Moderators: David Steffen, John Adaska

* Graduate student presentation

Effects of prolonged formalin fixation on diagnostic immunohistochemistry

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Immunohistochemistry is routinely used in diagnostic pathology for identifying infectious agents in lesions and immunophenotyping neoplastic diseases. With the development of antibodies cross-reacting in animal species and the identification of new markers, the use of immunohistochemistry will only increase in coming years.

Formalin fixation is considered a major limiting factor for diagnostic immunohistochemistry, as formalin can crosslink antigens and thereby mask epitopes. Prolonged formalin fixation is generally thought to result in loss of antigenicity; however, the relationship between the duration of formalin fixation and antigen detection has only been evaluated for a handful of antibodies. The goal of this study was to determine the effect of prolonged formalin fixation on the immunohistochemical detection of antigens using 30 commonly used antibodies.

Tissues collected from diagnostic submissions or from animals euthanized as part of a separate research project were serially sectioned and fixed in 10% neutral buffered formalin. Samples were processed at day 1 and at approximately 1 week intervals for 7-16 weeks. Two tissue sections at each time point were immunohistochemically stained with antibodies against the following cell antigens: protein gene product 9.5, chromogranin A + B, glucagon, insulin, lysozyme, Ki67, pan cytokeratin, cytokeratin 7, cytokeratin 5, high molecular weight cytokeratin, vimentin, glial fibrillary acidic protein, S-100, sarcomeric actin, smooth muscle actin, muscle specific actin, CD18, CD20, CD3, CD79a, and CD31. We also examined the effect of formalin fixation with antibodies for the following infectious agents: *Aspergillus sp*., porcine coronavirus, *Felid herpesvirus, Canine parvovirus, Porcine respiratory and reproductive syndrome virus, Mycobacterium avium ssp. paratuberculosis, Bovine herpesvirus 1, Porcine circovirus 2,* and type A rotavirus. In this study, we used immunohistochemistry protocols routinely used in our diagnostic service for each antibody. Sections were evaluated for amount and intensity of staining.

Canine parvovirus, Felid herpesvirus, and cytokeratin 7 staining was significantly decreased to near absence by 2, 3, and 4 weeks post-fixation, respectively. Although there was variation between time points, there was moderate to strong staining at all time points for all other antibodies.

In conclusion, these results suggest that **prolonged formalin fixation (up to 7 weeks) has minimal effects on antigen detection for most commonly used antibodies. However, the relationship between the length of formalin fixation and antigen detection is antibody dependent and each antibody should be evaluated independently.** These results further validate the use of immunohistochemistry in diagnostic pathology.

* Graduate student presentation

Sporadic congenital *Swinepox virus* infection in Germany and Spain: morphologic studies and detection and characterization of swinepox viruses

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Introduction. Swinepox (SP) is a mild, acute disease of pigs characterized by typical poxviral lesions in the skin. Although the disease has a worldwide distribution, its occurrence is relatively sporadic and mortality is usually low or very low. Congenital SP is of extremely rare presentation. Therefore, the purpose of this study is to describe 4 (1 from Spain and 3 from Germany) different cases of congenital SP in Germany as well as the characterization of the swinepox viruses (SWPV) involved.

Material and methods. A total of 21 piglets were studied by clinical and morphologic examination (18 from Southern Germany, 2006; 1 from Northern Germany, 2007; and two from Spain, 2007). In the 4 reported cases, some of the studied piglets were delivered dead or mummified, whereas others were apparently healthy although with skin lesions. In the tongue and skin of these piglets, multiple pustules and crusts of 1 cm diameter were observed all over the body. Similar lesions were also present in some of the live-born piglets. Portions of these lesions were taken for standard microbiological examination as well as for virological studies. Samples from most of the parenchymatous organs as well as skin were fixed by immersion in 10% buffered formalin and routinely processed for histological examination. For the specific detection of SWPV, a PCR method based on the thymidine kinase (TK) gene (546 bp) was used. PCR-amplicons were cloned, sequenced and compared among themselves and to reference strains.

Results. Microscopic examination of the skin and tongue revealed scattered ulceration and loss of epidermis with some crusts and pustules involving the full thickness of epithelium. Many epithelial cells presented ballooning degeneration with or without central nuclear clearing; moderate numbers of irregular eosinophilic intracytoplasmic inclusion bodies were observed. Moderate numbers of lymphoplasmacytic cells were also infiltrating the superficial dermis. No significant lesions were observed in the other examined organs. Microbiological cultures of skin yielded negative or inconclusive results for any pathogenic bacteria. SWPV infection was confirmed in all studied cases and piglets by means of PCR. Strains from southern Germany (n=2) and Spain (n=1) showed 100% nucleotide sequence identity compared to reference strains Kasza and Shope. The northern Germany strain (n=1) had one nucleotide exchange (T ➔ C) at position 132/374 of the amplicon (99.7 % identity) compared to previous strains, as well as another nucleotide exchange at position 306/374 (A ➔ T) compared to a southern Germany strain from 1971 (corresponding to a post-natal SP case).

Discussion. The present case report summarizes information concerning 4 cases of congenital SP from Germany and Spain. As far as we know, congenital SP is considered very rare in Europe and even its postnatal form is considered regressive or, alternatively, confounded by other diseases such as exudative epidermitis (chronic form). Although the diagnosis of SP is rather easy by histopathology, the use of PCR that specifically detects the TK region seems to offer a quick and specific molecular diagnosis. Moreover, this latter technique should permit molecular epidemiological studies, which are virtually missing at present.

Conclusion. Although it is a very rare presentation, **SP must be seriously considered within the differential diagnosis of skin diseases of neonatal piglets**. Moreover, **PCR offers a reliable and quick technique for the diagnosis of SWPV infection.**

* Graduate student presentation

Zinc-responsive dermatosis in a litter of Pharaoh hounds

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Three 11-week-old Pharaoh hound littermates were presented with 50-day duration of pruritus rapidly progressing to erythematous crusted papules affecting nearly every region of haired skin. Extremities and foot pads were exfoliative, erythematous and painful. Constitutional signs included inappetance, lethargy, mental dullness and inferior stature compared to unaffected pups in the litter. Two littermates identically affected were males and 1 less affected was female. The remaining 2/5 pups were unaffected. CBC and chemistry were normal for this age.

Histologically, the epidermis was hyperplastic and disorganized. Keratinized cells were present at various levels of the epidermis, with occasional prominent pallor of keratinocytes within the stratum spinosum. The stratum granulosum was disorganized with variable size and shape of keratohyaline granules. Marked surface parakeratotic to rarely orthokeratotic hyperkeratosis extended into follicular infundibula. Crusts contained scattered bacterial cocci and bipolar yeasts.

Blood zinc levels in two affected pups were low (<0.1-0.6 ppm, normal 0.8-2.0 ppm). Skin lesions and constitutional signs partially improved following treatment with per os zinc gluconate (10 mg/kg/day) and markedly improved after IV zinc sulfate (10 mg/kg). Serum zinc normalized following IV administration then decreased to subnormal levels despite oral zinc supplementation. One patient developed an intestinal intussusception following IV zinc sulfate and was euthanized. The two affected patients continue to require IV zinc supplement approximately every 3 weeks. At 7 months of age, the affected male was 11.4 kg and 50.8 cm at the withers, unaffected male 17.8 kg and 62.5 cm; affected female 10.4 kg and 50.8 cm, unaffected female 15.5 kg and 53.4 cm. Both affected pups have severe enamel hypoplasia. At 18 months of age, they are managed on approximate monthly IV zinc injections, with development of new skin lesions as an indicator for treatment.

This case represents a severe zinc-responsive dermatosis similar to lethal acrodermatitis in Bull terriers.

Spindle cell sarcoma of the meninges in a dog

José Ramos-Vara, Rebecca Packer, Patty Lathan, Brian Eyden, Craig Thompson, Margaret Miller

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A 33 kg, 9-year-old, neutered male Greyhound dog had a 2-month history of seizures with progressive ataxia and abnormal mentation. Image analysis revealed a large mass on the right side of the brain, invading the skull. Cytologically this mass revealed a large population of spindle-shaped cells that generally contained a single nucleus set in a scant to moderate amount of smooth blue cytoplasm. There was marked anisocytosis and anisokaryosis, moderate nuclear pleomorphism and the presence of multiple, variably sized prominent nucleoli. Due to the poor prognosis, the dog was euthanized.

On gross examination, the right dorsal and lateral aspect of the calvaria had a 3 x 3 x 1.5 cm firm mass partially replacing the frontal bone. This mass penetrated the skull and extended through the right cerebral hemisphere, forming a 3.5 x 3 x 2 cm well-circumscribed, firm mass displacing the left cerebral hemisphere. Histologically, the tumor was unencapsulated and composed of long intersecting bundles of tightly packed spindle cells with indistinct cell limits, pale eosinophilic cytoplasm, fusiform, hypochromic nuclei with single or multiple nucleoli and blunt ends. There were 19 mitotic figures/10 hpf. Thin cytoplasmic projections of the tumor cells extended into the neuropil. Approximately 40% of the mass was necrotic or hemorrhagic. The stroma was scant to moderate and usually with abundant collagen. Where the tumor invaded the skull, there was extensive lysis and remodeling. A diagnosis of spindle cell sarcoma was made. Metastatic disease was not observed.

Immunohistochemistry, using a panel for myogenic and neural differentiation, was performed on formalin-fixed, paraffin-embedded brain tissue. There was moderate to strong reaction with antibodies to smooth muscle actin, CD10, calponin, neuron-specific enolase (NSE), progesterone receptor, protein gene product (PGP) 9.5, and vimentin. Reactivity for muscle actin, claudin-1, desmin, and Glut-1 was weak. Approximately 30% of the neoplastic cells reacted with antibody to proliferation marker Ki67. No staining was detected with antibodies to pancytokeratins, cytokeratin 5, E-cadherin, glial fibrillary acidic protein (GFAP), KIT, p63, sarcomeric actin, and S100. Ultrastructurally, tumor cells contained abundant rough endoplasmic reticulum, peripheral filaments and showed cell surface fibronectin fibrils.

Based on immunohistochemistry and electron microscopy, this tumor is best regarded as a sarcoma with myofibroblastic differentiation. The moderate to strong immunoreactivity for smooth muscle actin, calponin, and CD10 suggests smooth muscle differentiation, but these markers can be present in other stromal cells including myofibroblasts. Endoplasmic reticulum is usually scant in smooth muscle cells; filaments are numerous. NSE and PGP 9.5 were also strongly positive, which would be unusual for a myofibroblastic tumor, but could be interpreted as “aberrant” immunostaining. The negative staining for S100 or GFAP would tend to rule out neural differentiation. Meningothelial origin was also excluded from the differential diagnosis by immunohistochemical and ultrastructural findings. Due to the lack of other masses on gross and microscopic examination, this tumor was considered to be primary and most likely originated from meningeal stromal cells.

Immunohistochemical characterization of canine meningiomas

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Meningiomas encompass a group of pleomorphic tumors. The current WHO classification of canine meningiomas includes meningotheliomatous, fibrous, transitional, psammomatous, angiomatous, papillary, granular cell, myxoid, and anaplastic. Some meningioma types can be confused with schwannomas, neurofibrosarcoma, and other spindle cell tumors. Current markers used in veterinary medicine for meningiomas are vimentin, NSE, and S100. However, these markers are detected in non-meningothelial tumors with similar phenotype.

In this study, we examined the immunoreactivity in 19 meningiomas of 2 novel markers used in the characterization of human meningiomas: E-cadherin and claudin-1. **Epithelial cadherin (E-cadherin)** is a protein present in cell-cell adherens junctions of epithelial and arachnoidal tissue, and has been detected in the majority of human meningiomas. **Claudin-1** is an important component of tight junctions and is present in nearly all carcinomas and in a variable number of human meningiomas.

Meningioma types included were 6 fibroblastic, 7 transitional, 3 microcystic, and 3 meningothelial, as determined by routine histopathology. Tumors had been fixed in formalin and embedded in paraffin following routine procedures. For E-cadherin, we used a mouse monoclonal antibody (BD Transduction, 610182) diluted at 1/100, incubated for 60 min. at room temperature and with LSAB+ as the detection system. For claudin-1, a rabbit polyclonal antibody (abcam, ab15098) diluted at 1/25, incubated for 60 min. at room temperature, and with EnVision+ as the detection system. Antigen retrieval using a decloaker and citrate buffer, pH 6.0 was used for both markers. Skin was used as positive control tissue. Tumor reactivity was graded as follows: 1+ (10-30% positive cells); 2+ (31-60% positive cells); 3+ (>60% positive cells).

Seventeen of 19 meningiomas had strong reactivity with antibody to E-cadherin. One case was weakly positive and one was negative. The staining was mostly localized in the cytoplasmic membrane with weaker and diffuse staining in the cytoplasm. There were no significant immunoreactivity differences amongst meningioma types. Only 5 meningiomas were positive for claudin-1; the immunoreactivity was weak to moderate and located mainly in the cytoplasmic membrane but also in the cytoplasm.

We conclude that E-cadherin is a good marker of meningothelial differentiation and can be used in conjunction with other markers to distinguish meningeal tumors with similar phenotypes.

Generalized nodular dermatofibrosis in the absence of renal neoplasia or cysts in an Australian heeler

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A 13-year-old, spayed female, Australian heeler dog had at least a 10-year history of numerous subcutaneous nodules of which fine needle aspiration and cytological evaluation was non diagnostic. Abdominal ultrasound 3.5 months prior to necropsy detected a small left kidney but no cysts or neoplasms.

Gross necropsy demonstrated innumerable, firm, round to oval, white, 0.25 to 2 cm masses throughout the subcutaneous tissues of the axial and appendicular skeleton, epimysium of numerous muscles and the visceral peritoneum of the lateral abdominal body wall. The left kidney was approximately half the size of the right, and there was severe bilateral renal papillary necrosis.

Histologically, the subcutaneous nodules were characterized by small, well-demarcated, collections of mature, hypocellular collagen that is consistent with previous reports of nodular dermatofibrosis associated with renal cystadenomas/adenocarcinomas (NDRC). Histologic evaluation of the kidneys demonstrated severe diffuse acute renal papillary necrosis and severe chronic lymphoplasmacytic interstitial nephritis.

This is the first report of nodular dermatofibrosis in a dog without concomitant renal cystadenomas/adenocarcinoma.

* Graduate student presentation

Pet-food nephrotoxicity in cats and dogs: towards a case-definition

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Nephrotoxicity in dogs and cats induced by contaminated pet foods has captured the attention of owners, veterinarians, and diagnosticians, causing nationwide alarm over these animal deaths. Yet, renal disease is a common finding in diagnostic laboratories and this new disease entity must be carefully distinguished from the many other causes of acute renal failure. As part of the AAVLD-led effort to survey the veterinary community for cases of pet food nephrotoxicity, many cases were reviewed. As a preliminary step towards a case definition, 5 necropsy cases were identified, (4 cats and 1 dog) for which data were available in 5 areas:1) history of consumption of contaminated pet food, 2) urinalysis, 3) serum clinical chemistry, 4) gross and/or histologic findings, and most importantly, 5) kidney or urine testing for melamine, cyanuric acid, ammeline, or ammelide.

A positive kidney or urine test for 1 or more of the 4 contaminants was required for inclusion in this group. More than 1 of the contaminants was present in 4 of the 5 animals. All cats and the dog had a history of ingestion of a contaminated food brand, though lot numbers could not always be confirmed. In addition all had markedly elevated blood urea and creatinine concentrations, in a range of 125-310 mg/dL (44.6-110.6 mmol/L) and 11.5-22.5 mg/dL (1,017-1,989 µmol/L), respectively. Serum phosphorus was 8.6-16.6 mg/dL (2.8-5.4 mmol/L). The appropriate reference intervals for blood urea, creatinine, and phosphorus are 7-41 mg/dL (2.5-14.6 mmol/L), 0.5-2.2 mg/dL (44-194 µmol/L), and 1.9-8.3 mg/dL (0.6-2.7 mmol/L), respectively. Urine was isosthenuric, with specific gravity of 1.010 – 1.014. Casts, leukocytes, erythrocytes, and yellow-to-brown crystals with radial striations resembling shattered round plates were often but not always present in the urine sediment. Histologically, the most common finding was the presence of characteristic yellow-brown crystals within the renal distal tubules and collecting ducts with or without microscopically visible tubular necrosis, degeneration, or sloughed epithelium. It was also very common to see, in addition, chronic interstitial nephritis, which may have predisposed the animals to intoxication and death. Two additional dogs and 2 cats lacked urinalysis results, but otherwise were similar in their clinical and necropsy presentation.

Further characterization of the clinical presentation and lesions in a much larger sample is underway using the database of cases in the AAVLD survey. Specifically, the temporal progression, the role of predisposing diseases, the prognostic value of any of the clinical values, and the association of all 4 contaminants to the presence of disease are important issues to address.

Nonsuppurative meningoencephalitis associated with Aleutian disease in farmed mink (*Mustela vision*)

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In October of 2004, a mink farm in southeastern Idaho experienced a severe and sudden outbreak of diarrhea, anorexia and lethargy that often resulted in death of adults and weaned kits. On gross necropsy of 4 such affected animals, the kidneys of 3 were bilaterally moderately enlarged, diffusely pale tan, irregularly roughened and speckled by dark tan to white pinpoint foci. These affected individuals also had variable degrees of segmental enteritis and pulmonary congestion and edema. The fourth animal, which had no gross renal lesions, had severe hepatic lipidosis.

Histologically in the 3 mink with gross renal lesions, there was severe lymphoplasmacytic interstitial nephritis with diffuse and global membranous glomerulopathy and variable mineralization of renal tubules. Other lesions included moderate periportal lymphoplasmacytic hepatitis and germinal center necrosis in each spleen. All **affected animals had mild to moderate segmental nonsuppurative meningitis with variable extension of inflammation into the gray matter predominantly around vasculature.** In 2 of the 3 affected mink, vasculature throughout the cerebrum and brainstem was cuffed by mild to moderate numbers of lymphocytes, plasma cells, histiocytes and fewer neutrophils. Endothelium of affected vasculature was prominently hypertrophied. Such lesions were commonly associated with gliosis and satellitosis.

Bacterial culture from brain sections was negative for all animals. Reverse transcriptase polymerase chain reaction (RT-PCR) and immunohistochemistry (IHC) for *Canine distemper virus* was negative. ***Aleutian mink disease virus*** **DNA was detected in sections of kidney and brain by PCR and confirmed by sequencing.**

Aleutian disease, caused by a parvovirus, is characterized by plasmacytic infiltration of multiple organs with immune- complex glomerulonephritis, as also observed in the mink here. Nonsuppurative meningitis and meningoencephalitis are rarely reported in cases of Aleutian disease. Thus, **this case series represents a unique manifestation of Aleutian disease in mink.** The actual prevalence of this lesion in cases of Aleutian disease is unknown. The authors of the only previously reported Aleutian disease outbreak in mink associated with meningoencephalitis suggested that the observed neuropathology may be virus strain specific. In addition, variations in lesion location and intensity may be at least in part depend on factors such as age, mink color strain, and immune status of the affected animal.

* Graduate student presentation

A serologic, histologic, and immunohistochemical survey of leptospirosis in Ohio raccoons *(Procyon lotor)*

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Leptospirosis is a zoonotic bacterial disease caused by pathogenic spirochetes of the genus *Leptospira*. The disease occurs worldwide and affects numerous hosts including humans, domestic animals, and wildlife. In order to gain insight into leptospirosis in a less-studied but common North American wildlife species, 115 raccoons (*Procyon lotor*) were captured as part of a nuisance control program in Ohio. Serum chemistry, including blood urea, creatinine, and urinalysis to evaluate presence and degree of azotemia, was performed in all animals. Serology for leptospiral antibody titers was also done in each case. Full necropsies were performed on all raccoons, and sections of liver and kidney were collected for routine microscopic evaluation. Microscopic renal pathology was classified by severity (mild, moderate, severe), distribution (focal, multifocal, diffuse), microanatomic location (interstitial, tubulointerstitial, tubular, glomerular), and nature of inflammation (plasmacytic, lymphoplasmacytic, mixed neutrophilic.) In addition, immunohistochemistry, with an antibody that cross-reacts with all virulent leptospiral serovars, was performed on sections of kidney from every raccoon.

Of the 115 kidneys surveyed, 16 (13.9%) were positive for the presence of leptospiral antigen by immunohistochemistry. All 16 kidneys exhibited microscopic pathology. The predominant microscopic pattern was mild to moderate plasmacytic to lymphoplasmacytic interstitial nephritis in 11/16 (68.75%) kidneys. Leptospiral antigen was detected within inflammatory foci in 13/16 (81.75%) of the kidneys surveyed. More intense immunohistochemical staining was observed in the most severely inflamed kidneys. In 3 kidneys, histologic lesions were mild and leptospiral antigen was not associated with inflammatory foci.

This is the first reported investigation to characterize renal pathology in raccoons and correlate it with the presence of pathogenic leptospiral antigen as well as serologic data and serum chemistry. **Leptospiral infection was confirmed in 13.9% of examined raccoons in the state of Ohio.** This incidence is similar to what has been reported based on the detection of leptospiral antibodies in numerous serologic surveys of raccoons in the United States and Canada. **The results of this survey further confirm that the raccoon has to be considered a potential reservoir of leptospirosis and may represent a health risk to dogs, humans, and other species.**

* Graduate student presentation

Equine giant cell tumor of soft parts: a series of 18 cases (2000-2007)

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In horses, giant cell tumors of soft parts are rare neoplasms, with the majority of reported cases occurring within the hindlimb muscles and soft tissues in older horses. The following report documents 18 cases of equine giant cell tumors of soft parts with the majority occurring in male horses aged 10 years or older.

Nine of the 18 cases (50%) arose within the hindlimbs. Histologically, the neoplasms were similar in appearance with the majority demonstrating liposarcomatous change, variable areas of necrosis and hemorrhage, and an intermediate number of mitotic figures. Key histologic features included numerous multinucleated giant cells and hemosiderin-laden macrophages admixed with a spindle cell proliferation.

Surgical excision was attempted in the majority of horses and considered clinically complete; recurrence of the neoplasm was documented in 1 horse. In 17 horses, surgical excision, regardless of margin integrity, appeared successful with no recurrence of disease documented within 2 months. Unfortunately, 6/18 horses were lost to follow-up within approximately 2 months of surgery. Therefore, long-term survivability and recurrence of the neoplasm at the previous surgical site could not be evaluated. **Within the 11 remaining horses that were available for follow-up evaluation, there has been no evidence of metastasis, and all 11 horses continue to do well.**

A larger case series with more controlled follow-up is necessary to further evaluate malignant potential and the importance of complete surgical excision.

Mineralization of the brain stem in horses with clinical neurological signs

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Six aged horses (older than 18 years) with a clinical history of neurological signs of 24 to 72 h duration were submitted for post-mortem examination to the San Bernardino branch of the California Animal Health and Food Safety Laboratory of the University of California, Davis, between 2004 and 2006. The horses originated in different properties of Southern California.

Post mortem examination revealed focal symmetrical areas of mineralization and malacia in the cerebellar white matter in areas corresponding to deep cerebellar nuclei. Histologically, severe mineralization of blood vessels (arteries and veins) and neuropil surrounding these vessels was prominent in these areas. A few neurons and glial cells were degenerate or necrotic in areas surrounding vascular and parenchymal mineralization. Mild to moderate mineralization of blood vessels, but not of brain parenchyma, was also observed in the internal capsule of these horses. Six additional age-matched horses dying of causes unrelated to CNS disease during the same period and originating in the same area were included in this study as controls. No gross abnormalities were observed in the brains of these horses.

Microscopically, only mild mineralization of blood vessels in the cerebellar white matter was noted. No inflammatory infiltrate was observed in any of the areas of mineralization or elsewhere in the central nervous system of any of the horses in both groups studied. Ancillary tests performed in most horses included virus isolation, PCR for West Nile virus and *Equid herpesvirus 1*, western blot for *Sarcocystis neurona*, serology for EEEV, WEEV, VEEV and SLEV, and determination of brain cholinesterase. Results of all these tests were unrewarding.

Mineralization of blood vessels in aged horses is usually considered an incidental finding without clinical significance. However, **the severe mineralization observed in the brains of the 6 horses with neurological signs in this study coupled with negative results for other possible etiologies suggests a relationship between mineralization and the clinical signs observed.**

Recent occurrence of the neurogenic form of *Equid herpesvirus 1* infection in horses in California

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This paper describes the recent occurrence of the neurogenic form of *Equid herpesvirus 1* (EHV-1) in 11 horses in California.

The first case, a 7-year-old Warmblood was one of the 15 horses shipped from Germany to the New York Animal Import Center in Newburgh, NY where horses were quarantined for 2 weeks. The other 14 horses were shipped to 7 different states. The Warmblood arrived in southern California in early December 2006 and within 2 days it developed fever, depression, ataxia, bladder paralysis and eventually became recumbent. The animal was unable to rise up again, became unresponsive to treatment, and subsequently it was euthanized and submitted for necropsy. In the following weeks, two carcasses of adult horses that were euthanized after showing ataxia, bladder paralysis and recumbence, two full-term male aborted fetuses, and samples of nasal swabs and blood from 6 other horses with history of fever, acute onset of ataxia and urinary incontinence were submitted for examination.

Gross pathology was not very remarkable, except 2 of the 3 horses that showed multifocal acute hemorrhage of the white matter in the cervical and thoracic spinal cord. In adult horses, common microscopic lesions were mild to moderate subacute encephalomyelitis, vasculitis and multifocal hemorrhages. Microscopic lesions in the aborted fetuses consisted of acute hepatic necrosis, thymitis, and interstitial pneumonia with abundance of amphophilic/eosinophilic intranuclear inclusion bodies. Tissues were positive by fluorescent antibody test and/or immunohistochemistry for EHV-1.

Confirmation of the presence of the neuropathogenic marker in EHV-1 was performed using a real-time PCR assay. **All 5 necropsy cases and the 6 clinical horses tested positive for the neuropathogenic marker on nasal swabs, whole blood and or tissue/brain pool.** The non-neuropathogenic form of EHV-1 was also detected by PCR on all clinical cases and 5 of 6 necropsy cases. No epidemiological link was found between the first case and the other 10 California cases.

Pathogenesis of proximal sesamoid bone fractures in Thoroughbred racehorses

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Introduction. Fractures of the forelimb proximal sesamoid bones incurred during racing and training result in up to 40% of California Thoroughbred racehorse deaths annually. Mid-body fractures, often of both bones within a limb, are most common. The pathogenesis of proximal sesamoid bone fractures is poorly understood. Trabecular microstructure anisotropy may increase with different training methods in equine proximal sesamoid bones. Active training may strengthen the suspensory ligament, leaving the proximal sesamoid bones as the weakest point of the suspensory apparatus and predisposed to fracture under high load circumstances. Since underlying pathology of bone precedes complete fractures of Thoroughbred racehorse humeri and third metacarpal bones, we hypothesized that proximal sesamoid bone fractures are acute events caused by microstructural maladaptation due to chronic, repetitive loading events (racing and training). Our objectives were to determine if sesamoiditis, exercise history, and bone microstructural features were associated with proximal sesamoid bone fracture.

Materials and methods. a) Forelimb proximal sesamoid bones were collected from 328 racing Thoroughbreds submitted to the California Animal Health and Food Safety Laboratories for the California Horse Racing Board Postmortem Program. Osteophytes, large vascular channels and fracture location were categorized from high-resolution contact palmarodorsal radiographs. Relationships between radiographic findings and fractures were examined by use of χ^2 and logistic regression techniques. b) Official timed race and workout records were compared between 121 horses with a proximal sesamoid bone fracture and 148 horses without a fracture. Logistic regression analysis was used to evaluate potential exercise risk factors for proximal sesamoid bone fracture. c) Fractured proximal sesamoid bones from 8 horses with a mid-body fracture, and intact proximal sesamoid bones from the contralateral limb of the same 8 horses with fracture and from 8 age/sex-matched horses without a fracture were examined. Undemineralized sagittal bone sections were dehydrated, embedded in methylmethacrylate, and hand-polished to produce sections 100 μm thick for microradiographs. Microradiographs were assessed for bone composition and fracture margin qualities.

Results. Odds of proximal sesamoid bone fracture were approximately 2 to 5 times higher in bones without radiographic evidence of osteophytes or large vascular channels, respectively. Horses with fractures spent more time in active training and racing, completed more events, trained and raced longer since their last lay-up period, had higher exercise intensities during the 12 months prior to death, and had greater cumulative distances overall than horses without proximal sesamoid bone fracture. Age/sex-matched control bones were more porous than fractured bones and intact contralateral control bones. Mid-body fractures appeared to begin within the palmar region of the bone.

Discussion. Fractures were less likely to occur in bones with evidence of sesamoiditis. Limiting exercise intensity and the continuous time spent in activity during a horse's career may decrease the frequency of proximal sesamoid bone fractures in Thoroughbred horses. Mechanical properties of the dense trabecular bone of proximal sesamoid bones may differ from human vertebral trabecular bone, that is generally weaker when more porous. Proximal sesamoid bone fracture appears to begin due to tension associated with suspensory and distal sesamoidean ligamentous forces, and propagates to the region of the bone normally in compression with the third metacarpal bone condyle.

Conclusion. Proximal sesamoid bone fracture in Thoroughbred racehorses is associated with radiographic, epidemiologic, and biomechanical features that are useful in the design of preventive strategies.

* Graduate student presentation

Necrotic enteritis caused by *Clostridium perfringens* type C in horses

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Three 2- to 7-day-old foals, 2 foals 3- and 5-months-old, 2 adult horses, and tissues from a 2-day-old foal, all with clinical histories of colic and/or diarrhea followed by acute death, and 2 with acute fatal illness were submitted for post-mortem examination to the San Bernardino branch of the California Animal Health and Food Safety Laboratory of the University of California, Davis.

Post-mortem examination revealed necrotizing enteritis in 5 cases and, in 1 case each, entero-typhlocolitis, typhloenteritis, or enterocolitis. Histological examination revealed necrotizing enteritis and/or typhlocolitis with acute coagulation necrosis of the superficial mucosa, mild neutrophilic infiltration, congestion and multiple thrombosis of lamina propria and submucosa, and large numbers of gram-positive bacilli in the lumen mixed with sloughed epithelial cells, neutrophils, fibrin and cell debris.

A rich, almost pure culture of *Clostridium perfringens* was obtained from the small intestine in all animals. *C. perfringens* beta (CPB) toxins were detected in intestinal content of all the animals and alpha (CPA) toxins were detected in 3 animals. Three isolates were typed by PCR, being 1 toxinotype C, 1 toxinotype A, and 1 toxinotype AE. The gene encoding for beta 2 toxin was present in 2 of these isolates (type C and type AE). All 3 isolates were negative for the gene encoding for *C. perfringens* enterotoxin. *Salmonella* culture was negative and no aerobic bacterial pathogens were isolated from intestinal or colonic content or a variety of tissues cultured from all of the animals.

Detection of CPB coupled with isolation of *C. perfringens* type C from intestinal contents of these horses confirmed a diagnosis of infection by this microorganism. Young foals are particularly sensitive to *C. perfringens* type C infection, probably due to the low level of trypsin in the intestine.

Toxicology Scientific Session

Sunday, October 21, 2007

Genoa

Moderators: Catherine Barr, Patricia Talcott

* Graduate student presentation

Determination of serum and tissue melamine and/or cyanuric acid concentrations in growing pigs

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In the spring of 2007 many of the country's top pet food producers were forced to recall over 1100 varieties of their most popular foods which were found to cause renal toxicosis in cats and dogs in the United States. In April of 2007, at least 56,000 market weight pigs were accidentally fed the contaminated food. The US Food and Drug Administration has proposed that melamine, a component used in the production of plastics and fertilizer, is a potential causative agent of the renal toxicosis. This pilot study was designed to answer some of the questions about melamine and its effects as a renal toxin.

Twenty-two, 5-week-old pigs entered the study and were split into 5 groups, each consisting of 2 males and 2 females, as well as 1 control group of 1 male and 1 female. The groups were gavaged with 200, 400, and 1000 mg melamine per kg BW; 400 mg cyanuric acid (a metabolite of melamine) per kg BW; and finally a mixture of 400 mg of melamine and 400 mg of cyanuric acid per kg BW. The vehicle used to dose controls and prepare the dose suspensions was a canned preparation of turkey gravy. After 10 days of gavaging, the control pigs and a randomly selected male and female from each dose group were euthanized and necropsied. Tissues, including urine, serum and feces, were saved for melamine analysis. The remaining 10 pigs then became a '2-week recovery group', at which time they were euthanized and necropsied to investigate the concentration and distribution of melamine and cyanuric acid in serum and various tissues.

From day 0 to day 10, the low- and mid-dose melamine and the cyanuric acid treatment group animal's blood urea and creatinine concentrations remained within normal limits, while 1 pig from the high dose melamine group and all 4 pigs in the combination melamine/cyanuric acid group showed elevated blood urea and creatinine levels. Common clinical observations in the affected pigs included intermittent loose stools, anorexia, dehydration and lack of fecal production. In more severely affected pigs, lethargy, anuria, anorexia and bright red blood on the outside of the stool were observed. After 7 days of dosing, all of the animals gained weight, except 1 in the high dose melamine group and 1 in the combination melamine/cyanuric acid group, both of which lost weight.

After 10 days of dosing, there was an increase in blood urea and creatinine levels in animals from the melamine/cyanuric acid combination group. Preliminary results suggest that the combination of melamine and cyanuric acid is a more potent nephrotoxin than either melamine or cyanuric acid individually.

Assessment of melamine and cyanuric acid toxicity in cats

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Introduction. The major recall of pet foods associated with numerous episodes of acute renal failure in dogs and cats focused initially on melamine as the suspect toxicant. Melamine, a chemical used for the production of melamine resins and as a fertilizer, was found in suspect pet foods. Melamine is considered to be relatively non-toxic, but specific data on toxicity, especially in cats is lacking. In the course of the pet food recall investigation, cyanuric acid was identified in addition to melamine. However, only limited data on the toxicity of cyanuric acid to mammals exist. Additionally, the possibility of the combination of toxicants, melamine and cyanuric acid, being responsible for renal failure in cats and dogs has not been established.

Materials and methods. In a pilot study, melamine was added to the diet of cats at 0.5% and 1%, 1 cat at each concentration. Cyanuric acid was added to the diet of 1 cat at 0.2% for 4 days, followed by 0.5% for 3 days, and 1% for 3 days. Subsequently, melamine and cyanuric acid were administered together at 0% (control), 0.2%, 0.5%, and 1% of each, to 1 cat per dose group. Clinical observations were made at least once daily. Routine serum chemistries and urinalyses were obtained immediately before feeding the spiked food and at various time points during the dosing periods. The 3 cats dosed with melamine and cyanuric acid were euthanized at 48 h after dosing and a full post-mortem examination was performed. The cat dosed with cyanuric acid for 10 days was euthanized 24 h after the last dose and a necropsy was performed. Urine and kidneys were analyzed for melamine and cyanuric acid using liquid chromatography/mass spectrometry.

Discussion. No effect on renal function was observed after feeding melamine at 0.5% or 1% for 10 days. Exposure to cyanuric acid of up to 1% in the diet for a total of 10 days had no effect on renal function. Approximately 12 h after dosing with melamine and cyanuric acid combined, the 3 dosed cats developed slight depression, vomiting and anorexia. The cats did not eat the second spiked food sample offered. Renal function was significantly impaired (marked increase in serum urea and creatinine) in all 3 cats when assessed at 36 h after the initial melamine and cyanuric acid exposure. Gross lesions were limited to the kidneys (all 3 cats) and lungs (2 cats). In all cats, fan-shaped, birefringent crystals were detectable by gross examination, urinalysis, touch impression, and histology. Histology on either alcohol-fixed or formalin-fixed tissue revealed crystals, often occlusive, within the distal nephron extending from the renal papilla to the cortex. Renal interstitial edema was severe, and present throughout the kidney. At the corticomedullary junction, hemorrhage and tubular damage were present in regions remote from detectable crystals. In 2 cats, there was pulmonary edema. The kidneys contained estimated concentrations of 110 to 120 mg melamine /kg wet weight and 215 to 245 mg cyanuric acid /kg wet weight. These estimated concentrations are comparable to levels detected in diagnostic cases.

Conclusion. The results of this pilot study demonstrate that neither melamine nor cyanuric acid alone had an observable effect on the renal function of cats. However, **the combination of melamine and cyanuric acid caused acute renal failure in cats.**

A method for the analysis of melamine-related compounds in kidney tissue

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Introduction. Ingestion of melamine adulterated pet food has been implicated in kidney failure and death in cats and dogs. Melamine and the related compounds ammeline, ammelide, and cyanuric acid have been detected in suspect food and its constituents. **Crystalline material found in the kidneys and urine of affected animals has been determined to consist of melamine cyanurate, a salt of melamine and cyanuric acid.** In order to support diagnostic investigations into melamine toxicity, a method was needed to analyze for melamine cyanurate in kidney tissue. This analysis was complicated by the fact that the compound is insoluble in water and in most organic solvents.

Materials and methods. Melamine cyanurate, melamine, ammeline, ammelide, and cyanuric acid were extracted from 1 g of kidney tissue by homogenization in 25 mL of 50/40/10 acetonitrile/water/ diethylamine, a solvent mixture that dissolves melamine cyanurate; 2.5half mL of the liquid extract was diluted to 8 mL by addition of 5.5 mL of acetonitrile. The diluted extract was sonicated and centrifuged in order to precipitate and remove proteins; 4 mL of the supernatant were transferred to a separate tube and evaporated dry under nitrogen. The extract was reconstituted in 0.5 mL of 20% acetonitrile in water and the internal standard $^{15}N_3$-melamine was added. The extract was analyzed on an Applied Biosystems 4000 Qtrap LC-MS/MS system fitted with a Microm Biosystems HPLC and an Applied Biosystems atmospheric pressure chemical ionization source. Analysis was performed using selective reaction monitoring for two separate precursor-product ion transitions for each analyte. Ammeline, ammelide, and cyanuric acid were analyzed in negative ion mode, whereas melamine and $^{15}N_3$-melamine were analyzed in positive ion mode. Method performance was evaluated by analysis of control tissue samples fortified with crystalline melamine cyanurate as well as separate control samples fortified with the 4 analytes in solution. The method was also used for routine analysis of tissue taken from animals suspected of having died from ingestion of contaminated food.

Discussion. Recovery of melamine cyanurate from fortified control samples was measured as the sum of the melamine and cyanuric acid levels vs. the amount of melamine cyanurate fortified into the sample. Recoveries were between 80 and 90%, demonstrating that the extraction procedure effectively separated the complex into its constituent molecules and that it efficiently removed them from the matrix. Recoveries from samples fortified with the 4 analytes in solution ranged from75 to 90% for melamine, ammelide, and cyanuric acid. They were somewhat lower for ammeline. **This indicates that this method is effective at extracting any of the free analytes present as well as the melamine cyanurate salt**. The detection limit for this method was 2.5 ppm for each separate analyte (equivalent to 5 ppm of melamine cyanurate).

Phosphine poisoning in an equine boarding facility

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Nine hours after the morning feeding, horses at a boarding facility began to exhibit generalized muscle tremors, profuse sweating, and ataxia. Over the course of the next 6 hours, clinical signs progressed to recumbency, seizures, and cardiovascular shock despite treatment with atropine, diazepam, xylazine, and detomidine during the rapid, large scale response of veterinary hospital personnel. Sera taken from several animals 15 hours post-exposure revealed severe hypoglycemia, lactic acidosis, and elevated blood ammonia concentrations. Six hospitalized animals appeared to stabilize with glucose therapy. However, ammonia, GGT, AST, and AP levels rose as treatment progressed, and signs of hepatoencephalopathy began 8-15 hours later. Four of the 6 died or were euthanized.

As differential diagnoses were postulated, it was revealed that pelleted feed stored in a grain bin at the facility had been treated with aluminum phosphide tablets to reduce the weevil population. Instead of being sealed for several days then aired prior to use, the feed was distributed among 66 horses living on site the morning after it was treated.

Six horses were necropsied, including 2 of those that died at the boarding facility. Phosphine gas was detected in the stomachs of the first 3 horses that were necropsied. The major gross finding was petechial to ecchymotic hemorrhages in multiple organs. Histologically, the most consistent findings were edema and neuronal necrosis in the cerebrum, congestion in multiple organs, and hepatocellular lipidosis. Three horses that lived at least 24 hours following exposure had centrilobular necrosis in the liver. **The pathologic findings, although nonspecific, were similar to those described in a previous report of zinc phosphide toxicosis in a horse and in multiple reports of phosphine toxicosis in humans.**

Of the 66 horses reportedly given the treated feed, 27 died or were euthanized. Of the 29 horses that showed clinical signs, only 2 recovered. Based on post-exposure monitoring of clinical chemistry parameters, there were no apparent physiological sequelae to the incident.

An overview of the use of GFAAS, ICP-AES and ICP-MS instrumentation for the analysis of metals in a veterinary diagnostic toxicology laboratory

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From toxicological and nutritional perspectives, metals play a critical role in evaluating animal health. Historically, as with practically all analytical toxicology methods, analysis of metals in veterinary samples has been conducted using relatively insensitive methods that were both time and energy consuming. In recent history, analytical systems for the detection and quantification of metals have become much more sensitive, accurate and efficient and have progressed from detecting one metal at a time with atomic absorption spectrometry (flame AAS or graphite furnace AAS [GFAAS]), to detecting dozens of metals with one analysis using inductively-coupled argon plasma atomic emission spectrometry (ICP-AES), to potentially pinpointing a source for metal exposure by measuring metal isotopes utilizing inductively-coupled plasma mass spectrometry (ICP-MS).

The Perkin-Elmer AAnalyst 800 GFAAS, the Fisons Accuris ICP-AES and the Agilent 7500ce ICP-MS analytical systems are utilized collectively on a daily basis for the analysis of metals at the veterinary diagnostic toxicology section of the California Animal Health and Food Safety Laboratory System (CAHFS). Factors such as sensitivity, accuracy, precision, robustness, speed and ease of use, and versatility are considered when deciding which instrument or combination of instruments to use for the analysis of a particular metal or array of metals. For example, each day, as part of a rapid ICP-AES screen for an array of metals, lead is analyzed semi-quantitatively in a large number of tissues. Subsequently, rapid, quantitative, confirmatory analysis of any suspect lead-positive tissues is carried out by GFAAS or ICP-MS. If desired, the ICP-MS can also then be utilized to measure lead isotope ratios to potentially help identify the source of lead exposure.

The collective use of GFAAS, ICP-AES and ICP-MS systems provides great depth and breadth of analytical capability for the identification, detection, quantification, and confirmation of metals in the veterinary diagnostic toxicology lab setting.

Hepatocellular necrosis associated with arsenic poisoning in cattle

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Arsenic poisoning was diagnosed in a group of 160 Brahman-cross cattle aged 15-18 months. The cattle had been transported 1,200 km and denied access to food for 72 h and water for 18 h. After being unloaded from the trucks, they were given access to water and then placed overnight in a holding yard which contained an old arsenical dip site. The following morning the cattle were moved to a paddock containing pasture. Clinical signs and deaths commenced approximately 24 h after placement in the holding yard. Some animals were found dead. Clinical signs were characterized by diarrhea, a high-stepping gait, ataxia and recumbency. The mortality rate was approximately 35%, with the majority of deaths occurring in the first 24 h of the clinical episode.

Serum biochemical changes in clinically affected animals included elevations in glutamic dehydrogenase, gamma-glutamyl transpeptidase, total bilirubin, urea and creatinine. Histologically there was hepatocellular necrosis and mild renal tubular necrosis. The hepatic lesion varied from patchy single cell necrosis to confluent necrosis of small groups of centrilobular hepatocytes. Arsenic poisoning was confirmed by determination of arsenic levels in liver (7-19 mg/kg wet weight) (n=4) and kidney (15-17 mg/kg wet weight) (n=2). Some soil immediately beside the old dip site appeared to be missing, suggesting the cattle had licked it.

Possible contributing factors were the use of salt licks prior to the cattle being transported, the prolonged periods without food and water, and the stress of transport. The latter 2 factors may have influenced the metabolic state of the cattle at the time of their exposure to the arsenic.

Noteworthy features of this episode were the prominent hepatocellular necrosis and the very long interval (40-50 years) since previous use of arsenic in the dip. During the intervening years, there had been no recognized poisoning of other cattle that had access to the holding yard.

This episode highlights the ability of heavy metals such as arsenic to persist in the environment and the subsequent danger posed to animals and humans.

Concentration of arsenic in milk and meat products of dairy cattle exposed to elevated water arsenic concentrations

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Introduction. The United States Environmental Protection Agency reduced the allowable concentration of arsenic in public water supplies from 50 ppb to 10 ppb. All large municipal water supplies should have been in compliance as of January 2007. This regulatory effort by EPA is part of a much larger initiative to reduce exposure of United States citizens to arsenic from all sources due to its possible epidemiological association with cardiovascular disease, diabetes, cancer, and other diseases of humans. Minnesota Dairy producers asked about the potential impact of this arsenic initiative on their industry.

Materials and methods. Twenty dairy herds were enrolled in the study. These herds were stratified as low, medium, and high herds ingesting water containing less than 10 ppb, from 10 to 40 ppb, and greater than 40 ppb arsenic, respectively. The range of water concentrations was 0.5 to 114 ppb. Urine was collected from 6 cows per herd. Water, feed and bulk tank milk were collected from each herd. Milk was collected from the low and high herds and made into cheese. Skeletal muscle, heart, liver, kidney, and pancreas were collected from cull cows in the low and high groups. Samples were analyzed for arsenic using inductively coupled plasma mass spectroscopy with a limit of quantitation of 5 ppb from most samples.

Results. Arsenic was present in urine in a dose responsive manner correlated with water arsenic concentration. Arsenic was not detected in milk, cheese, whey, liver, heart, skeletal muscle, or pancreas, but was detected in kidney.

Conclusion. Elevated arsenic concentrations were not present in milk or most edible tissues derived from dairy cattle drinking water containing the elevated concentrations of arsenic found in Minnesota wells.

Rapid screening of feed samples for mycotoxins by LC-MS/MS

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Introduction. Mycotoxins are secondary metabolites produced by fungi, typically from the *Aspergillus*, *Penicillium* or *Fusarium* genera. About 300 secondary metabolites have been isolated from agricultural products during all stages of crop production and storage. Mycotoxin accumulation in food and feeds creates a worldwide problem for livestock and humans. They are associated with various adverse health effects such as feed refusal, vomiting, induction of cancer and immunotoxicity. Several analytical methods have been published for determination of mycotoxins in cereals, feeds, foods and other biological samples. In recent years, liquid chromatography coupled with tandem mass spectrometry (LC-MS/MS) has become the technique of choice for rapid, unambiguous screening of feed samples for these compounds.

Materials and methods. Rapid, sensitive and selective analytical methods for detection of mycotoxins including aflatoxins, DON, DAS, T-2, zearalenone, ochratoxin A, fumonisin B_1, lolitrem B, penitrem A, roquefortine C in feed and other biological samples using liquid chromatography coupled with triple quadrupole/linear ion trap mass spectrometry (LC-MS/MS) have been developed. Feed samples for the main mycotoxin screen, including aflatoxins, DON, DAS, T-2, zearalenone and ochratoxin A, were prepared as follows: stein-milled feed samples (10 g) were extracted with 40 mL of acetonitrile:water (84:16, v/v), an aliquot of each sample was cleaned-up using the MycoSep226 extraction columns (Romer Labs ^{R}). Concentrated extract was filtered through a 0.45 µm filter into an autosampler vial and analyzed. Procedures for fumonisin B_1, lolitrem B, penitrem A, and roquefortine C required sample extraction using other solvents such as methanol:water (fumonisin B1,) or methylene chloride (penitrem A, roquefortine C, lolitrem B). All sample extracts were 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). Twenty µL of standards in matching matrices or in sample extracts were injected into the LC-MS/MS. Each set of sample runs contained a reagent blank, control and fortified samples.

Discussion. The LC-MS/MS methodologies developed in this study are suitable for rapid analyses of feed samples for a variety of mycotoxins. The method detection limits are at or below the detection limits offered by other screening techniques, such as ELISAs, allowing for confirmatory testing on suspect samples. The advantage of the LC-MS/MS approach over the conventional methods and ELISAs is the reduced length of sample preparation and guarantee of unambiguous results.

Conclusion. The LC-MS/MS methods have been successfully applied to analyses of many diagnostic samples for aflatoxins, DON, DAS, T-2, zearalenone, ochratoxin A, fumonisin B_1, lolitrem B, penitrem A and roquefortine C, providing rapid turn around times and reliable results. The LC-MS/MS approach requires less sample clean-up and provides sufficient number of identification points typically required for confirmation of results.

Diagnostic and residue support of an acute organophosphate toxicosis in cattle

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On April 10 at approximately 7 AM, approximately 162 feedlot cattle were fed a total mixed ration (TMR) prepared in a truck-mounted mixer and delivered to a fence-line bunk. Within 1-3 hours, cattle were exhibiting profuse drooling, dyspnea, diarrhea, muscle tremors, ataxia and incoordination, and recumbency. By approximately 10 AM, an estimated 100 animals were dead or recumbent. Within 3 days, 162 animals in the lot had died. Attending veterinarians reported blood was normal in color. Feed in the affected pen had an odor different from other pens. Affected animals responded favorably to parenteral treatment with atropine unless their clinical condition was beyond recovery.

Initial samples were delivered to the Iowa State University Veterinary Diagnostic Laboratory on April 11 and scheduled for immediate analysis. Whole blood cholinesterase was determined and found to be more than 95% inhibited in 2 samples taken from clinically affected cattle. Caudate nucleus cholinesterase was within the normal range. Two specimens of TMR pooled as one sample revealed 1430 μg/g (ppm) of fonofos, identified by gas chromatography and subsequently confirmed by mass spectroscopy. In addition, rumen contents from one of the dead cattle contained 56 ppm fonofos as determined by gas chromatography. Fonofos is O-ethyl S-phenyl ethylphosphonodithioate formerly registered in the United States as a granular corn rootworm insecticide. Presently it is not marketed for that application. Laboratory results, correlated with the clinical history of acute ataxia, trembling, drooling, dyspnea and diarrhea were compatible with and supported a diagnosis of organophosphate poisoning from fonofos. This product is extremely toxic to cattle (LD_{50}=1.3 mg/kg) and causes clinical effects typical of those described by the referring veterinarians.

There was no known usage or storage of fonofos at the feedlot site, and the cattle producer indicated fonofos had not been used on the farm within the last 5-7 years. In an attempt to show the origin of the contaminated TMR, initial sequential TMR samples from the affected pen were analyzed for fonofos. A residual sample from the mixing truck contained 1120 ppm fonofos. Eight sequential bunk samples ranged from 8 ppm to 26 ppm fonofos. Individual feed ingredients from which the TMR was mixed were not found to contain fonofos.

Sixty days subsequent to the poisoning episode, samples of blood, liver, kidney and muscle were requested by regulatory authorities for preliminary analysis to clear the remaining animals for slaughter. Five blood samples had normal cholinesterase activity. Two samples each of liver and muscle were analyzed for fonofos by gas-liquid chromatography. None contained detectable residues of fonofos, with a detection limit of 0.1 ppm. These results are consistent with other reports for fonofos toxicosis where a period of 30 days or more was associated with no detectable residues of the pesticide.

For comparison to the acutely poisoned animals, liver and heart muscle from the original affected cattle contained 1.7 ppm (liver) and 0.2 ppm (heart) fonofos. Skeletal muscle was not submitted from the acutely affected animals. **Low levels of fonofos found during the acute episode were cleared to below detection limits for the cattle tissues. Diagnostic laboratory assays supported both the acute diagnosis and the clearance of surviving animals to move to slaughter.**

Desorption electrospray ionization (DESI) for determination of terbufos in stomach contents

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A 1 ½ year old neutered male Pit Bull dog was presented for examination. It was salivating excessively, had muscle fasciculations and pinpoint pupils (miosis). Despite treatment which included atropine therapy, the dog died the morning following presentation. Two other dogs on the farm had been found dead the previous day. One of the dogs was submitted for necropsy to the Animal Disease Diagnostic Laboratory, Purdue University. No gross or microscopic lesions were noted. The brain cholinesterase activity was decreased to 26% of normal. The organophosphate insecticide, terbufos (mass/charge 289), was detected in the stomach contents by GC/MS. In addition, a small subsample of the stomach contents was applied to filter paper and directly analyzed via desorption electrospray ionization mass spectrometry (DESI-MS), using methanol:water (1:1) as the spray solvent. DESI mass spectra showed the presence of terbufos, as well as sodium and potassium adducts of terbufos due to the complex chemical matrix provided by the stomach contents.

For DESI-MS, ionization takes place in the ambient environment, which could be at the laboratory, a hospital, or in the field. The surface being tested is analyzed as it is received, often with no sample treatment (e.g., solvent extraction) which is commonly needed for most confirmatory analytical techniques. A solvent is electrosprayed at an angle onto the surface from which neutral molecules are released as secondary ions are transferred through air to the vacuum interface of the mass spectrometer. DESI-MS can work with a spray of water alone, but generally an organic solution is employed, such as aqueous methanol in this case. The lack of need for sample preparation and ability of direct ionization makes DESI-MS a rapid and sensitive method for chemical identification. For these studies, a commercially-available DESI source (Prosolia, Inc., West Lafayette) was used with a ThermoFinnigan LTQ mass spectrometer.

In this case, terbufos was detected in the stomach contents of a dog that was part of a group that had clinical signs and reduced brain cholinesterase activity consistent with organophosphate insecticide toxicosis. **Unique to this case was the direct detection of terbufos in the stomach contents through use of desorption electrospray ionization (DESI), which produced the protonated molecule at mass/charge 289. In the future, use of DESI will enable the rapid detection of toxicants in stomach contents and other diagnostic samples under ambient conditions.**

Comparative toxicity of tumbleweed shield lichen (*Xanthoparmelia chlorochroa*) collected from various locations throughout Wyoming

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The vagrant, foliose lichen *Xanthoparmelia chlorochroa* has been identified as the putative etiology in the death of an estimated 400-500 elk (*Cervus elaphus*) on the Red Rim-Daley Wildlife Habitat Management Area (RRWHMA) in Wyoming during the winter of 2004. This particular species of lichen was first reported to affect Wyoming cattle and sheep in the 1930s. There is a paucity of information regarding the toxicity of this lichen, and no other cases have been reported until the elk mortality in 2004. The roughly 70 years between reported *X. chlorochroa* poisonings may indicate that the lichen is only toxic during certain climatic/environmental conditions. The objective of this feeding study was to determine if *X. chlorochroa* collected from various locations throughout Wyoming and/or seasons would induce the specific syndrome observed in Red Rim-Daley poisoned elk.

Four treatment groups consisting of 3 adult Rambouillet ewes were fed lichen collected from 1) RRWHMA in March 2004, 2) RRWHMA in October 2006, 3) Bureau of Land Management McCulloch Peaks (MP) grazing area in February 2006, and 4) Monolith Ranch Hunter Management Area (MHMA) in May 2006. During the 2-week acclimation period, sheep were offered enough ground hay to fulfill a 2% basal body weight diet requirement. The first lichen ration consisted of 10% lichen mixed with ground hay. Each day the amount of lichen in the diet was increased by 10%. Once the diet reached 100% lichen, ewes remained on lichen until it was no longer available or they became moribund.

Ewes were observed multiple times each day for clinical signs. Serum chemistries and complete blood counts were performed on all blood samples collected. Using a metabolism crate, urine was collected from each ewe at least once while on the 100% lichen diet. A complete post-mortem examination was performed on all ewes immediately following euthanasia. Ewes were examined for congruence of signs, clinical pathology and post mortem lesions among lichen groups and with the Red Rim-Daley elk.

Clinical signs varied considerably among lichen groups. The first and most severely affected ewes were in the RRWHMA 2004 group. Clinical signs consisting of red urine and progressive muscular weakness began after the third day on 100% lichen. Red urine was also observed in the 3 remaining groups after the third day on 100% lichen. However, muscular weakness was not observed until after they had been on the 100% lichen diet for several more days. Ewes from both the MP 2006 and MHMA 2006 groups displayed incoordination and muscle weakness. Ewes in the RRWHMA 2005 group did not display the dramatic muscular weakness observed in the other groups, and red urine was the primary clinical sign. **All ewes exhibited a transient spike in serum creatine kinase activity as well as mild appendicular skeletal myopathy.**

The objective of this feeding study was to determine if *X. chlorochroa* varied in toxicity by location and/or season. The RRWHMA 2004 group was the most severely affected out of all the lichen groups, and best represented what was observed in the RRWHMA poisoned elk. All the lichen groups displayed various locomotor clinical signs, but the severity of the clinical signs differed among each lichen group. The apparent variation in toxicity may be due to fluctuating secondary metabolite concentrations, as concentrations of these compounds can vary in response to certain environmental conditions.

* Graduate student presentation

Development of a quail embryo model for the detection of botulinum toxin type A activity

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Clostridium botulinum is a ubiquitous microorganism that under certain anaerobic conditions can produce botulinum toxins. Due to concerns in regards to both food-borne illness and the potential use of botulinum toxin as a biological weapon, the capability to assess the amount of toxin in a food or environmental sample efficiently is critical. Currently the mouse inoculation assay is the method used for assessment of botulinum toxin activity. However, the mouse model requires 36 h for completion of the assay. In addition, there are safety concerns for handling needles and moving animals with samples containing undetermined amounts of toxin. Mouse colonies are also expensive to maintain. The objective of the study was to develop and evaluate a new screening assay for determination of biologically active botulinum toxin utilizing incubating quail embryos (*Coturnix coturnix japonica,*).

Fertile quail eggs were incubated for 15 days under standard conditions and embryo viability was assessed by both light candling and with motion detection (digital egg monitor). Eggs with viable embryos were marked at the margin of the air cell closest to the blunt end of the egg, and a 2 mm hole was ground through the eggshell. The embryos were then given injections into the neck/shoulder area with varying concentrations (1 to 250 ng / 0.05 mL) of botulinum toxin type A. Following injection, eggs were returned to the incubator and embryo viability was reassessed at 1, 2, and 3 days post-injection. At 1 day post-injection, embryos injected at 20 ng or higher with type A toxin had more than 75% of the embryos determined to be not viable. The 5 and 10 ng injection doses had 53% and 50%, respectively, of the embryos remaining viable at 1 day post-injection. After 3 days post-injection, both the 5 and 10 ng injection dosages had less than 50% viability, the 1 ng injection dose had 73% viable embryos, while the control group (0 ng of toxin) retained a high viability at 93%.

Premixing of the toxin for 1 h with serotype A specific antibody (in excess) demonstrated that the depression in the ability of the quail embryos to pip and hatch was indeed attributable to biologically active type A toxin. Embryos given injections of toxin from 1 to 50 ng that had been preincubated with serotype A specific antibody maintained a viability of greater than 80% after 3 days post-injection compared to less than 14% viability for those embryos injected at the same dosages without preincubation of the toxin with type A antitoxin antibody.

These experiments have demonstrated that the Japanese quail embryo at 15 days of incubation is an effective vertebrate animal model to detect the activity of botulinum type A toxin. Based on these data, the LD50 for quail embryos after 1 day is approximately 1 ug of toxin per kg of body weight and after 3 days is approximately 500 ng of toxin per kg of body weight. Utilization of a quail embryo may be a beneficial model for the analysis of botulinum toxin activity, while decreasing assay assessment time, and enhancing personnel safety.

Virology Scientific Session

Sunday, October 21, 2007

Ponderosa A

Moderator: Tim Baszler, Kyoung-Jin Yoon

***** Graduate student presentation

Genetic variation of canine papillomaviruses in domestic dogs and African wild dogs

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Human papillomaviruses are very diverse, with more than 100 different isolates and clones. Until recently, only one canine papillomavirus (*Canine oral papillomavirus*, COPV), had been characterized, although canine warts were recognized over 100 years ago. Two new canine papillomaviruses have recently been reported, *Canis familiaris papillomavirus type 2* (CfPV-2) (1) and CPV3 (2). Other novel canine papillomaviruses have been described in a variety of canine cancers (3).

In this study, 3 African wild dogs (*Lycaon pictus*) and 4 domestic dogs of various breeds that had been identified with papilloma-like lesions were chosen for further characterization and confirmation of the presence of papillomavirus. In the African wild dogs, lesions were noted in the gingival, cervical and cranial thoracic skin, behind the elbows, and medial thigh. Each dog had multiple lesions. Most of the lesions were located in the cranial thoracic area. Interestingly, this is the same area that incurs the most damage during intraspecific aggression and may serve as a mode of direct transmission. Initial sequence analysis of the L1, E1 and E7 regions of the papillomavirus in the African wild dogs revealed that although the animals are housed together, they do not appear to be affected by the same viral strain. Sequence analysis also suggests the presence of a novel canine papillomavirus, with the E1 region of one African wild dog having no GenBank match using nucleotide sequence but a 70% amino acid match to CPV3. The 4 domestic dogs have also been shown to be PCR positive for canine papillomaviruses. Interestingly, the canine papillomavirus sequence from a Chihuahua demonstrated the presence of an L1 nucleotide sequence suggested to be associated with canine pigmented epidermal nevus (PEN), which has been described only in Pugs.

Further sequence analysis will add to the identification and molecular characterization of canine papillomavirus. **This study is the first report of canine papillomavirus in African wild dogs and demonstrates that the genetic diversity of canine papillomavirus has been largely underestimated.**

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Canine parvovirus genotypes (CPV-2b and CPV-2c) circulating in the USA, 2006-2007

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Canine parvovirus 2 (CPV-2) is the most common cause of viral diarrhea in young puppies up to 6 months of age and is associated with high morbidity and mortality. Parvoviruses are single-stranded DNA viruses that cause segmental enteritis associated with crypt dilation, necrosis and villus atrophy. There is anecdotal evidence of the increase in CPV-2 infection in the South-Central USA in the last few years.

In the last year, we have examined many canine enteritis cases for CPV-2 infection. These cases were submitted from 9 different states in the USA. We received a variety of samples (feces, intestinal loops, tongue) for CPV examination. Parvoviral infection was confirmed by fluorescent antibody testing of fresh tissues and by immunoperoxidase testing of fixed tissues. For genotyping CPV-2 isolates, DNA was extracted from fecal samples or tissue samples, subjected to PCR amplification of a portion of the VP 2 gene DNA, subjected to BLAST analysis, and the genotype was determined by using Clustal W alignment. The CPV-2 positive samples were inoculated in Crandell feline kidney cell line and the amount of the CPV was quantified by a hemagglutination test with swine erythrocytes (0.8% suspension in buffer, pH 7.2).

Based on sequence analysis performed on clinical specimens, 14 CPV-2c, 13 CPV-2b and only 1 CPV-2 were detected. We were able to isolate about 40% of the CPV-2 samples by virus isolation in Crandell feline kidney cell line, and virus recovery was affected by sample quality and not the genotype of the CPV-2 isolate.

To our knowledge, this is the first report of detection of CPV-2c in American canine populations. However, CPV-2c has been reported in other countries (Italy, Spain and Vietnam) in dogs. **Based on this study, CPV-2b and CPV-2c are co-circulating in several states in the USA.** Many of these cases from this study had a history of CPV-2 vaccination. The factors that affect the efficacy of the current vaccines are not understood. The role of emerging novel CPV genotypes (CPV-2c) in viral enteritis in the American canine population needs further study.

The emergence of Canine parvovirus 2c in the United States

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Canine parvovirus 2 (CPV-2) emerged around 1978 as a major pathogen of dogs worldwide. In the mid-1980s, the original CPV-2 evolved and was completely replaced by two variants, CPV-2a and CPV-2b, which continue to co-circulate in various proportions among dog populations worldwide. In 2000, a new variant of CPV (named CPV-2c) was detected in Italy and now co-circulates with types 2a and 2b in that country. The CPV-2c has also been reported from single outbreaks in Vietnam and Spain.

Merial Inc. obtained 33 fecal samples from vaccinated (31 samples) or unvaccinated (2 samples) dogs to support epidemiological surveying. All samples were tested for CPV DNA using real-time PCR. Conventional PCR, MGB PCR, and DNA sequencing were performed on the 27 samples that were positive for CPV. The PCR products of the CPV-positive samples in this study were sequenced using the VP2 region spanning nucleotides 4002 – 4585 of the CPV genome and compared to GenBank sequences of CPV-2, CPV-2a, CPV-2b and CPV-2c. Based on the occurrence of the codon GAA at position 4062-4064 (encoding for glutamine at residue 426), **7 of the 27 positives were determined to be CPV-2c from 5 states: Arizona, California, Georgia, Oklahoma, and Texas.** The dogs were of seven different breeds, ranged in age from 3-8 months, and had all received 2-3 commercially available CPV vaccines prior to onset of clinical signs, which included nausea, vomiting, and diarrhea. Four of the 7 CPV-2c isolates differed from European CPV-2c isolates by exhibiting 2 further mutations in the target region: G→A at nucleotide 4076 that involves the third base of codon 430 and thus does not cause a change in residue; A→G at nucleotide 4104 that affects the first nucleotide of codon 440, changing it from ACA to GCA, thus altering amino acid residue 440 from threonine to alanine (Thr→Ala). Of the remaining CPV-positive samples (20), 19 were determined to be CPV-2b and 1 was determined to be CPV-2a. Among the 19 CPV-2b isolates, 17 originated from clinically sick dogs of various breeds, ranging in age from 2-14 months, which had received 2-4 doses of various commercially available CPV vaccines, whereas 2 originated from unvaccinated dogs. Of all 27 CPV-positive samples detected in this study, 16 were determined by MGB-PCR analysis to be field strains rather than vaccine strains. The other 11 strains were not differentiated for vaccine or field origin. However, since all of these 11 strains were either CPV-2b or 2c, they could not be of vaccine origin since vaccines containing these sub-types are not currently used in the United States.

The findings from this study indicate that CPV-2c is probably widely distributed in the United States, since the 7 isolates originated from geographically widely dispersed locations, with no evidence of prior contact between any of the dogs. The finding of mutations in 4 of the 7 American isolates that do not occur in European CPV-2c isolates may mean that the 2 groups of CPV-2c isolates evolved independently. However, antigenic analysis and further sequence comparison involving more of the genome of both groups of isolates are required before a definitive statement can be made on their evolution.

* Graduate student presentation

Exotic bluetongue viruses identified from ruminants in the southeastern USA from 1999-2006

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World-wide, 24 serotypes of *Bluetongue virus* (BTV) have been identified, and five (BTV-2, BTV-10, BTV-11, BTV-13, BTV-17) are considered endemic in the United States. Isolation and identification of BTV isolates is routinely performed at the National Veterinary Services Laboratories (NVSL), Ames, IA. From 1999 to 2005, several isolates of BTV originating from Florida sheep, cattle or deer could not be identified at the NVSL as one of the U.S. BTV serotypes. Virus neutralization tests conducted on the isolates using type-specific reagents to BTV serotypes that had been identified in the Caribbean and Central American regions were inconclusive.

For BTV, the serotype identification is conferred by the major outer capsid protein, VP2. Until recently, genetic sequences for the VP2 region of all 24 serotypes of BTV were not available. Using newly published sequences of all 24 VP2 genes, PCR primers were developed for the exotic BTV VP2 regions. Subsequent PCR testing and sequencing of the PCR products were performed with the previously untypable isolates. The archived Florida isolates, as well as recent isolates from Florida and Mississippi, were successfully identified. Several of the isolates were also submitted to the Institute for Animal Health, Pirbright, United Kingdom for identification and/or confirmation of the NVSL results.

BTV serotypes previously believed to be exotic to the United States that have been identified are:

BTV-3: Highlands County FL, 1999 (sheep); Martin Country FL, 2001 (deer); Alachua County FL, 2002 (deer); Okeechobee County FL, 2002 (cattle); Manatee County FL, 2003 (cattle); Yalobusha County MS, 2006 (deer).
BTV-5: Manatee County FL, 2003 (cattle).
BTV-6: Okeechobee County FL, 2006 (cattle).
BTV-14: Marion County FL, 2003 (sheep)
BTV-19: Manatee County FL, 2003 (cattle)
BTV-22: Okeechobee County FL, 2002 (cattle); Marion County FL, 2005 (sheep)

Culicoides insignis, the common vector of BTV in Caribbean and Central American regions and extreme southeastern United States, has traditionally been restricted to those areas. *Culicoides sonorensis* is considered the BTV vector for midwestern, western and other southern regions of North America. The limited range of *C. insignis* in the United States may account for the initial isolation of these exotic BTV serotypes only from southeastern animals, however, it is not known if the range of *C. insignis* is expanding as a result of global warming. Additionally, the potential for infection of *C. sonorensis* with any of these viruses is not known.

Influence of PrP genotype 96SS on susceptibility to chronic wasting disease (CWD) and survival of CWD-positive white-tailed deer in Wyoming

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Chronic wasting disease (CWD) is a transmissible spongiform encephalopathy of mule deer (*Odocoileus hemionus*), white-tailed deer (*Odocoileus virginianus*; WTD), Rocky Mountain elk (*Cervus elaphus nelsoni*), and moose (*Alces alces*). Evidence suggests that polymorphisms of the host prion (PrP^c) gene can influence the susceptibility of individual WTD to acquiring CWD and can affect the clinical course of CWD in affected deer. Serine (S) has been found to substitute for glycine (G) at codon 96 in the WTD PrP^c gene. This may have relevance to free-ranging populations as there were significantly fewer hunter-harvested WTD in Wisconsin that were homozygous or heterozygous for 96S in the CWD-positive population compared to the CWD-negative population. Despite the reduced susceptibility to CWD, the 96S allele did not prevent CWD infection, but appeared to slow the progression of disease. The slower progression of disease in WTD with the 96S allele may have significant influence on survivorship of deer that are infected with CWD and theoretically could prolong the time infected deer are shedding the putative prion agent into the environment.

One of the specific objectives of this study is to monitor CWD-positive and CWD-negative WTD throughout their lifespan via radio-telemetry to determine differences in survival rates and the influence of the 96S allele on length of time from testing positive for CWD until death. Deer were captured as fawns, tested for CWD by tonsil biopsy and immunohistochemistry, blood was collected for genetic analysis, deer were marked with ear tag radio-transmitters, and recaptured on a yearly basis to re-test for CWD and replace radio-transmitters with global positioning system collars.

In 5 years, we have captured, marked and CWD tested 154 WTD. The overall CWD prevalence is 27% (37/136). Based on Kaplan-Meier annual survival analysis, survival rates are lowest for adult CWD-positive deer (0.29; female = 0.22; male = 0.40). **Annual survival rates are significantly lower for adult CWD-positive deer than CWD-negative deer ($X^2_1 = 8.85$, $p = 0.00293$).**

We used a Chi-squared test to determine if the genotype at codon 96 had a significant influence on the probability of WTD being CWD-positive based on the overall CWD prevalence of 0.28 within the genotyped population. **Our results indicate that there is no influence of codon 96 on the probability of CWD infection ($X^2_2 = 2.38$, $p = 0.305$)**. **The average length of time known alive with CWD (number of months from testing positive for CWD until death) is longer for 96SS WTD ($\overline{x} = 23.3$, $n = 3$, range = 12-29) than for 96GG/GS WTD ($\overline{x} = 7.5$, $n = 21$, range = 1-19).** The WTD from the 96GG/GS sub-population that lived the longest (19 months) lived 4 months less than the average time lived by 96SS WTD. The 96SS WTD that lived the fewest months with CWD (12) was hit by a vehicle and still in excellent physical condition, indicating that he was in the pre-clinical stages of CWD. The 2 remaining 96SS WTD have lived for 29 months with CWD and were still alive at time of submission.

The protracted lifespan of 96SS CWD-positive WTD could mitigate the population effects of CWD caused by the significantly lower annual survival rates of CWD-positive WTD. Further, CWD-positive WTD that live longer are more likely to reproduce or reproduce in more reproductive seasons, which may lessen the population level effect of CWD and contribute to higher 96SS prevalence, especially in high CWD prevalence areas. We encourage state wildlife agencies and diagnostic laboratories to analyze PrP^c genotypes of deer submitted for CWD testing to build a genetic database of free-ranging deer populations to determine the selective pressure of CWD on 96SS and ultimately the influence of 96SS on mitigating the impact of CWD.

* Graduate student presentation

Vesicular disease of unknown etiology in swine

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A shipment of 185 6- to 8-month-old barrows and gilts from 6 farms in Manitoba, Canada arrived for slaughter at a processing plant in Austin, MN during June 2007. Upon ante-mortem examination, 80% of the pigs showed lameness, 25-50% had vesicular lesions on coronary bands, and 1 pig had ruptured vesicles on the snout. Because the clinical signs were highly suggestive of vesicular diseases including foot-and-mouth disease (FMD), swine vesicular disease (SVD), vesicular stomatitis (VS), and vesicular exanthema of swine (VES), a foreign animal disease investigation was initiated by the Veterinary Services office in MN. Epithelial, blood and serum samples were collected and sent to the Foreign Animal Disease Diagnostic Laboratory (FADDL) on Plum Island for analysis. Vesicular diseases including FMD, SVD, VS, and VES were presumptively ruled out within 6 h of sample arrival on the basis of serology, real time RT-PCR, and antigen ELISA. Lymph nodes (LNs) and tonsils from the affected pigs were submitted the following day to the National Veterinary Services Laboratories (NVSL) in Ames and Plum Island (FADDL) for additional analysis.

Animals were confirmed negative for FMD, VS, SVD and VES viruses upon completion of virus isolation. However, **multiple agents were isolated from the epithelium and LNs from the majority of affected animals.** The agents identified by electron microcopy and confirmed by PCR were ***Porcine parvovirus*****, *Porcine circovirus 2*****, and *Porcine teschovirus 1*** (excluding Teschen disease virus). In addition, the ***Seneca valley virus*** (SVV), a *Picornavirus*, recently shown to be related to the *Cardiovirus* genus, was isolated from the tonsils, epithelium and LNs of the affected animals. RT-PCR and sequence analysis of the VP1 and 2A regions of the viral genome revealed a 95% nucleotide sequence identity with the GenBank sequence for SVV. It is important to note that viruses antigenically and/or genetically-related to *Porcine teschovirus 1* and SVV have periodically been isolated over the last 20 yr from swine in the USA. These animals have been presented with a variety of clinical signs, including vesicular-like lesions. However, inoculation of these viruses in conventional pigs did not produce infection or disease (unpublished data).

Much remains unknown concerning the role of these multiple isolates in causing these vesicular lesions at such a high morbidity rate. In order to demonstrate the significance of these agents, an animal inoculation study will be designed to fulfill Koch's postulates. These experiments will be performed in germ-free pigs. **An attempt will be made to reproduce disease and re-isolate these agents from the lesions to demonstrate that they are indeed the underlying cause of this clinical manifestation.**

Porcine teschovirus and Pseudorabies virus infections in a Wisconsin swine herd

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Serum samples collected from a Wisconsin swine herd testing positive for Pseudorabies virus (PRV) antibodies were forwarded to the National Veterinary Services Laboratories (NVSL) for confirmation testing. The samples tested positive for PRV antibody by the latex agglutination test, gB ELISA, and gI ELISA. No clinical signs typical of PRV were noted in the adults, but respiratory and neurological signs were noted in pigs approximately 2 weeks of age. The herd was depopulated, and adult heads, fetuses, tissues from young pigs, and blood samples were submitted to the NVSL for diagnostic testing.

Tonsils and trigeminal ganglia were harvested from the heads of the adult animals and analyzed by a nested polymerase chain reaction (PCR) for TK and gI nucleic acid. Brain was taken for histologic evaluation. The remaining tissues were analyzed for the presence of virus using PK15 and fetal porcine kidney cell cultures for virus isolation. Serum samples were tested by the latex agglutination test, gB ELISA, and gI ELISA.

Sera from some of the adults and young pigs were positive for PRV antibodies on all 3 serologic tests. PRV was not identified by virus isolation or by PCR in any of the tissues tested. Virus was isolated from lung and brain of younger pigs and neutralized with antisera to *Porcine teschovirus 1* or *11* (PTV-1/PTV-11) (formerly *Porcine enterovirus 1*). PCR typing of the isolates showed CPE group 1 and PTV-1/PTV-11 positive amplicons. The PTV-1/PTV-11 amplicons from the PCR conducted on neurologic tissue isolates were sequenced and found to be PTV-11 genotype. Mild perivascular lymphocytic encephalitis suggestive of a viral infection was observed in brain tissue from adult pigs. **Use of multiple diagnostic techniques resulted in the detection of both PRV and PTV exposure in this herd.**

Isolation of reassortant H2N3 avian/swine influenza virus from pigs in the United States

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In April 2006 and September 2006, outbreaks of respiratory disease occurred in growing pigs housed in 2 separate multi-site commercial swine farms, Farm A and Farm B. The swine farms were located 4 miles apart and did not share pigs, feed, personnel, or transportation sources. At both farms, pigs had gross lesions of pneumonia at necropsy. The attending veterinarians submitted formalin-fixed and unfixed sections of lung tissue with coolant to the Minnesota Veterinary Diagnostic Laboratory (MVDL).

At the MVDL, the formalin-fixed tissue was routinely processed for histopathology, and the unfixed tissues for bacteriology, molecular diagnostics (RT-PCR and PCR tests), and virus isolation. Pig lungs from both farms had lesions of influenza virus and bacterial co-infection characterized by subacute bronchopneumonia and interstitial pneumonia with metaplastic and necrotizing bronchiolitis. Swine influenza virus was detected in the lungs by RT-PCR and virus isolation. The swine influenza viruses were untypable by hemagglutination inhibition serotyping tests with reference antisera against A/Sw/IA/1973 H1N1, A/Sw/NC/2001 H1N1, and A/Sw/TX/1998 H3N2. Multiplex subtyping RT-PCR tests for swine H1N1, H3N2, and H1N2 were negative. Hemagglutinin (HA) gene sequencing attempts with H1 and H3 specific primer sets were also negative. The viruses, A/Swine/FarmA/22454/2006 and A/Swine/FarmB/49644/2006, were forwarded to the NADC for sequencing and to St. Jude for serotyping. **Through full genomic sequencing of the eight RNA segments of each virus, the viruses were subtyped as H2N3 influenza viruses with HA and neuraminidase (NA) genes of avian origin.** Serotyping with ferret anti-duck H2N3 antisera was positive for both viruses.

This is the first description of avian/swine H2N3 influenza virus isolated from pigs in the United States. The introduction of the virus into the swine farms is thought to be through the use of surface (pond) water used in the swine barns. The ponds used as water supplies for both Farms A and B are frequented by migrating waterfowl in the spring and fall of each year. The H2N3 viruses continue to circulate in both Farms A and B as indicated by positive (>1:40) H2N3 antibody titers found in sera collected from replacement breeding gilts and weaned pigs 6 months or more after onset of disease. The impact of this virus on swine health and disease control strategies will be monitored through expanded swine influenza virus surveillance and characterization efforts.

Characterization of the novel H2N3 influenza virus subtype isolated from US pigs

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Pigs have been suggested to be the mixing vessel for avian and human influenza viruses because the porcine trachea contains binding receptors with preferences for human and avian influenza viruses. In the pig, genetic reassortment to create novel influenza subtypes by mixing avian, human and swine influenza viruses is possible.

Here, we report the characterization of two novel reassortant H2N3 viruses isolated from pigs with respiratory disease. Molecular and phylogenetic analyses of these viruses revealed that the HA segment is similar to an avian influenza virus (AIV) H2N3 isolated from mallards and the NA sequence is similar to an AIV H4N3 isolated from blue-winged teal. The HA protein revealed a Q226L mutation when compared with the putative parental avian HA protein, indicating a preferential binding to SAα2,6Gal, the mammalian influenza receptor. All internal genes except PA were similar to influenza virus gene segments found in contemporary triple reassortant (human, swine, avian) SIVs in the United States. The PA segment has high homology to the PA of an AIV H6N5 isolated from mallards.

Experimental infection of swine showed that the H2N3 virus is virulent for pigs, replicating in the lung and causing macroscopic and microscopic lung lesions. All experimentally inoculated pigs seroconverted to H2N3 and seroconversion was demonstrated in sentinel contact pigs as well. In addition, the H2N3 virus infected mice, inducing lung lesions, clinical disease and death. The H2N3 virus also infected ferrets and transmitted efficiently to contact sentinel ferrets. **These data indicate that the novel reassortant H2N3 subtype virus has the ability to infect various mammalian hosts.**

Validation of a new avian influenza virus antibody blocking ELISA using avian serum samples from different countries around the world

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The IDEXX-FlockChek* MultiS-Screen AI Ab Test Kit is a new blocking enzyme-linked immunosorbent assay (ELISA) for the detection of antibodies to avian influenza virus (AIV). The test is based on nucleoprotein (NP) antigen coated on the solid phase that binds avian serum antibodies against AIV. Well characterized serum samples from ostriches, ducks, quails, chickens and turkeys were obtained from commercial operations in several countries, including Brazil, Mexico, China, United States, and Canada. Samples were evaluated at the Research and Development facilities of IDEXX Laboratories.

The overall specificity of the ELISA was 99.7%. For chickens, specificity was 99.8% in a population of 2,431 serum samples from birds of different ages that were highly vaccinated against several poultry infectious agents. Specificity was 98.6%in ducks, and 100%for turkeys, ostriches and quails.

The overall sensitivity of the ELISA was 97.5% based on analysis of more than 750 positive avian serum samples that were exposed to different avian influenza (AI) subtypes.

Overall, these results show a very good level of correlation between the IDEXX-FlockChek* MultiS-Screen AI Ab Test Kit and AGID (kappa of 0.96) or homologous HI results (kappa of 0.73) for detection of antibodies to AI.

Comparison of the pathogenicity of different H5N1 HPAI viruses in chickens and ducks

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Contrary to what is observed in chickens where infection with highly pathogenic avian influenza (HPAI) viruses produce fatal disease, the Asian H5N1 HPAI viruses have changed from producing mild respiratory infections in ducks to some strains causing systemic disease and death. In order to further understand the difference in pathogenicity observed between chickens and ducks in their response to infection with H5N1 HPAI viruses, we studied the clinical disease, gross and microscopic lesions, the tissue distribution of viral antigen, and the cytokine profile in 2-week-old white Pekin ducks and White Leghorn chickens inoculated intranasally with 4 different strains of Asian origin H5N1 HPAI viruses: A/Ck/HK/220/97, A/Egret/HK/757.2/02, A/Ck/Indonesia/7/03 and A/duck/Vietnam /203/05.

Chickens inoculated with any of the 4 viruses were severely depressed the day after inoculation and died with a mean death time (MDT) between 1.6 and 2 days post inoculation (dpi). None of the ducks inoculated with A/Ck/HK/220/97 died, contrary to all ducks inoculated with A/Egret/HK/757.2/02 or A/duck/Vietnam /203/05 which died, with MDTs of 5.5 and 3.9 dpi respectively. Six of 10 ducks inoculated with A/Ck/Indonesia/7/03 died. Sick ducks were depressed and displayed neurological signs.

Microscopically, lesions and presence of viral antigen in tissues was similar in all the infected chickens and the ducks infected with A/Egret/HK/757.2/02, A/Ck/Indonesia/7/03 and A/duck/Vietnam/203/05, the severity of the lesions correlating with viral replication in tissues. Lesions in the lung were more severe in chickens, and virus replication was observed in vascular endothelial cells, which was not observed in the ducks. These differences may explain in part the differences in pathogenicity observed between the chickens and the ducks when inoculated with the same viruses.

No difference in body temperature was found between control chickens and chickens inoculated with any of the 4 viruses. Conversely, an increase in body temperature was observed in the ducks infected with A/Egret/HK/757.2/02, A/Ck/Indonesia/7/03 and A/duck/Vietnam /203/05, and this increase was proportional to virulence of the viruses. Innate responses differed also between chickens and ducks. In general, cytokine expression in chickens was suppressed following infection when compared to controls. **Understanding the mechanisms for cytokine induction and suppression following HPAIV infection will provide insights into the pathogenicity of AIV in different avian species.**

White spot syndrome virus responsible for significant mortalities in Louisiana crayfish

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A Louisiana crawfish operation experienced significant mortalities in the spring of 2007 in 4 production ponds stocked with *Procambarus clarkii* (red swamp crayfish) and *Procambarus zonangulus* (Southern white river crayfish). High mortality, lethargy, and predominant loss of large crayfish were the initial findings. The affected ponds were stocked with red swamp crayfish from a 40-acre pond located in a neighboring parish. Stock pond crayfish originated from wild stocks collected from the Atchaflaya Basin floodway or from privately owned crayfish ponds. The stock pond had been experiencing decreased production that was attributed to overstocking by the owner, as there were no increases in daily mortalities observed in the stock pond.

Crayfish were collected from the index pond for a diagnostic work-up. Gross examination revealed fouling of the gills and soft cuticles. Histologic examination revealed intranuclear viral inclusions in the epithelium of the cuticle, gastrointestinal tract (sans midgut), sperm duct, seminiferous tubules, and antennal gland. Inclusions were also present in the connective tissue, gills, hematopoietic tissue, glial cells, heart, and muscle. Rod shaped enveloped virions approximately 320-350 nm long and 80-100 nm wide were observed in the hindgut and gill epithelium by scanning electron microscopy. Based on these characteristics, *White spot syndrome virus* (WSSV) was suspected. DNA was extracted from pooled pleopod tissues and evaluated by PCR (OIE protocol) using the OIE prescribed nested set primers. PCR results were positive for the detection of WSSV. In addition, an in situ hybridization assay with a WSSV specific probe on histologic sections was positive. A shrimp bioassay was performed using 12 SPF *Litopenaeus vannamei*. Shrimp were injected intramuscularly with a purified crayfish gill homogenate. All 12 shrimp were moribund or dead within 48 h. No evidence of disease occurred in the control shrimp. Histologic findings for the test shrimp were consistent for WSSV infection and confirmed by in situ hybridization and PCR.

In an effort to confirm the WSSV diagnosis, a second set of samples were collected from the index pond, the stock pond and 2 private ponds that were also experiencing increased mortalities. Pleopods from 5 crayfish were pooled and gills from the same 5 crayfish were pooled. Each pool was evaluated independently by PCR for the presence of WSSV DNA. PCR amplicons were sequenced for final confirmation of WSSV. Surveillance efforts have been initiated to determine how far the virus has spread in both the farmed and wild stocks of crayfish. WSSV has been detected in wild *P. clarkii* in the Basin, but prevalence of the virus in the basin wild stocks hasn't been determined.

WSSV is thought to infect all decapod crustaceans. WSSV is a World Organization for Animal Health (OIE) notifiable pathogen. The use of SPF *L. vannamei* broodstock and the practice of strict biosecurity measures have limited disease introduction in US shrimp mariculture. Currently the U.S is considered free of WSSV disease in farmed shrimp populations, although rare outbreaks have occurred. Spread of the virus has been controlled through early diagnosis and depopulation, decontamination, and fallowing of ponds. **The occurrence of clinical disease and the detection of WSSV in multiple farm populations and wild crayfish stocks in Louisiana is a significant finding, as the current husbandry practices of crayfish farming and the ecology of the crayfish present major challenges to disease biosecurity.**

A pandemic strain of calicivirus threatens rabbit industries in the Americas

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Rabbit hemorrhagic disease (RHD) is a severe acute viral disease specifically affecting the European rabbit *Oryctolagus cuniculus*. As the European rabbit is the predominant species of domestic rabbit throughout the world, RHD contributes to significant losses to rabbit farming industries and endangers wild populations of rabbits in Europe as well as predatory animals that depend upon rabbits as a food source. *Rabbit hemorrhagic disease virus* (RHDV), the etiologic agent, is a genus *Lagovirus* in the family *Caliciviridae*.

Typically, RHD is seen as an outbreak of sudden death in 70% to 95% of infected animals. There have been 4 separate incursions of RHDV in the USA, the most recent of which occurred in the Indiana in June of 2005. Animal inoculation studies confirmed the pathogenicity of the Indiana 2005 isolate, which caused acute death and pathological changes characterized by acute diffuse severe hepatic necrosis and pulmonary hemorrhages. Complete viral genome sequences of all USA outbreak isolates were determined, and comparative genomics revealed that each outbreak was the result of a separate introduction of virus rather than from a single virus lineage.

All of the USA isolates clustered with RHDV genomes from China, and phylogenetic analysis of the major capsid protein (VP60) revealed that they were related to a pandemic antigenic variant strain known as RHDVa. Rapid spread of the RHDVa pandemic suggests a selective advantage for this new subtype. **Given its rapid spread, potential for further incursions, pathogenicity, and the possibility to broaden host range, RHDVa should be regarded as a threat to the Americas.**

Real-time PCR detection and differentiation of *Equid herpesvirus 1* with and without the neuropathogenic marker in California horses

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Enhanced surveillance efforts following the detection of *Equid herpesvirus 1* (EHV-1) carrying the neuropathogenic marker (NEHV-1) in 2 unrelated horses with neurologic disease in California during November and December of 2006 allowed for field evaluation of a newly introduced real-time PCR assay. The assay is based on differential hybridization of 2 specific oligoprobes identifying a single nucleotide change in the EHV-1 polymerase gene (1).

The assay was used in a 3-month surveillance effort that included 352 horses on 4 California racetracks. The assay was also used on a population of private horses (n = 136) submitted to the diagnostic laboratory for differential diagnosis or routine health monitoring during the same time period. In addition to 425 non-clinical horses, horses showing neurologic disease (n = 23) and febrile or respiratory disease (n = 40) were evaluated.

Among the non-clinical horses submitted from the racetrack, 6% were positive for EHV-1 from nasal swab specimens and 7% were EHV-1 positive from blood specimens. NEHV-1 was detected from 11% of nasal swabs and 5% of blood specimens in the same group of horses. Among the non-clinical private horse population, 3% were positive for EHV-1 from nasal swabs and none from blood, compared to 5% of non-clinical horses that were PCR positive for NEHV-1 from nasal swabs and 4% positive from blood specimens submitted. Seventeen percent of horses with febrile or respiratory disease tested positive for NEHV-1 from nasal swabs, whereas only 2% tested positive for EHV-1 from nasal swabs. Neither EHV-1 nor NEHV-1 was detected from the blood samples submitted from horses with a history of febrile or respiratory disease. Of the 23 horses with a history of neurologic disease, 3 (13%) tested positive for NEHV-1 from nasal swabs and 4 from blood (17%), whereas only 1 of the neurologic horses tested positive for EHV-1 from a nasal swab specimen and none from blood**.**

In the current evaluation, EHV-1with the neuropathogenic marker had approximately half the neurologic-disease association compared to the previously reported 5-fold increased risk. Additionally, the differential PCR assay was positive for both virus subtypes in a small subset of racetrack horses, with diagnosis of dual infection being critically dependent on the stringency established for rule-out of non-specific cross-reactivity between the 2 viruses in the assay system.

The ability to differentially detect EHV-1 and NEHV-1 shows promise for the further study of equine herpesvirus-1 prevalence, latency reactivation versus re-infection, and association of the 2 viral subtypes with clinical disease. Additional field studies are required to understand and document diagnostic application of this *Equid herpesvirus 1* differential assay.

Reference

1. Allen GP. Development of a real-time polymerase chain reaction assay for rapid diagnosis of neuropathogenic strains of equine herpesvirus-1. J Vet Diagn Invest. 2007;19:69-72.

Molecular diagnostic tools for early detection of arthropod-borne animal viruses

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Outbreaks of *West Nile virus* in the United States and *Rift Valley fever virus* (RVFV) in East Africa have highlighted the need for validated early detection tools for arthropod-borne viruses. This need is further supported by the ongoing *Bluetongue virus* (BTV) epidemic in Europe and by the recent detections of exotic BTV strains (BTV-1 and BTV-3) in the United States.

Bluetongue virus causes disease in sheep and cattle and has significant economic impact due to trade barriers. Although US strains of *Epizootic hemorrhagic disease virus* (EHDV) have not been experimentally proven to cause disease in cattle, there is serologic evidence of widespread infection in cattle. The Arthropod-Borne Animal Diseases Research Laboratory has been involved with the development of rapid nucleic acid detection tests for BTV and the related EHDV for several years. We have developed rapid real-time reverse transcriptase-polymerase chain reaction (qRT-PCR) tests that detect prototype strains of indigenous and exotic BTV and EHDV RNA. The EHDV qRT-PCR detected all 40 field strains available. In addition, the RVFV outbreak has prompted the evaluation of existing qRT-PCR assays on livestock samples. **The EHDV qRT-PCR was evaluated against 105 clinical samples, and detected 90% of all VI positive clinical samples, which was 12% better than our previously published EHDV PCR protocols.**

Bacteriology Scientific Session

Sunday, October 21, 2007

Ponderosa B

Moderator: Lorraine Hoffman

* Graduate student presentation

Mastitis pathogens from 3164 dairy goats and 591 dairy sheep, and farm characteristics from 1993-2004

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Intramammary pathogens of dairy sheep and dairy goats are similar to those of dairy cattle, but the most common isolates, their prevalence and mean somatic cell count (SCC) in bulk milk are not the same as in cattle. Adoption of mastitis control practices by small ruminant producers is not well documented.

Milk samples were aseptically collected for milk culture from 3164 dairy goats and 591 dairy sheep from 1993-2004 by personnel from the Quality Milk Production Services. The 36 goat herds and 6 sheep flocks were located in New York State. Mean number of lactating does was 88 (range 4 - 583, median 30.5). Mean number of milking ewes was 99 (range 52 -193, median 83.5). The most common goat breeds, when specified, were 1009 French Alpines in 7 herds and 242 Saanens in 13 herds. Mean number of dry does was 23, and of dry ewes was 258.

Five goat herds were visited because of SCC > 750,000/mL, the legal limit, with mean SCC of 1,260,000/mL in bulk milk. The remaining herds were visited because the owners wished to monitor milk quality and mastitis control practices. **Sheep flock mean SCC was 425,000/mL and "monitoring" goat herd mean SCC was 753,577/mL, indicating that even among goat farms not expressly concerned about SCC, the mean for goats was above the legal limit**. When all goat herds were included, mean bulk milk SCC was 835,258/mL.

Mean estimated milk production per 305 d was 1102 lb (500 kg) for sheep and 1856 lb (843 kg) for does. **Pathogens were isolated from milk of 64 ewes (10.8%) and 864 does (27.3%)**. No *S. agalactiae* or *Mycoplasma* spp. were isolated. The most common pathogens were coagulase-negative staphylococci (CNS), from 6.8% of ewes and 23.0% of does, non-agalactiae streptococci (*Strep* spp.), from 3.2% and 0.8%, and *S. aureus* from 1.2% and 1.5%, respectively. Among the 5 goat herds visited because of illegal SCC, 36.1% of does had positive milk cultures, mainly CNS (31.8%).

Two of the 6 sheep farms did no udder preparation, 1 dry wiped teats, 2 stripped for abnormal milk, and 1 dry wiped and stripped before milking. All broke vacuum before unit removal and used postdip. Pipelines were used on 3 farms, and lowline parlors on 3. Mean teat end vacuum was 12.5" Hg (range 10.4" - 13.5"). Pulsation ratios were between 50:50 (4 farms) and 70:30, and rates were between 60 and 123/min. Five of 6 vacuum controllers met standards. Mean number of milking units was 9, ranging from 2-24. All farms used narrow-bore inflations, 5 rubber and one silicone. All farms used antibiotic IMM during lactation, and 4 of 6 dry treated ewes.

Thirty-three of the 36 goat farms (92%) used udder preparation; 50% predipped, most with 0.5% titratable iodine dip, 31% used udder wash, 11% dry wiped, and 69% stripped for abnormal milk. 89% broke vacuum and 83% postdipped, most with 0.5% iodine or 0.5% chlorhexidine. Bucket (19%), lowline parlor (25%), and pipeline (56%) milking systems were used. Teat end vacuum averaged 13.7" Hg (range 10" – 15.5"). Pulsation ratios were between 50:50 and 70:30, with 70% either 50:50 or 60:40. Rates were between 50 and 120/min; most were 60 or 90/min. 81% of vacuum controllers met standards. Mean number of milking units was 6 (range 1-24, median 5). All farms used narrow-bore inflations, 79% rubber and 21% silicone. 78% infused antibiotics during lactation, 78% dry treated all does and 6% selectively dry treated.

The most common mammary pathogen isolated from both sheep and goats was CNS, accounting for 63% and 84% of isolates, respectively. The prevalence of mammary pathogens in goats was low compared with dairy cows, despite the fact that goats have markedly higher mean bulk milk SCC than cows. Adoption of mastitis control practices was similar to that for cow farms, except that sheep owners did minimal udder preparation before milking.

Farm outbreak of equine botulism associated with *Clostridium putrificum*

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An Amish farm with about 20 horses (draft horses, Standardbreds, and ponies) lost 10 head in 8 days at the end of June 2006. Beginning one month later and continuing over a 27-day period, the farm lost another 7 head including some Standardbreds purchased to restock. The deaths occurred following a 24 - 48 h period of marked weakness and recumbency, without muscle tremors or dysphagia. A few of the affected animals died, but most were euthanized.

Tremetol (toxic principle in white snakeroot), organophosphate and carbamate insecticides, heavy metals, bromide, and malicious poisoning were ruled out. Ionophore antibiotic testing indicated that dairy feed fed occasionally to the horses was contaminated with monensin, but at too low a concentration to be consistent with toxicosis. Furthermore, the last 7 deaths occurred after horses were no longer exposed to the dairy feed. Oats (which were grossly moldy) and corn were tested for fumonisins; oats tested negative and corn contained about 5 ppm fumonisin B. Given that corn was mixed 1:10 with oats and no suggestive lesions were found, fumonisin toxicosis was also ruled out.

Anaerobic culture of gastric, colonic, or fecal samples from horses (4/4) and environmental samples (6/6) yielded a *Clostridium sp*. Bacterial overgrowth was prevented by heat shock (70°C, 15 min). Based on clinical signs, lack of morphological changes, negative toxicology results, and consistent isolation of a *Clostridium sp.*, botulism was diagnosed. Isolates were identified as *C. putrificum* by the MicroID system (BioLog^{R}), and the identity was confirmed by sequencing an ~ 900 bp fragment of 16S ribosomal DNA. Originally, any clostridial species producing neurotoxins was classified as *C. botulinum;* however, other non-*C. botulinum* species have caused botulism. In the case isolates, specific toxin genes were not identified by PCR assays with primers for types B, C, or D *C. botulinum* neurotoxin genes. Culture enrichments of gastroenteric samples from 2 horses were tested in the mouse bioassay, and although initial results suggested toxicity, it was not reproducible. Supernatant fluids from cultures of colonic and environmental isolates were tested by FRET-based and gel shift activity assays with substrates specific for neurotoxin serotypes A, C, or E. The samples did not have proteolytic activity specific for the 3 neurotoxins, but were selectively proteolytic.

The working diagnosis for the outbreak is botulism caused by *C. putrificum*, likely encoding a novel neurotoxin. A retrospective study suggested that the original source of the *C. putrificum* might have been a neighbor's horse that had died following a brief episode of colic. As a favor to the neighbor, the horse had been composted on the case farm in a large manure pile in the horse pasture. Most of the carcasses of the affected horses were composted in the same manure pile, likely amplifying the problem.

Bacteriologic findings and lesions in piglets affected or not affected by *Clostridium perfringens* type A enteritis

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Clostridium perfringens type A is controversial as a cause of porcine enteritis. Piglets develop diarrhea in natural and experimental infections, but pathologic changes are often mild or absent. Diagnosis is typically by exclusion of other etiologic agents and demonstration of large numbers of beta2 toxin (CPB2)-producing type A organisms in jejunum. We examined piglets displaying signs typical of type A enteritis, and included controls that were normal or, based upon nature of clinical signs, likely to be affected by other diarrheagenic agents. In most piglets, we obtained specimens from stomach, rectum, and 12 equally-spaced locations along the entire small intestine. Duplicate specimens were fixed in 10% phosphate-buffered formalin and subjected to semi-quantitative culture for *C. perfringens*. Lesions, presence of morphologically-compatible gram-positive rods, and numbers of *C. perfringens* were scored (0-4), and all isolates from each specimen were toxinotyped by multiplex PCR.

Extent of bacteriological culture positivity was significantly higher in principals than in controls (2.39 ± 0.4 vs 0.64 ± 0.31; $p < 0.01$), but there was no significant variation in scores from proximal to distal small intestine. A surprising finding was the extent of culture positivity in stomach (average score 2.58). Average **percent of isolates producing CPB2 was higher in principals** than in controls (87.2% vs 53.8%), but the difference was not significant, due to substantial variability among control samples (ranging from 0 - 100% *cpb2* positive). Nearly all isolates from stomachs of principals and controls were *cpb2* positive.

Principal pigs had higher average lesion scores throughout small intestine (average score 0.52 ± 0.22) than controls (average score 0), but variation in lesion scores from proximal to distal small intestine was not significant. **Gram-positive rods were noted in both principal and control pigs, although the average score was much higher in principals** (1.12 vs 0.33). These organisms were seen in only 3 segments of small intestine from all control pigs, but this variability eliminated statistically significant differences between groups.

These results suggest that (a) dominance of CPB2-producing *C. perfringens* type A probably begins in stomach, (b) small intestinal tissue morphologic changes are mild, but are on average distinct from controls, (c) numbers of type A are higher in affected than in unaffected piglets, and (d) observation of gram-positive rods in small intestine may be a positive predictor of type A disease.

These findings must be confirmed by examination of more piglets; however, results to date suggest that, given the current state of knowledge, ***C. perfringens* type A enteritis may be diagnosed with greater accuracy on a herd basis, rather than in individual piglets.**

Development of a duplex PCR for the simultaneous detection of *Actinobacillus suis* and *Actinobacillus pleuropneumoniae* in clinical samples from swine

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Introduction. *Actinobacillus suis* and *Actinobacillus pleuropneumoniae* are gram-negative microorganisms that colonize the upper respiratory tract of swine. Both organisms can cause severe necrohemorrhagic pleuropneumonia and affect mainly finishing pigs. The differential diagnosis of the infection caused by these pathogens is critical for the development of effective control strategies. Diagnosis of *A. pleuropneumoniae* and *A. suis* infection has traditionally been based on isolation of these pathogens from clinical samples. However, the isolation of these pathogens from swine tissues can be impaired by antimicrobial treatments of clinically affected animals and poor handling of samples prior to submission to the diagnostic laboratory. To overcome the limitations of traditional bacterial isolation for the differential diagnosis of *A. suis* and *A. pleuropneumoniae*, we developed and validated a duplex PCR test for the simultaneous detection of these pathogens in clinical samples of swine. The PCR test has the advantage of detecting live or dead microorganisms, hence improving the sensitivity of detection.

Materials and methods. We designed primers for the detection of *A. suis* to target the 16S rRNA gene using the GenBank sequence AY362899. Primers for the detection of the *A. pleuropneumoniae* species-specific Apx IV toxin gene have been described previously (1). PCR reactions were initially standardized individually. The analytical sensitivity and specificity for the single PCR tests were defined. Following individual standardization, we combined primers targeting the 16S rRNA gene of *A. suis* and the Apx IV gene of *A. pleuropneumoniae* in a duplex PCR using a Qiagen multiplex PCR kit, and defined the analytical sensitivity and specificity of the duplex PCR. Clinical samples of pigs suspect for *A. suis* (n=118) and/or *A. pleuropneumoniae* infection (n=115) were tested by the standardized duplex PCR and results were compared with bacterial isolation.

Results and discussion. The analytical sensitivity of the single *A. suis* and *A. pleuropneumoniae* PCR tests was 7 x 10^2 CFU/ml and 8 x 10^1 CFU/ml, respectively. The sensitivity of the duplex PCR test was 1 x 10^2 CFU/ml for *A. suis* and 2 x 10^3 CFU/ml for *A. pleuropneumoniae*. Single and duplex PCR tests were negative when using DNA extracted from 12 unrelated bacterial species that are commonly isolated from swine tissues. When we tested DNA from *A. suis* and *A. pleuropneumoniae,* we obtained bands of the expected sizes, specific to each organism. *A. suis* primers did amplify the DNA from *A. equuli,* which is a closely related equine pathogen. None of the tissues tested was positive for both pathogens, indicating that dual infection by *A. suis* and *A. pleuropneumoniae* is unlikely to occur or is infrequently observed in the field. In 19 samples, *A. suis* was detected by isolation and PCR, and in 13 samples both techniques detected *A. pleuropneumoniae*. Eighty samples were negative by both tests. PCR was more sensitive than isolation for both species. For *A. suis*, 14 samples tested positive by PCR and negative by isolation, and for *A. pleuropneumoniae*, 18 samples tested positive by PCR and negative by isolation.

Conclusions/relevance. The duplex PCR test developed in this study was more sensitive than bacterial isolation for the detection of *A. suis* and *A. pleuropneumoniae* in clinical samples. **The test proved to be a reliable tool for differential diagnosis of the infection by these pathogens.**

Reference

1. Schaller A, et al. Identification and detection of *Actinobacillus pleuropneumoniae* by PCR based on the gene apxIVA. Vet Microbiol 2001;79:47-62.

Actinobacillus sp. biochemically and phenotypically similar to *Actinobacillus pleuropneumoniae* can be differentiated by genomic fingerprinting, toxin profiling, and sequencing of the 16S rRNA gene

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Introduction. *Actinobacillus pleuropneumoniae* colonizes the upper respiratory tract of swine and causes severe fibrinous and necrohemorrhagic pleuropneumonia in susceptible animals. Naïve populations may experience high morbidity and mortality, with death occurring between 24 and 72 hours post-infection. Many swine herds in the United States have eradicated this pathogen from their populations. The negative status of these herds is monitored using serology, PCR testing, and bacterial isolation. The correct identification of suspect bacterial isolates is critical for the definition of the herd's health status, and an equivocal result may harm the ability of these herds to supply animals to negative herds. Recently, at the University of Minnesota Veterinary Diagnostic Laboratory an *Actinobacillus sp*. isolate biochemically and phenotypically similar to *A. pleuropneumonia*e was isolated from the pleura of a pig with no lesions. Initially, this isolate was identified as *A. pleuropneumoniae* based on biochemical tests and was classified as serotype 10 at the University of Montreal. However, additional testing by genotyping, toxin profiling, and sequencing of the 16S rRNA revealed that this isolate was distinct from *A. pleuropneumoniae* and more closely related to *A. minor, A. porcinus* and *A. indolicus,* early non-pathogenic colonizers of the upper respiratory tract of swine. Molecular diagnostic tools were critical in identifying the *A. pleuropneumoniae*-like isolate as a non-pathogenic *Actinobacillus sp*., and for the accurate assessment of the herd's negative status.

Materials and methods. The *A. pleuropneumoniae*-like isolate was recovered from the pleura of a 14-week-old finishing pig with no lesions. This isolate's identification as *A. pleuropneumoniae* was based on the following characteristics: V-factor requirement (+), X-factor requirement (-), catalase (-), hemolysis (+), urease (+), CAMP test (+), esculin (-), indole (-). Serotyping was performed at the University of Montreal using indirect hemagglutination as previously described.[1] Genotyping was performed by ERIC-PCR,[2] and the *A. pleuropneumoniae*-like isolate was compared with the reference strains for the 15 known serotypes of *A. pleuropneumoniae*. Toxin profiling was performed by PCR detection of the following genes: apxIA (723bp), apxIB (811bp), apxII (965bp), apxIII (396bp), and apxIV (1600, 2000, 2400, and 2800 bp) as previously described.[3] The 16S rRNA gene from this isolate was sequenced[4] and compared with sequences available at GenBank (http://www.ncbi.nlm.nih.gov/BLAST/).

Results and discussion. The isolate recovered from the pleura was biochemically identical to *A. pleuropneumoniae*. This isolate was classified as a serotype 10 by indirect hemagglutination, suggesting a cross-reaction between non-pathogenic *Actinobacillus sp*. and *A. pleuropneumoniae*. Genotyping by ERIC-PCR revealed that the *A. pleuropneumoniae*-like isolate was distinct from all *A. pleuropneumoniae* reference strains, with only 10.9% similarity with the cluster of reference strains. Toxin profiling revealed the presence of apx II and the absence of the remaining toxin genes characteristic of *A. pleuropneumoniae* (apxIA, apxIB, apxIII, and apxIV). The partial sequence of the 16S rRNA gene was 100% similar to *A. minor, A. porcinus,* and *A. indolicus.* These bacterial species, however, were negative for all toxin genes.

Conclusions/relevance. Genotyping by ERIC-PCR, toxin profiling, and sequencing of the 16S rRNA can be used to differentiate non-pathogenic *Actinobacillus sp.* that are biochemically and phenotypically similar to *Actinobacillus pleuropneumoniae.* The correct identification of these bacterial species is critical for the accurate definition of health status for swine herds.

References: 1. Vet Microbiol 1992;32:135–148. **2.** Nucleic Acids Res 1991;19:6823-6831. **3.** J Vet Diagn Invest 2005;17:359-362. **4.** LaPara TM, et al. Appl Environ Microbiol 2000;66:3951-3959.

Actinobacillus suis molecular epidemiology

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Introduction. *Actinobacillus suis* is an early colonizer of the upper respiratory tract of swine. This pathogen has recently emerged as an important cause of mortality in swine herds. Currently, there are no commercial vaccines available for the control of *A. suis*, and most field veterinarians rely on autogenous vaccines and antimicrobial treatments to control disease. The present study describes the molecular epidemiology of *A. suis* field isolates that were recovered from tissues submitted to the University of Minnesota Veterinary Diagnostic Laboratory. We identified important trends regarding the genetic variability, prevalence of strains, geographic distribution, age of affected animals, association with lesions, and antimicrobial resistance profiles of *A. suis* isolates recovered from North American swine herds.

Materials and methods. We genotyped 56 *A. suis* isolates recovered from swine tissues submitted to the University of Minnesota Veterinary Diagnostic Laboratory using the repetitive element-based PCR (1). We compared the genomic fingerprints obtained using primers targeting the enterobacterial repetitive intergenic consensus (ERIC) and boxA sequences and calculated the discriminatory power of each set of primers using the Simpson's index of diversity (2). Genotyping results were analyzed using the GelCompare software, and dendrograms based on genomic fingerprints were constructed using the Pearson's Product Moment Correlation and the unweighted pair group method with arithmetic mean (UPGMA) methods. We also evaluated the date of isolation, location of affected herd, age of affected animals, tissues that yielded *A. suis* isolation, lesions associated with isolation, and antimicrobial resistance profiles for each isolate that was genotyped.

Results. Using the ERIC-PCR technique, we identified 2 main clusters of strains among the 56 *A. suis* isolates. The ERIC-PCR strain group 1 contained 51 isolates, and strain group 2 contained 5 isolates. The Simpson's index of diversity for the ERIC-PCR was 0.166, therefore a random isolate has a 16.6% chance of being classified as a new strain. Using the box-PCR technique, we identified 6 clusters of strains: groups 2, 3, and 4 each had 1 isolate each; groups 1 and 5 had 8 isolates; and group six had 37 isolates. The Simpson's index of diversity for the box-PCR was 0.531, therefore a random isolate has a 53.1% chance of being classified as a different strain. The 56 isolates analyzed in this study were obtained from 25 different swine herds from 9 different states. Finishing pigs (between 10 and 20 weeks of age) were the main affected population (n=29), followed by 6-month to 2-year-old pigs (n=8), 0 to 3 week-old piglets (n=6), and nursery pigs (n=4). Age was not available for 9 pigs. *Actinobacillus suis* was isolated from 26 lung/pleura samples, 18 systemic sites (liver, pericardium, lymph node, spleen, and skin), and 4 tonsil samples. The isolation site was not available for 8 isolates. Forty isolates were recovered from tissues with lesions, whereas 6 isolates were from animals with no apparent lesions. Lesion information was not available for 10 animals. Thirty-two different antimicrobial resistance profiles were identified among the 56 *A. suis* isolates genotyped, with no clear predominance of a single profile. Isolates from the same genotype group had different antimicrobial resistance profiles.

Discussion. Although 6 different strains of *A. suis* were identified among 56 field isolates using the box-PCR, this organism is still highly clonal compared with *Haemophilus parasuis*, for example, for which more than 40 different strains were identified from 120 field isolates. Although *A. suis* can affect pigs of any age, finishing pigs are the main affected population in North American swine herds. The fact that isolates sharing similar genomic fingerprints have different antimicrobial resistance profiles indicates that resistance genes may be harbored in plasmids. We have recently confirmed this hypothesis by detecting tetracycline and beta-lactam resistance genes in plasmids isolated from *A. suis* (data not shown).

Conclusions/relevance. *Actinobacillus suis* affecting North American swine herds are highly clonal. Further studies are needed to evaluate if isolates sharing similar genomic fingerprints also share similar protective antigens. This information may be important for the selection of vaccine strains to be used in the control of this emergent swine pathogen.

References 1. Versalovic et al. Nucleic Acids Res 1991;19:6823-31. **2.** Simpson. Nature. 1949;163:688.

Mycobacterium avium subsp. *paratuberculosis* (MAP) infection in cull cows from Johne's disease herds

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Introduction. The distribution of MAP in tissues of cattle culled from known infected herds has not been well characterized, especially relating the MAP tissue burden to previous fecal cultures over time. The objective of this project was to better correlate fecal shedding to MAP tissue bio-burden and to determine what proportion of fecal culture negative cows would have culture positive fecals and tissues at slaughter.

Materials and methods. Tissues and fecal samples were harvested from cows culled from 3 dairy herds. Samples were collected from a total of 174 animals. Five samples were collected from each animal; 2 intestinal lymph nodes, ileum, ileocecal (IC) valve and a fecal sample. Fecal samples were processed using the 3-day incubation method with HPC/BHI, and tissues were processed using 2-day method in HPC. All samples were plated on a solid medium – Herrold's egg yolk with mycobactin J.

Discussion. Of the 174 cattle with harvested tissues, 17 (9.8%) were previously fecal culture positive for MAP. Fourteen of 17 animals were positive at slaughter (82%) and 3/17 (18%) cattle had all 5 samples culture negative. Each of the 3 cows with negative tissues was previously fecal culture positive with only one cfu of MAP on 1 of 4 tubes of HEYM. The 3 positive fecal samples may have represented transient "pass-through" MAP from other cattle in the herd.

Of the 20 fecal culture positive cattle at the time of slaughter, 9 were characterized as heavy shedders with over 100 cfu MAP/tube and the same 9 cows were massively infected in each of the 4 tissues examined with more than 300 cfu MAP/tube. No other fecal culture positive or negative cows had similar massive tissue infection with MAP. Interestingly, 5/20 (25%) fecal culture positive cows at slaughter had negative cultures on all 4 tissues. Three positive fecal culture cows (very low shedders) had only 1 colony on the 4 tissues cultured, suggesting infections as adults. The other 3 cows had modest levels of MAP in several tissues.

Of the 156 cattle with all negative fecal cultures prior to culling, 58/156 (37%) had at least 1 positive sample at slaughter. Of these 58 cattle, 25 were culture positive on 1 sample, 11 on 2 samples, 12 on 3 samples, 6 on 4 samples and 4 cows were positive on all 5 samples. An intestinal lymph node was positive most frequently, followed closely by ileum and IC valve. There were 26/58 (45%) cattle that had less than 10 total cfu of MAP on 20 tubes of HEYM for the 5 samples, which suggests a more recent infection. Thirty one percent (18/58) of the cattle had the next higher tissue level of MAP ranging between 10 and 100 total cfu MAP for the 20 tubes, suggesting a heavier and or repeated doses over time as adults.

Conclusions. Cattle shedding more than a few colonies of MAP or those with multiple positive fecal cultures had multiple tissues positive at high MAP concentrations. An estimated 35% of consistently culture negative cattle will have positive tissues at slaughter with a wide spectrum of MAP concentrations, suggesting a moderate level of adult infections.

Acknowledgement Financial support for this work was provided by a cooperative USDA Agricultural Research Service (58-1265-3-115) grant.

Semi-quantification of *Mycobacterium avium* subsp. *paratuberculosis* in bovine feces based on a days-to-detection method by a liquid culture system, ESP II

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Quantifying *Mycobacterium avium* subsp. *paratuberculosis* (MAP) organisms in bovine feces is challenging because of their slow growth, clumping property, fastidious nutritional requirements, and contamination by other microorganisms. Traditionally, colony counting on solid agar medium has been the gold standard to enumerate viable MAP organisms in fecal samples. A simple days-to-detection method was developed for the enumeration of MAP cells in bovine feces using a liquid culture system in conjunction with quantitative PCR. Culture of a total of 300 bovine fecal samples was performed in comparison on a conventional solid agar medium, Herrold's egg yolk medium (HEYM), and a liquid culture medium, ESP II, respectively. All heavy shedders (71/71) and moderate shedders (15/15) were identified by the two culture systems. However, there were significant differences with low shedders between the two systems. Of 68 low shedders, HEYM detected 39 (57.3%) whereas ESP II detected 62 (91.2%). After acid fast staining and PCR confirmatory steps, ESP II detected an additional 17 positive samples. The total number of positive samples by HEYM was 125 (42%) and by ESP II, 165 (55%).

The numbers of viable MAP cells, determined by HEYM slants, were compared to the days-to-detection by ESP II. The mean days-to-detection were for heavy shedders were 13.81 (SD = 3.49; range = 3.4 - 23.06) for heavy shedders, 21.17 (SD = 5.06; range = 15.99 - 37.03) for moderate shedders, and 30.84 (SD = 6.93; range = 19.99 - 49.71) for low shedders. The numbers of colony forming units (CFUs) of 30 heavy shedders were measured by inoculating serial dilutions of each sample on HEYM: the range was between 10^2 to 10^6 CFUs per g of feces, and for the majority (73%) of heavy shedders was 10^4 to 10^5 CFUs per g of feces. A linear relationship between days-to-detection and actual viable MAP cell numbers was demonstrated with a correlation coefficient of 0.78 ($y = -3.7158x + 32.298$, $P<0.001$).

During the recent 6 years, we have used the semi-quantification days-to-detection method to group 224,254 dairy cattle into 3 shedding categories: heavy (<22 days; n = 7,026; 3.1%), moderate (22 to 29 days; n = 4,158; 1.8%), and low shedders (>29 to 35 days; n = 13,310; 5.9%). There were 943 (0.4%) fecal cultures that were acid fast positive bacilli but not confirmed as MAP.

This study indicates that our categorization of field samples into 3 epidemiologically significant shedding levels based on quantitative estimates using a days-to-detection method with the ESP II system provides a greatly improved tool for diagnosis of *MAP* and for disease management by reducing total incubation time by 35 days. Except for special needs, such as export requirements and Johne's negative herd status, the identification of low shedders did not have practical implications in control of Johne's disease in our study.

Effects of culture conditions and tuberculin source on interferon-γ production in whole blood cultures from *Mycobacterium bovis* infected cattle

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The BOVIGAM® interferon (IFN) - γ assay constitutes an ante-mortem, *in vitro* laboratory-based tuberculosis test and is widely used complementary to the tuberculin skin test. The assay is performed in two stages: first, whole blood is cultured with antigens stimulating blood leukocytes to produce IFN-γ which is quantified by ELISA in a second step. Environmental conditions before and during the culturing of the leukocytes influence the efficacy of *in vitro* IFN-γ production. Optimal conditions are therefore essential.

In this study we analyzed the effect of stimulation vessel geometry, temperatures during stimulation, and the stability of antigens stored at different temperatures. Blood from experimentally infected cattle and from tuberculosis-negative cattle was stimulated in 24-well tissue culture trays (standard), 48-well and 96-well culture plates with the following antigens: purified protein derivative from *Mycobacterium bovis* (PPD-B) and from *Mycobacterium avium* (PPD-A), a fusion protein from early secretory antigenic target 6 (ESAT-6) and culture filtrate protein 10 (CFP-10), and pokeweed mitogen.

Stimulation was equally efficient in all 3 plate formats. The results with specific antigens correlate with mitogen induced stimulation. CO_2 is not required during incubation, as cultures from an incubator with 5% CO_2 produced similar amounts of IFN-γ as without CO_2. However, the temperature used for stimulation was critical. **Stimulation at 37°C and 33°C was equally efficient**, but a culture temperature of 29°C reduced IFN-γ production significantly. At 25°C and 22°C no stimulation was detectable.

Antigens are usually stored at 2-8°C (tuberculins) or at -80°C (recombinant proteins) until usage. We tested in parallel antigen storage of recombinant proteins (ESAT-6:CFP-10 fusion protein, TB10.4, TB27.4, MPB83) at 4°C for 24 h or at 20°C for 8 h prior to use in cell culture. Our results show that **antigens may be stored at either of these conditions without affecting the efficacy of stimulation.**

Finally, we compared the activities of tuberculins from 5 different sources in naturally infected cattle (n=10). Matched PPD-B and PPD-A tuberculins were used at 8 dilutions each. Relative potency 30 (RP30) was defined as the tuberculin concentration required to induce 30% of the peak response values. RP30 differed by a factor of more than 10 between the PPD-B with the highest and lowest potency. **Therefore, tuberculins of different sources may give different results and the overall assay performance may be improved by optimizing tuberculin concentrations.**

Preliminary evaluation of the potential shedding of *Mycobacterium bovis* by coyotes and raccoons

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Bovine tuberculosis (bTB) is endemic in white-tailed deer (*Odocoileus virginianus*) in the northeast corner of Michigan's Lower Peninsula. At least 7 wildlife species have been found positive for bTB in that area. In addition to white-tailed deer, bTB has been found in black bears (*Ursus americanus*), bobcats (*Felis rufus*), coyotes (*Canis latrans*), raccoons (*Procyon lotor)*, red fox (*Vulpes vulpes*), and North American opossums (*Didelphis virginiana*). Each animal infected with bTB has the potential to be a transmission host to other animals, depending on whether or not the animal sheds the infectious organism, *Mycobacterium bovis*. Previous research indicates bTB prevalence in coyotes as high as 33% in the endemic area and raccoons as high as 4.7%. These species, especially raccoons, often frequent cattle feed areas and therefore could serve as a source of infection for cattle. The USDA, Wildlife Services, National Wildlife Research Center (NWRC) is conducting 3 studies to examine the shedding potential in coyotes and raccoons: 1 captive study and 2 field studies.

In the captive study, 4 coyotes were orally inoculated with 1.0×10^5 CFU/ml of ***M. bovis.*** Samples being taken consist of oral and nasal swabs and feces. All 3 samples are being cultured. Fecal samples are also being tested by polymerase chain reaction (PCR) and by exposing guinea pigs to the coyotes' feces. As of June 2007, this study has been ongoing for 12 weeks. To date, culture results consisting of pre-inoculation samples and day 17 samples have been negative. No effect has been observed on the guinea pigs. Near the end of the study at approximately 16 weeks, coyotes and guinea pigs will be necropsied and tested for ***M. bovis*** infection.

In the field, coyotes and raccoons are being trapped in select counties in northern Michigan. Oral/nasal swabs, fecal and tissue samples are being collected. Histology and standard culture techniques are being performed at the USDA, National Veterinary Services Laboratory (NVSL) on all tissue samples. The associated oral, nasal and fecal samples of tissue samples positive under histopathology or culture at NVSL are being cultured at the Microbiology, Immunology and Pathology lab at Colorado State University using modified culture technique. The fecal samples are also being tested by PCR at NWRC. As of June 2007, 49 coyotes have been sampled from three counties. Of these, 4 coyotes were mycobacteriosis compatible under histopathology and of the 21 samples for which culture results are available, 1 concurred with the histopathology results and 2 additional samples were found ***M. bovis*** positive, despite being negative under histopathology. The related swabs and fecal samples are currently being cultured. As of June 2007, 42 raccoons have been sampled. Histopathology results on these raccoons were negative for bTB, but culture results are still pending.

Although still too early to reach any concrete conclusions, **culture results from the captive study suggest that coyotes do not shed *M. bovis* within a 17-day incubation period**. It is apparent from the field study that there are coyotes infected with bTB in the endemic area of Michigan. The final findings will provide information on the transmission risk of coyotes and raccoons, and thus their role in the spread of bTB.

Detection and speciation of *Leptospira sp*. in clinical samples using PCR followed by sequencing of amplicons

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Introduction. Leptospirosis is a zoonotic bacterial infection that causes stillbirths, abortions, and infertility in swine and cattle. At least 200 *Leptospira* serovars have been identified as pathogenic and over 60 as non-pathogenic (1). The main pathogenic species affecting swine and cattle in the USA are *Leptospira interrogans* serovars *hardjo, pomona, bratislava, canicola, icterohaemorrhagiae*, and *grippotyphosa*, whereas *L. biflexa* serovars *patoc I* and *andamana* are recognized as an ubiquitous non-pathogenic group. Diagnosis of leptospirosis can be performed by bacterial isolation and/or serology by microscopic agglutination test (MAT). Both tests have limitations, such as low sensitivity and occasional cross-reactions, respectively. The objective of this study was to validate a PCR test to detect *Leptospira* sp. in clinical samples and to further characterize positive samples by sequencing the obtained amplicons.

Materials and methods. The PCR test described by Merien et al (2) was standardized using a pure culture of *L. interrogans* serovar *hardjo*. The sensitivity of the test was evaluated using 10-fold dilutions of the pure culture in PBS, swine fetus tissue homogenate, and bovine urine. The specificity of the test was evaluated by testing pure cultures of *L. interrogans* serovars *hardjo, bratislava, pomona, icterohaemorrhagiae, canicola*, and *grippotyphosa*. Additionally, DNA from 15 unrelated bacterial species commonly isolated from porcine and bovine tissues were tested. Amplified fragments were sequenced, aligned using the MEGA3 software, and compared with those available at the GenBank using the BLAST software. The minimum number of leptospiral bacterial cells needed for sequencing was defined by amplifying the DNA extracted from 10-fold dilutions of a pure culture followed by sequencing the bands with different intensities.

Results. The PCR test was positive for the 6 *Leptospira* serovars tested and negative for the 15 unrelated bacterial species. Sensitivity of the test using pure culture and spiked clinical samples was as follows: 1 fg of DNA for the pure culture in PBS and bovine urine, and 1 pg in swine fetus tissue homogenate. Two main clusters were identified by sequencing of the PCR amplicon. Cluster I included the following pathogenic species: (Ia) *L. canicola*, *L. hardjo*, and *L. pomona*; (Ib) *L. icterohaemorrhagiae*, *L. bratislava*, *L. grippotyphosa*; (Ic) *L. noguchii* serovar *panama*; (Id) *L. borgpetersenii* serovar *hardjo-bovis* and *L. borgpetersenii* serovar *tarassovi*; and (Ie) *L. santarosai* serovar *shermani*. Cluster II included the *L. biflexa* serovars *patoc 1* and *andamana*, which are both non-pathogenic. A minimum of 0.4 pg/µL of *Leptospira* DNA is needed for accurate sequencing of amplicons obtained from clinical samples.

Discussion. This newly validated PCR test proved to be highly sensitive for the detection of *Leptospira* sp. in clinical samples from swine and cattle. Sequencing was able to differentiate between pathogenic and non-pathogenic *Leptospira sp*. A PCR-RFLP for the simultaneous detection and speciation of *Leptospira sp*. has been described in the literature (3). The PCR and sequencing approach evaluated in this study is less complex than the PCR-RFLP method, while giving similar results.

Conclusion/relevance. PCR and sequencing can be used for detection and speciation of *Leptospira* sp. in porcine and bovine clinical samples.

References: 1. Soto F, et al. Brazilian J Microbiol 2006;37:582-586. **2**. Merien F, et al. J Clin Microbiol 1992;30:2219-2224. **3**. Heinemann M, et al. Vet Microbiol 2000;73:261-267.

Yersiniosis in farmed deer caused by a distinct O-genotype of *Yersinia pseudotuberculosis*

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Yersinia pseudotuberculosis infects a variety of avian, mammalian, and reptilian species. It is a major cause of mortality in wild and captive cervids. The common manifestations of the disease in cervids are necrotizing, ulcerative enteritis and mesenteric lymphadenitis, with or without the involvement of visceral organs. Of the 21 known O-serotypes or O-genotypes, O:1 to O:3 are usually isolated from deer clinical cases.

Between December 2006 and February 2007, acute death of 10 deer occurred in an enclosed deer farm with approximately 400 head of various species of deer. Necropsies of dead animals performed by the attending veterinarian did not reveal any significant gross lesions. A serum sample and postmortem tissue specimens collected from a 6-month-old white-tailed deer and lung tissue from an 8-month-old Axis deer that had been dead approximately 24 hours were submitted to the Mississippi Veterinary Research and Diagnostic Laboratory (MVRDL) for laboratory diagnosis.

Histological examinations showed that the white-tailed deer had marked diffuse cholangiohepatitis, mild multifocal suppurative cystic colitis, mild myocarditis, marked multifocal hemorrhage with mild interstitial pneumonia, and nematodiasis. Marked congestion and mild pulmonary edema with hemorrhage were seen in the lung tissue of the Axis deer. ***Y. pseudotuberculosis* was isolated from lung tissues of both animals and colon contents of the white-tailed deer**. Serology and molecular virology results indicated that the deer were negative for evidence of bovine viral diarrhea, brucellosis, bluetongue, epizootic hemorrhagic disease, Johne's disease, leptospirosis, and malignant catarrhal fever.

PCR-based virulence potential studies indicated that the isolates were positive for *virF, inv*, *yopB,* and *yopH,* 4 genes essential for invasion and colonization of host intestine and lung by *Y. pseudotuberculosis.* O-genotyping demonstrated that the *Y. pseudotuberculosis* isolates from both deer were genetically identical to each other, but distinct from all O-serotypes (or genotypes) reported previously. In addition to all O-antigen genes possessed by the members of O:3, **a gene encoding mannosyltranferase-like protein (*wbyK*) was detected in these isolates.** The *wbyK* gene of these isolates showed 94% of DNA sequence homology with the *wbyK* gene of serotype O:1b. A follow-up O-serotyping test identified the isolates as serogroup O:3.

In summary, we report an outbreak of fatal yersiniosis in farmed deer due to infection with a distinct O-genotype of *Y. pseudotuberculosis* that had the virulence potential to invade and colonize host intestine and lung.

Consolidation of virulence and antimicrobial resistance genes on plasmids of *Salmonella* Dublin

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Introduction. Infection with *Salmonella enterica* serotype Dublin is associated with a spectrum of disease in calves including diarrhea, septicemia, interstitial pneumonia and meningoencephalitis. Prior to 2002, all isolates of *S.* Dublin tested by our laboratory were susceptible to ceftiofur. Since 2003, greater than 50% of *S.* Dublin tested have been resistant to ceftiofur as well as at least 4 other antimicrobial drugs. Because multidrug resistance in Enterobacteriaceae is frequently plasmid-encoded, we sought to characterize the plasmid content of several Multidrug-resistant isolates of *S.* Dublin.

Materials and methods. Ceftiofur-resistant isolates of *S.* Dublin (n=7), each isolated from a different herd during 2002-2003, were characterized. Plasmid profiles were determined using the method of Kado and Liu. Plasmids that transferred ceftazidime resistance to a laboratory *E. coli* strain were analyzed by restriction fragment length polymorphism analysis (RFLP) using *Pst*I, followed by Southern hybridization using a full-length $bla_{\text{CMY-2}}$ probe to identify the fragment(s) containing this cephalosporin resistance determinant. Plasmids were additionally characterized using a mixed-plasmid fingerprinting microarray.

Results. Two plasmid profile types were recognized: one type containing two plasmids (90 kb and 165 kb) and a second type containing a single 165 kb plasmid. The 165 kb plasmid encoded multidrug resistance in both profile types. Mixed-plasmid microarray analysis revealed that singly carried 165 kb plasmid encoded genes (*spvRA, spvC*) that are required for *S.* Dublin to cause invasive disease and that are typically carried by the Dublin serovar associated virulence plasmid. Incompatibility gene probes on the microarray demonstrated that the singly carried plasmid belongs to group IncA/C, rather than IncFII, to which the typical serovar-associated virulence plasmid belongs.

Conclusion. In some clinical isolates of *S.* Dublin, resistance genes and genes characteristic of the serovar-associated virulence plasmids have become co-integrated, which may increase the association of multiple drug resistance with this serovar.

* Graduate student presentation

Abortion and stillbirth in a western Wyoming cattle herd vaccinated with *Brucella abortus* strain RB51

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Brucella abortus strain RB51 vaccine was licensed for use in cattle by the United States Department of Agriculture, Animal and Plant Health Inspection Service (APHIS) in 1996 as part of the cooperative State-Federal Brucellosis Eradication Program. Bovine heifer calves are traditionally vaccinated with a calfhood dose of 1.0 - 3.4 X 10^{10} organisms at 4-12 months of age. Adult cattle or those in high-risk areas, such as western Wyoming, can be vaccinated or booster vaccinated with permission of the state veterinarian and the APHIS area veterinarian in charge. Strain RB51 typically does not produce any clinical signs of the disease; the organism is cleared from the blood stream within 3 days and is not spread from vaccinated to non-vaccinated cattle. However, little is published on losses associated with the use of strain RB51 in pregnant cattle. The incidence of abortion due to RB51 is likely dependent upon the previous vaccination history, dose administered, stage of gestation when vaccinated, presence of concurrent disease processes, and the animal's present immune and nutritional status.

In Sublette County, WY, 1,400 head of pregnant adult cattle were vaccinated on 4 separate ranches with a calfhood dose of strain RB51 in early November 2006. Calf losses were observed in one of the vaccinated herds beginning on February 10, 2007 and ending on April 3, 2007. Most losses were reported between March 6-15, 2007 and involved mostly 5 - 6-year-old cows. In this vaccinated herd of 280 cattle, 7 abortions, 3 stillborn calves, 3 premature weak live calves, and 5 open cows were reported. These calf losses accumulate to approximately 6.4% (18/280). One aborted calf and 1 premature weak calf from this herd were submitted to the Wyoming State Veterinary Laboratory (WSVL) for necropsy. *Brucella abortus* was cultured from lung, liver, rumen and abomasal contents, and was identified as strain RB51 by standard biochemical strain typing procedures and *Brucella abortus* typing PCR tests. Subsequently, the National Veterinary Services Laboratories (NVSL) isolated and confirmed strain RB51 in tissues collected from 3 additional abortions. **Strain RB51 was isolated from tissues of 4/7 (57%) aborted calves and 1/3 (33%) premature weak calves.** Gross lesions were lacking in the calf and fetus submitted to the WSVL, and microscopic changes were restricted to very mild neutrophilic bronchopneumonia. Mild pneumonia also was observed in the three fetuses submitted to NVSL. Placenta was not available for examination on any of the cases. *Bovine viral diarrhea virus*, *Bovine herpesvirus 1* (IBRV), and other viruses were ruled out as a cause of abortion or premature birth by virus isolation and fluorescent antibody tests, and no other significant bacteria were isolated from fetal or calf tissues on these cases. Toxicology tests on the premature calf additionally ruled out nitrate poisoning. These laboratory analyses suggest strain RB51 as the causative agent of calf losses in the affected herd.

The incidents of bovine abortion, stillborn and premature calves associated with *Brucella abortus* vaccine strain RB51 represent a rarely reported aspect of brucellosis vaccination of pregnant cattle. **Findings suggest that losses may occur due to vaccination with strain RB51 and that using reduced dosages of the vaccine should be considered when vaccinating pregnant cattle.** Vaccination strategies should consider concurrent disease, immune status, nutritional factors and vaccination history. *Brucella abortus* strain RB51 should be recognized as a potential cause for abortion in vaccinated cattle, and diagnostic laboratories need the ability to differentiate between vaccine and field strain.

* Graduate student presentation

Identification of a new O group derived from non-serotypable Shiga-toxin-producing *Escherichia coli* strains by restriction analysis of O-antigen gene cluster

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Introduction. O-antigens are components of lipopolysaccharides that are present on the outer surface of *Escherichia coli* and other gram-negative bacteria. O-antigens are important virulence factors that are targets of both the immune system and bacteriophages. Conventionally, O serotyping is based on agglutination reactions using antisera raised in rabbits against O standard reference strains for qualitative O determination. However, serotyping is laborious and challenging as cross reactions often take place giving ambiguous results. Some strains may not react with any known antisera and are thus, non-serotypable or O negative.

Materials and methods. Non-serotypable Shiga toxin-producing *E. coli* (STEC) strains (n = 56) from the collection of the E. coli Reference Center were O typed by a PCR-RFLP method.

O-typing: PCR amplification of O-antigen gene cluster was carried out using primers at the JUMPstart end and the *gnd*-end using Expand Long Template PCR kit. PCR reaction mixes comprised of 500 μM final concentration of four dNTPs, 5 μL of PCR buffer containing 2.75 mM $MgCl_2$, 0.75 μL enzyme mix, 300 nM primers and 500 ng template DNA. Amplification was performed by denaturing at 94^{o}C for 2 min, followed by 30 cycles of denaturation at 94^{o}C 10 min, annealing at 64^{o}C for 30 sec, followed by final elongation at 68^{o}C for 15 min. Restriction analysis of the amplified fragment was performed by digesting with *Eco*RV for 2 h at 37^{o}C. The RFLP profile was compared with all of the standard reference strains belonging to O1-O181 serogroups.

Results and discussion. A new STEC group was identified with a unique RFLP profile of the O-antigen gene cluster. This group of strains (n=17) were all isolated from pigs and were highly pathogenic, carrying genes for Shiga toxin 2e (100%), heat-stable toxins, STa and STb (70% and 76% respectively), and F18 fimbriae (82%). Some of them were related, as determined by MLST. A PCR assay for this group is being developed based on the sequence of the O-antigen gene cluster. The O group will be designated following antiserum production in rabbits.

Conclusion. This group of STEC strains had not been recognized as pathogenic as they were O-negative, non-serotypable strains. **This PCR assay will allow detection and identification of highly pathogenic strains of *E. coli* that are associated with pigs.**

USAHA /AAVLD Scientific Session

Monday, October 22, 2007

Rose Ballroom

Co-chairs: Grant Maxie, President-elect, AAVLD; Jim Leafstedt, President-elect, USAHA

Examining the roles of AAVLD and USAHA concerning major diseases

07:30 AM **Welcome and announcements**
Jim Leafstedt, President-elect, USAHA
Grant Maxie, President-elect, AAVLD
Moderator - Will Hueston

07:45 AM **Control of *Viral hemorrhagic septicemia virus* (VHSV) of fish** - Gary Egrie..... 148

08:15 AM **Will we ever eliminate *Salmonella* from poultry?** - Stephen Roney 149

08:45 AM **Control and eradication of the scrapie prion from sheep** - Cindy Wolf 150

09:15 AM BREAK

09:30 AM **Comments on control of *Bovine viral diarrhea virus* in the United States** - Dale Grotelueschen..... 151

10:00 AM **Control and eradication of *Porcine reproductive and respiratory syndrome virus* (PRRSV)** - Scott Dee..... 152

10:30 AM **Control of equine infectious anemia should take new directions** - Charles Issel..... 153

11:00 AM **Building an infrastructure to address emerging diseases: the Johne's disease template** - John Adams..... 154

11:30 AM Summation

11:45 Adjournment

Control of *Viral hemorrhagic septicemia virus* (VHSV) of fish

P. Gary Egrie

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Riverdale, MD 20737

The purpose of the VHS Federal Order that APHIS issued was primarily driven to prevent the spread of VHS from wild to aquacultured populations. Current surveillance evidence of the VHS epizootic has shown that the disease is restricted to wild fish populations in the Great Lakes watershed. However, APHIS recognizes that the health of farmed and wild aquatic animal populations are linked, and at times resources may need to be applied for activities not traditionally thought to be the purview of an agriculturally based agency in order to appropriately structure Federal regulations. The intent is to protect farmed populations while minimizing the impact of those regulations on farmers, and to address pathogen vectors not easily controlled by regulatory actions.

Will we ever eliminate *Salmonella* from poultry?

C. Stephen Roney

National Poultry Improvement Plan, Conyers, GA 30094

When speaking of *Salmonella* in poultry, several categories of disease should be considered:

1) Those that are devastating to the production of poultry,
2) Those that cause disease in people but not in poultry,
3) Those that may cause disease in poultry and /or people and,
4) Those that cause no disease in poultry or people.

The reason to categorize these is to examine the incentives that apply for the control of each group. *Salmonella* biovar Pullorum and *Salmonella* serovar Gallinarum fall into the first group. The National Poultry Improvement Plan was established in 1935 specifically to set programs to control *S.* Pullorum and hence pullorum disease. Because these 2 strains were ovarian transmitted and could result in mortalities as high as 50% in progeny, the success of the entire poultry industry depended on controlling these diseases. Serological diagnostic tests were discovered that made the monitoring of these 2 species possible, and **the industry lowered the infection rate of *S.* Pullorum and *S.* Gallinarum from 15% to essentially 0% in just a few years**. This is an example of high incentive to control a disease.

Some of the paratyphoids, or motile serotypes, of *Salmonella* may exist with no signs of disease in poultry but cause illness if transferred to people. These are difficult to control since the poultryman might not even know that he had such an infection in his birds. The incentive for controlling this group is obvious from a public health standpoint, and research is ongoing to help to identify these scenarios and eliminate these bacteria that might enter the human food chain.

Salmonella serovar Enteritidis (SE) causes disease in people but may or may not cause disease in poultry. However, when SE became associated with commercial eggs a few years ago, the incentive to control this disease in the egg industry became very high. An SE clean category was added to the NPIP, and with testing and vaccination, **the incidence of SE in commercial layers dropped dramatically**. Meat-type chickens followed suit shortly thereafter, and the SE monitored program was implemented.

Those salmonellas that cause no disease in poultry or people present a different incentive to control than the others. Since FSIS currently does not speciate those isolates found on broiler carcasses at processing, producers do not always know if these isolates are of public health concern. However, the isolation percentages allowed by FSIS are decreasing, and **the incentive is rising to control all *Salmonella* species in poultry**. As often happens, the primary breeders lead the way in controlling all species of *Salmonella* and most of the multiplier breeding stock is being supplied to the production companies free of all *Salmonella* species. There is still some infection that occurs in the multipliers after they arrive in the hands of the production companies, and this is the next area of incentive to control that will need to be strengthened. Much new information is becoming available regarding the control of the paratyphoids in production units, and NPIP programs such as US Salmonella Monitored have been established to aid in their control.

Control and eradication of the scrapie prion from sheep

Cindy Wolf

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Comments on control of *Bovine viral diarrhea virus* in the United States

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Bovine viral diarrhea virus (BVDV) is recognized as a cause of substantial production and economic loss to the cattle industry in the United States. Losses are associated with a range of clinical entities and include reproductive and respiratory as well as secondary disease from BVDV-induced immune suppression. Reduced pregnancy rates in cows have been documented in herds with persistently infected (PI) calves present, and modeling including pregnancy rate loss, increased death loss and reduced weaning weight suggests about $15-25 cost per female if PI calves are present at the cow/calf level. Mixed results as to effects of PI animal presence in feedlots have been found, although BVDV in feedlots is regarded as a major pathogen. Persistent and transient (acute) infections are components of BVDV disease. PI calves are born as a result of infections at approximately 1½ to 4 months gestation and as progeny of PI females. PI BVDV animals are the primary reservoir of the virus in populations and shed high amounts of virus to other contacts and the environment. Elimination and prevention of PI BVDV animals in cattle herds are critical components for controlling BVDV.

Organizations including the Academy of Veterinary Consultants (AVC), American Association of Bovine Practitioners (AABP), and the National Cattlemen's Beef Association (NCBA-Cattle Health and Well-Being Committee) have endorsed the need for higher levels of effective BVDV control. Additionally, the United States Animal Health Association (USAHA) passed a resolution in 2006 supporting the livestock industries in adopting measures to control and target eventual eradication of BVDV from North America. Discussions by organizations have focused primarily on control strategies and education as the primary focus of efforts. BVDV *control* can be defined as the implementation of planned strategies to maintain negative status, reduce incidence or eliminate BVDV from a unit of interest, including documentation and/or monitoring of progress. BVDV *eradication* can be defined as the implementation of planned strategies to eliminate BVDV from a unit of interest, including documentation of that status.

Biosecurity, biocontainment, vaccination to prevent birth of PI calves and surveillance plans to assess herd BVDV status are generally recognized as the fundamental components of BVDV control plans. Control strategies embraced by all interests, including scientific disciplines, veterinary practitioners, and cattle producers with broad participation will enable successful control at levels targeted by the industry as a whole. Diagnostic laboratories have developed excellent tests for PI BVDV identification and are offering testing services. Use of scientifically valid, cost effective surveillance is needed for better detection of BVDV-infected herds.

The cattle industry is involved at various levels with BVDV control. Goals related to BVDV control are evolving and may or may not include eradication as an ultimate target. Control plans must be effective and economically beneficial to present and changing production systems. Participation by all aspects of the industry will expedite accomplishment of goals related to control of BVDV.

Control and eradication of *Porcine reproductive and respiratory syndrome virus* (PRRSV)

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Porcine reproductive and respiratory syndrome virus (PRRSV) is an economically significant pathogen of swine, estimated to cost the US swine industry approximately $560 million per year. The majority of this cost (88%) occurs post-weaning, secondary to reduced growth rates and elevated mortality. This is a conservative estimate, based on the fact that it is calculated on direct costs only and does not include indirect costs, such as the cost of vaccinations, medications, as well as additional diagnostic and biosecurity costs that come into play post-infection. PRRSV is a single-stranded positive-sense enveloped RNA virus and is a member of the genus *Arterivirus*. Characteristics of PRRSV infection in the pig include persistent infection, prolonged viremia and infection of macrophages. It has been shown to undergo extensive genetic change (mutation and recombination) as it replicates within pigs and is transmitted from pig-to-pig.

These characteristics have made control via traditional methods, such as vaccination and animal flow challenging, and inconsistent results have been reported across farms. The inability to consistently control the disease of PRRS has led to discussion regarding the feasibility of area-based eradication programs. The annual cost of PRRS is significantly higher than the cost of 2 recently eradicated diseases of swine, specifically, hog cholera and pseudorabies. It has been well-documented that the elimination of PRRSV from individual farms through a number of time-tested techniques is possible; however, re-infection by an unrelated isolate via an unknown route (area spread) is a frequent event. This problem has plagued swine producers and veterinarians over the last 5 years and has been extremely costly, emotionally devastating, and has stymied large-scale eradication events.

Frustration with PRRS eradication experiences has stimulated the unique, collaborative efforts across the North American industry. In 2005, the American Association of Swine Veterinarians (AASV) published a position statement that *"Eradication of PRRS from the North American pig population is the long-term goal"*. Through its membership, the AASV has begun to organize PRRS eradication working groups at the continental level (North American PRRS Eradication Task Force), the provincial level (Ontario Swine Health Advisory Board), and at the state level (Minnesota PRRS Eradication Task Force). In addition, collaborative groups of PRRS researchers have also come forward (PRRS CAP 1) and the National Pork Board has dedicated significant resources to solving the problem of PRRS (NPB PRRS Initiative).

Challenges to achieving the AASV long-term goal will be to gain/maintain acceptance of the various constituents across the industry and the ability of researchers to discover cost-effective, real-time information, tools, and techniques to meet the needs of the industry. However, despite these seemingly insurmountable challenges, **it is universally acknowledged that the industry can no longer afford to live with the disease.**

Control of equine infectious anemia should take new directions

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Effective control of equine infectious anemia (EIA) first became possible in 1970 with the advent of the agar gel immunodiffusion (AGID or Coggins) test that was shown to correlate with horse inoculation tests for *Equine infectious anemia virus*. Even today, the USDA officially recognizes only the AGID and horse inoculation tests for the diagnosis of EIA. The activities of the USAHA Infectious Diseases of Horses Committee have played a dominant role in the formulation of guidelines for and implementation of standards for the control of EIA. Their activities, fostered by a subcommittee on EIA, have helped guide the USDA in educational efforts, in adoption of Uniform Methods and Rules and, recently, in discussions and recommendations for changes in the control program. **The changes indicated at this time include: 1) uniform acceptance of negative ELISA results for EIA, 2) adoption of a 3-tiered laboratory system, 3) increasing testing intervals for the over-tested mobile population, with regionalization, and 4) requirement for a negative test for transfer of ownership.**

- The first change is indicated because there are fewer false-negative reports using the ELISA than the AGID test, i.e., the power of a negative ELISA result is greater than a negative AGID test result.
- A 3-tiered laboratory system would adopt the ELISA as a first test at a primary laboratory, followed by ELISA and AGID tests on suspect cases at referral laboratories, and further testing on problem samples at reference laboratories.
- The third change, increasing the time between tests in previously tested horses, is indicated because in areas of the country where EIA is expected to occur at a rate of less than 0.01% in the untested population, the chance of encountering an infected horse is essentially zero. As most states fit this category, it should be relatively easy to convince owners and regulators to regionalize the country based on prevalence of infection.
- The fourth change, a negative test for change of ownership, is recommended because states who have adopted this strategy have found it a most effective method for finding new cases of EIA in previously untested horses.

Eradication of EIA in the US, theoretically possible, is hampered at the present time by our inability to test those equids that have eluded our current testing strategies/rules/regulations. As this untested population is estimated to exceed 40% in many regions of the country, the challenge is formidable. Eradication of EIA may require compensation or options to encourage more extensive testing, e.g., quarantine sites for housing test-positive horses. **The adoption of uniform standards, regionalization and implementation of meaningful state-federal cooperative programs for the control of EIA are long overdue and are essential if more effective control and, perhaps, eradication is to be realistically achieved.**

Building an infrastructure to address emerging diseases: the Johne's disease template

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Johne's disease has not been designated as a so-called "program disease" by USDA, unlike the designations of bovine tuberculosis and brucellosis. As Johne's disease emerges as a disease of major economic concern to the cattle industry, the development, funding, and management of a voluntary program become critical for meaningful disease control.

AAVLD Poster Session

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Foyer

***** Graduate student presentation

Metastatic ganglioneuroblastoma in a dog

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A 13-month-old mixed breed Shepherd-type dog was presented to the referring veterinarian with a primary complaint of progressive lethargy and dyspnea that was followed by emaciation, pain, and ultimately recumbency.

At necropsy, the cranial thoracic cavity was filled by a firm, gray-white and mottled red, soft tissue mass. The mass compressed the lungs caudoventrally and surrounded the esophagus, trachea, and great vessels but did not compress their lumens. The spleen was markedly enlarged, firm, friable, and did not exude blood on section. The liver contained numerous gray-white, solid or blood-filled nodules varying in size from a few millimeters to 2 cm.

Histologically, the tumor consisted of at least 2 populations of cells; sheets of small, polygonal to round cells (neuroblasts), and nests or individualized large, polygonal cells resembling neurons (ganglion cells). The latter were associated with a somewhat abundant, neuropil-like stroma. Hepatic metastases contained both cell types in similar proportions and distribution, however the spleen consisted almost exclusively of the small cell type.

Ganglioneuroblastomas are rare tumors that originate from sympathetic nervous system ganglia and have been reported in a number of domestic animal species. They are generally solitary tumors that arise in the retropleural and retroperitoneal space, mediastinum, and adrenal medulla where sympathetic ganglia are normally found. **Thoracic ganglioneuroblastomas have been described in dogs, but to the authors' knowledge, there have been no previous reports of metastatic ganglioneuroblastomas in the veterinary medical literature.**

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Diagnosis of swine abortion/stillbirth in south of Brazil

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Various infectious agents have been implicated in reproductive failure in sows, resulting in serious economic losses. The objective this study was to diagnose causes of abortion/stillbirth in swine fetuses submitted to the Federal University of Rio Grande do Sul, Brazil between July 2005 and March 2007.

An abortion was considered of bacterial etiology when a bacterial species was isolated in pure culture from the fetal tissues in association with histological lesions characterized primarily by neutrophilic infiltration and necrosis. These were of particular value to differentiate bacterial contamination from infection. The criteria used to classify viral abortion was polymerase chain reaction assay (PCR) and detection of viral antigen by immunohistochemistry. The diagnosis of *Porcine circovirus 2* (PCV-2) was based on the presence of multifocal myocarditis associated with a positive immunohistochemistry result. Diagnosis of *Porcine parvovirus* (PPV) infection was done through PCR associated or not associated with the presence of mononuclear infiltration in heart and kidney as well as hepatic congestion and necrosis. Abortions were considered to be idiopathic when no lesions or agents were identified. Fetuses that had significant histologic lesions, but in which infectious agents were not isolated or identified, were classified as "possibly infectious but of unknown etiology".

Fetuses from 121 cases of porcine abortion, stillbirth and mummification were examined for gross lesions, bacterial infections, and PPV or PCV-2 infection. **Bacterial abortion was identified in 5 cases (4.1%).** *Escherichia coli* (both hemolytic and nonhemolytic) was found twice, and *Erysipelothrix rhusiopathiae*, *Corynebacterium* sp. and *Actinobacillus suis* once each. *E. coli* abortion was characterized by accumulation of fluid in body cavities and fibrin. Microscopically, multifocal suppurative pneumonia and focal suppurative myocarditis were seen. *E. rhusiopathiae* was isolated from samples of lung, liver, stomach content and skin in 1 case. The main gross lesions were characterized by well-circumscribed white areas in the skin of the neck, hindquarters and ocular region. Microscopic lesions were characterized by mild mononuclear perivasculitis associated with gram-positive rods. Abortions caused by *Corynebacterium* sp and *Actinobacillus suis* had no macroscopic lesions. Microscopically, the lesions were characterized by diffuse suppurative pneumonia in both cases.

PPV infection was diagnosed in 33 cases (27.2%). Characteristic pathological changes were seen in some maturely stillborn and mummified piglets with PPV infection. Mononuclear myocarditis and nephritis were the most prominent features observed associated with fetal mummification.

PCV-2 was identified in 7 cases (5.7%). In 3 of these 7 cases, co-infection with PPV was also confirmed by nested PCR. The most consistent gross lesions found in the PCV-2-infected fetuses were ventricular dilation, myocardial pale areas, and mesocolonic edema. Microscopically, multifocal mononuclear myocarditis was the main lesion observed.

Two cases (1.65%) with lesions of unknown etiology had nonsuppurative encephalitis and mild-to-severe suppurative pneumonia respectively.

Overall, **idiopathic abortion accounted for 61.1% of the cases,** which is similar to data from Switzerland (52%) and South Dakota (61.2%).

* Graduate student presentation

A case of bovine rabies without inflammatory reaction in the brain

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A 10-year-old cow exhibited signs of CNS disorder (rubbing shoulders and head on wire fence) was euthanized and submitted for necropsy. On gross examination the animal had moderately enlarged and yellow discolored liver and markedly distended gall bladder. Other significant gross lesions were not present in the brain or other internal organs.

On histologic examination of the brain, many magenta, up to 7 μm diameter, intracytoplasmic inclusion bodies were observed in neurons, especially in the cerebellum. The inclusions closely resembled **Negri bodies.** Degeneration of neurons was also observed; however, **there was no histological evidence of inflammation in any sections of central nervous system tissue.**

Rabies virus antigen was detected in the brain by direct fluorescent antibody examination.

Nasal acinar adenocarcinoma in a European brown bear (*Ursus arctos)*

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A captive 120 kg 19-year-old intact-female European brown bear (*Ursus arctos arctos)* was bred in a zoo in Lleida (Spain). The animal was anesthetized due to epistaxis and difficulty in breathing and swallowing. On physical examination, a 5-cm mass was observed in the soft palate. Three weeks later, rhinoscopy was performed and a large mass was observed in the left nostril; the mass was biopsied and diagnosed as a nasal adenocarcinoma. No surgical treatment could be performed due to the extent of the mass. The animal was euthanized 4 months later due to poor health status.

Twenty hours after being euthanized, computed tomography (CT) images of the bear were taken in the transverse plane at the level of the nasopharynx. CT images showed a large soft tissue mass (10x8x7 cm) that filled the nasopharyngeal, laryngeal and oral cavities. There was not extensive turbinate destruction or osteolysis.

At necropsy, a large, white, soft and multilobulated mass was observed growing from the left lateral nostril wall, with no apparent bone destruction, and markedly protruding into the caudal oral cavity.

Histopathology revealed a nodular, well-demarcated, densely cellular, expansive and mildly encapsulated neoplastic proliferation. Neoplastic cells were arranged in nests separated by fibrovascular septa of variable thickness. Cells were cylindrical, medium-sized, with indistinct borders. In a few cells, small cilia were observed in the apical border. They had a moderate amount of eosinophilic and homogeneous cytoplasm. The nuclei were central, oval, with finely stippled chromatin with a single and eosinophilic nucleolus. Anisocytosis and anisokaryosis were very marked, with karyomegaly in some cells; however the mitotic index was low (0-1/40X). In some focal areas, neoplastic cells invaded the thin connective capsule and partially affected the osseous wall. Extensive and multifocal areas of necrosis with cholesterol granulomas with multinucleated giant cells and hemorrhages were observed within the neoplastic proliferation. Moderate and scattered lymphoplasmacytic foci were also observed. Tumor cells were not observed in the regional lymph nodes.

These results were suggestive of a nasal acinar adenocarcinoma. Neoplasms are one of the most frequent causes of morbidity and mortality in captive bears. **To our knowledge, this is the first description of this tumor in bears in conjunction with a CT study**.

* Graduate student presentation

Burn scar squamous cell carcinoma (Marjolin ulcer) with osteosarcoma in a Boxer dog

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A 4-year-old Boxer was presented with a mass on the mid-dorsum at the site of a previous burn scar. The burn had occurred approximately 2 years previously. Excisional biopsy of the mass revealed an ulcerated squamous cell carcinoma. An unexpected finding was the presence of osteosarcoma within the dermis immediately adjacent to the squamous cell carcinoma. At the time of this report, no metastatic lesions have been detected.

Marjolin ulcer is a well-described syndrome of humans in which tumors arise at the site of a burn scar, often decades after the original insult. Squamous cell carcinoma is the most common type of tumor in this location, followed by basal cell carcinoma and malignant melanoma Very rarely, multiple tumor types may occur in the same site. In humans, burn scar squamous cell carcinomas have been reported to occur with melanoma and malignant fibrous histiocytoma. Scar-associated squamous cell carcinoma has been associated with branding in cattle and horses, burns in dogs, and laceration in a llama. However, to our knowledge, this is the first report of multiple tumor types in a burn scar in any domestic animal species.

The etiology of burn scar carcinoma is not well understood. Burn scars may be immunoprivileged and therefore not subject to immunosurveillance. Fas gene mutations, and loss of p53, both of which may lead to decreased apoptosis and increased tumor cell proliferation, have been reported in human cases of burn scar carcinoma. Although too few cases are reported in the veterinary literature to establish prognosis, these types of tumors tend to be more aggressive than their counterparts arising in non-scarred regions in humans.

Although burn scar carcinomas are very rare in dogs, lesions arising within scars up to several years after a burn are potentially malignant, and multiple tumor types may be present within the same lesion. Such lesions warrant immediate attention, diagnosis, and treatment.

Alimentary tract duplications in veterinary species

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Alimentary tract duplications (ATD) have been reported as rare congenital anomalies in humans and animals. In the human literature, they are divided into foregut, midgut and hindgut duplications and commonly occur in conjunction with anomalies of the neural system or genitourinary tract. ATD are often asymptomatic, but some can produce a mass effect leading to obstruction or rupture. Most ATD lie in close proximity to the wall of the viscus, have gastrointestinal-type mucosa, and are surrounded by 2 muscular layers. Communication with the gastrointestinal lumen is variable and the ATD may be tubular or cystic. The most common location for duplication in humans is the ileum, followed by the esophagus, stomach-duodenum, jejunum and colon. Similar duplications are reported in dogs, cats, horses, rats and poultry.

We present 1 case each of alimentary duplication in a cat, a horse and a llama.

- Clinically, the cat had a suspected vascular ring anomaly (VRA) at the base of the heart leading to megaesophagus and chronic regurgitation. The cat died from cardiac arrest following attempted corrective surgery.
- The horse had been clinically diagnosed with a ventral neck abscess, which after lancing resulted in a draining fistulous tract and severe myonecrosis. The horse was euthanized due to poor prognosis.
- The llama had a history of colic with possible obstruction. During surgery, a large mesenteric mass was discovered with intestinal perforation and the animal was euthanized.

In each case, at necropsy there was a non-communicating, cystic cavity located adjacent to the esophagus or colon.

Alimentary tract duplications, although rare, should be considered in the differential list for esophageal and colonic disorders.

* Graduate student presentation

High incidence of glomerulonephritis associated with inclusion body hepatitis in meat-type chickens: routine histopathology and histomorphometric studies

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During the routine histological evaluation of an outbreak of inclusion body hepatitis in Mississippi poultry, a high incidence of glomerulonephropathy was observed in the birds manifesting with classical hepatic pathology and unusual renal enlargement. Characteristic intranuclear adenoviral inclusion bodies were usually demonstrated in the livers of these birds. *Fowl adenovirus* was recovered from the livers of many of the groups that were also positive with PCR testing. The kidneys of severely affected birds were also different from most uninfected age-matched controls.

The glomerular lesions were consistent with proliferative or membranoproliferative forms of glomerulonephritis. While the glomerular lesions were dramatic in many of the kidneys, a spectrum of glomerular morphology was appreciated in the infected birds, which ranged from normal to severely affected. Histomorphometric evaluations were performed in order to generate a more quantitative analysis of alterations in glomerular size and cellularity, to detect statistically significant alterations in the borderline changes, and to get a clearer insight into the overall incidence of the glomerular pathological alterations.

In pilot studies, a marked increase in both the average size and total cellularity was observed for the affected glomeruli. For example, employing the ImageJ morphometric program, the average glomerular area values for normal glomeruli in the subcapsular cortical and central kidney regions were of 2 and 6 square image units respectively. However, glomerular measurements for kidneys exhibiting glomerulonephritis by routine histology demonstrated average values for the two regions of 8 and 18 sq. units. The average glomerular cell counts for the two regions in normal kidneys were 33 and 69 cells/glomeruli, whereas averages obtained for birds with glomerulonephritis were 100 and 220 cells/glomeruli. Relative to the normal kidneys, the average percent increase for all measured parameters in kidneys with glomerulonephritis was ~330%.

Granulomatous lesions in ferrets associated with group 1 coronavirus infection are not related to *Feline coronavirus*

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Introduction. Systemic granulomatous lesions in ferrets (*Mustela putorius furo*), similar to those observed in feline infectious peritonitis in cats, have been correlated recently to coronavirus infection. This hypothesis had been supported by the detection by immunohistochemistry of group I coronavirus antigen within these lesions. The objective of this work was to investigate, by molecular biology methods and immunohistochemistry, in 3 ferrets with granulomatous lesions, the possible presence of *Feline coronavirus* (FCoV) and other viruses belonging to Group I such as *Canine coronavirus* (CCV), *Transmissible gastroenteritis virus* (TGEV) and *Porcine respiratory coronavirus* (PRCV).

Material and methods. Three cases of ferrets suspected of suffering granulomatous lesions associated with coronavirus infection were followed. The animals were privately and separately owned in the city of Barcelona and aged 6 to 18 months. Clinical signs were non-specific and included enlarged lymph nodes, splenomegaly, anemia and hypergammaglobulinemia. Serology for *Aleutian mink disease virus* was negative in all 3 cases. The ferrets were anesthetized and biopsies from the mesenteric lymph nodes were taken from all 3 animals. Two portions of each biopsy were obtained and immediately immersed in formalin solution and RNA-later, respectively. Formalin-fixed tissues were routinely processed and stained with hematoxylin-eosin (HE). Immunohistochemistry for the identification of coronavirus group 1 antigen, using FCV3-70 as monoclonal antibody, was also performed in all three cases. RNA was purified from tissues in RNA-later solution using a commercially available kit; negative controls were concomitantly prepared with each extraction to monitor cross-contamination. RNA was tested by a 1-tube fluorogenic reverse transcription-polymerase chain reaction (RT-PCR) that detects all the strains of FCoV and also CCV, TGEV and PRCV. To monitor the quality of RNA extraction, each sample was tested with a commercially available 18S rRNA real-time RT-PCR assay. With each PCR run, 2 negative and 2 positive controls were amplified and analyzed.

Results. Histopathology of the lymph nodes revealed severe granulomatous lymphadenitis in all 3 cases. Four main types of coexisting lesions were observed: diffuse granulomatous inflammation on serosal surface, granulomas with areas of necrosis, granulomas without necrosis, and granulomas with neutrophils. Immunohistochemistry for the identification of coronavirus group 1 antigen was also positive in the 3 cases. The 1-tube fluorogenic RT-PCR was negative for the 4 analyzed viruses in all 3 cases.

Discussion. Granulomatous lesions in ferrets associated with group 1 coronavirus infection did not appear to be related to FCoV, CCV, TGEV, or PRCV infection. The quality of purified RNA was good as revealed by the 18S rRNA quantification. Hence, negative FCoV RT-PCR results cannot be explained by poor RNA quality. Further studies must be envisioned to investigate the role of other coronaviruses, mainly *Ferret enteric coronavirus* (FECV), as causative agents of this new disease.

* Graduate student presentation

The effect of route of inoculation on induction of mucosal disease in calves naturally persistently infected with *Bovine viral diarrhea virus*

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Introduction. Infection with *Bovine viral diarrhea virus* (BVDV), a *Pestivirus* in the family Flaviviridae, may result in a variety of outcomes, ranging from subclinical infection to fatal mucosal disease (MD). Severe-to-fatal MD can be induced in cattle persistently infected (PI) with noncytopathic (ncp) BVDV strains by superinfecting them with cytopathic (cp) BVDV strains. The objective of this study was to evaluate the route of inoculation on induction of MD in PI calves.

Material and methods. Two male Korean native PI calves (calves 1, 2), which had been infected with ncp BVDV-1 naturally, but were clinically normal, were superinfected experimentally with cp BVDV-1 (946 strain) intranasally (calf 1) or intravenously (calf 2). Both calves were moribund and euthanized at 30 and 38 post-inoculation day (PID), respectively. Necropsy was performed, followed by histopathology, immunochemistry (IHC), RT-PCR, and virus isolation.

Results. Serum neutralizing antibody to cp BVDV-1 was detected at PID 5 and PID 9 and developed a peak at PID 12 and PID 23 in calf 2 and calf 1, respectively. BVDV-1 was detected in feces, blood, nasal discharge, and saliva by RT-PCR in both calves.

Grossly, lesions were not prominent in calf 1. Histologically, small numbers of lymphoplasmacytic cells were infiltrated in mucosal layers of the abomasum and intestine, and the white pulp of spleen was atrophic. cp BVDV could not be isolated from samples from calf 1.

In calf 2, mild ulceration of oral mucosa, erosion of abomasum, and mild congestion and hemorrhage of small intestine were observed. There were more severe upper gastrointestinal lesions in calf 2 compared to calf 1, and these included deep ulceration and lymphocytic inflammation in the epithelium and submucosa of tongue, erosion in the abomasal mucosa, and submucosal hemorrhage and congestion in the small intestine. In addition, activation of lymphoid nodules in the spleen, giant cell infiltration and mild lymphoid depletion in lymph nodes were observed in calf 2. Although the biotype of BVDV could not be distinguished by IHC, viral antigen was detected in affected organs. Moreover, cp BVDV-1 was isolated from calf 2 from esophagus, abomasum, jejunum, lung, spleen, lymph nodes, kidney, adrenal gland, and thyroid gland.

Conclusions. The characteristic histologic lesions of MD were observed in PI calf 2 that had been infected intravenously with cp BVDV-1. Viral antigens were detected in affected organs by IHC, and cp BVDV-1 was isolated from organ/tissue samples of calf 2. **The selection of route of inoculation could be one of the important factors in induction of MD in PI calves.**

* Graduate student presentation

PCR evidence of co-infection of hamsters with *Clostridium piliforme* and enteropathogenic attaching and effacing *E. coli*

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Tyzzer's disease is an enterohepatic infection caused by *Clostridium piliforme*, an obligatory, intracellular, gram-variable, spore-forming, anaerobic, filamentous rod-shaped bacterium that is difficult to isolate in a cell-free medium. It has been documented in a wide variety of mammals, including rodents, lagomorphs, humans, and non-human primates.

Enteric lesions caused by attaching and effacing *E. coli* (AEEC) have been reported in calves, pigs, rabbits, and humans. Intestinal lesions caused by AEEC are termed 'attaching and effacing' because of their intimate attachment to the enterocyte and effacement of the microvillus border. A chromosomal gene, *eae*A, encodes the protein intimin, which is involved in AE activity.

Until now, a definitive diagnosis of Tyzzer's disease was based on clinical signs, post mortem findings, and histologic lesions. Recently, the use of a polymerase chain reaction assay has greatly enhanced the accuracy of detection of the causal agent leading to confirmatory diagnosis.

Our study included 13 hamsters presented to TVMDL–Amarillo from August 1999 to April 2007 that were screened for the presence of *C. piliforme* by PCR. The primary target organ of *C. piliforme* is intestine, and all of the animals had a clinical history of severe diarrhea and depression followed by death. Because bacteriology results on all of them revealed enteropathogenic *E. coli* as a potential pathogen, we decided to pursue additional screening for *E. coli* typing for virulence-associated genes by PCR.

Available samples from fresh liver and intestine, formalin-fixed tissues, and fresh feces were subjected to PCR amplification by 3 primer sets specific for *C. piliforme* and 6 primer sets specific for *E. coli.* Interestingly, a primer pair targeting a 270 base pair product on the 16S r RNA gene of C. *piliforme,* and a primer pair targeting a 425 base pair region on the *eae*A chromosomal gene of enteropathogenic *E. coli* were consistently amplified in 3 of 7 hamsters confirmed positive for *C. piliforme* infection by histopathology. Also, the *eae*A gene was detected in all of the samples collected from fresh feces from these 3 hamsters. Hence, our PCR results were consistent with the histological findings.

Although, *eae*A infection has been reported in rabbits, **dual infection of hamsters with these 2 pathogens has not been reported previously.** Primary infection with enteropathogenic *E. coli* may increase the susceptibility of hamsters to subsequent *C. piliforme* infection and also augment the enteric lesions in synergy.

The role of *Mycoplasma ovipneumoniae* in respiratory disease of bighorn sheep (*Ovis canadensis)*

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Pasteurellosis in Rocky Mountain bighorn sheep in the Hells Canyon region is characterized by high morbidity/mortality pneumonia in lambs during the summer and sporadic pneumonia in adult sheep in fall or winter. *Pasteurella* and/or *Mannheimia* sp. are typically isolated from the lungs of affected animals, although the predominant isolate may vary among affected animals both within and between outbreaks. As pasteurellosis may frequently be secondary to infections or stresses that impair pulmonary bacterial clearance, the goal of this study was to seek potential primary bacterial infections preceding pasteurellosis in bighorn sheep.

In the summer of 2006, 7 lambs with signs of respiratory disease were collected from 3 Hells Canyon populations for necropsy and collection of diagnostic specimens. Methods applied included non-culture based (16S library) pulmonary bacterial population assessment, bacterial species-specific PCR tests, and serology. DNA sequence analysis of 16S clone libraries prepared from pneumonic lungs demonstrated the consistent presence of *Mycoplasma ovipneumoniae* in the lambs with least advanced pneumonia. The agent was subsequently confirmed in all the study lambs and in a proportion of adult bronchopneumonia cases. Bacterial culture confirmed the presence of *M. ovipneumoniae* in 3 of the lambs. Serology (indirect hemagglutination) demonstrated a strong association between exposure to *M. ovipneumoniae* and the occurrence of respiratory disease in bighorn sheep herds at several sites in the western US and Canada, whereas herds without observed respiratory disease were seronegative.

***Mycoplasma ovipneumoniae* is a candidate primary infectious agent that may be associated with both outbreaks of pneumonia in bighorn sheep lambs and sporadic pneumonia in adult bighorn sheep**, presumably by impairing bronchociliary clearance and thereby predisposing infected animals to *Mannheimia haemolytica* and other secondary bacterial infections.

Improved specificity for detection of *Mycobacterium bovis* in fresh tissue using IS6110 real-time PCR

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Introduction. Currently accepted methods of detecting *Mycobacterium bovis* (*M. bovis*) in fresh tissues requires months for definitive culture results. It has been proposed that direct detection of *M. bovis* DNA in fresh tissues could provide more rapid results. These data would complement the current method of PCR detection of *M. bovis* in fixed tissues. One of the most significant impediments to the development of this technique is differentiation of *M. bovis* from environmental mycobacteria. PCR primers that amplify regions of the IS6110 region of the genome have been developed to differentiate members of the *Mycobacterium tuberculosis* complex from environmental mycobacteria. Currently, primers developed by Eisenach KD, et al. (J Inf Dis 1990;161:977-981) named IS6110 are the most widely used in veterinary diagnostic applications. Another primer pair that, in preliminary experiments, was more sensitive in our hands was IS41/43 developed by Plikaytis BB, et al. (Mol Cell Probes 1990;5:215-219). To evaluate specificity of these primer pairs, mycobacterial reference strains were obtained from ATCC.

Materials and methods. DNA was isolated from the following mycobacteria; *M. smegmatis*, *M. terrae*, *M. goodii*, *M. fortuitum*, *M. kansasii*, *M. avium* ssp. *paratuberculosis*, *M. wolinskyi*, *M. simiae*, *M. peregrinum*, *M. intracellulare*, *M. chelonae*, *M. avium* and *M. bovis*. Previously published primers IS41/43 and IS6110 were assayed for detection. PCR positive samples were sequenced using standard techniques. New primers and a TaqMan probe were designed based on sequences obtained from sequencing this region of each genome using Primer3 v0.3 (frodo.wi.mit.edu/primer3/). Real-time PCR was carried out using TaqMan Master Mix (ABI, Foster City, CA) according the manufacturers directions with 0.4 μM of each primer (IS6110_T) and 0.2 μM probe (IS6110_T_probe). To test these new primers and probe, tissue samples from 48 cattle from a naturally infected herd were tested for *M. bovis* by real-time PCR.

Results. The IS41/43 primer pair produced positive results with the following mycobacteria; *M. terrae*, *M. goodii*, *M. fortuitum*, *M. wolinskyi* and *M. simiae*. The IS6110 primer pair generated positive PCR results from *M. terrae*, *M. goodii*, *M. wolinskyi*, *M. peregrinum* and *M. chelonae*. The IS6110 region of these genomes was sequenced and real-time primers and probe developed. Using the newly designed primers in a real-time PCR format, these primers improved specificity by only detecting *M. bovis*, *M. bovis* BCG and *M. wolinskyi*. Based on sequencing data, *M. wolinskyi*'s PCR product was 100% identical to that of *M. bovis*.

To test the ability of the improved PCR primers and probe, tissue samples from a naturally infected herd were tested for *M. bovis* infection. Of these samples, 30 were *M. bovis* culture positive and 18 were *M. bovis* culture negative. We detected 20 of the 30 culture positive samples. None of the *M. bovis* culture negative samples were PCR positive using this assay. *M. smegmatis* was cultured from several of the *M. bovis* culture negative samples. These samples were PCR negative in this assay.

Conclusion. Using this real-time assay, fewer false positives occur when assaying tissues from animals infected with mycobacteria other than *M. bovis*. Inclusion of this assay in the diagnostic scheme would provide rapid evidence of *M. bovis* infection in suspect animals.

Antibiotic control of gram-negative contaminants in BD BACTEC™ MGIT™ 960 Para TB System liquid cultures of *M. avium* subsp. *paratuberculosis*

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Introduction. Decontamination during fecal processing and antimicrobials in culture media are relied upon to prevent the overgrowth of contaminant organisms during *Mycobacterium avium* subsp. *paratuberculosis* (MAP) culture for the diagnosis of Johne's disease in cattle. Recently, 2 gram-negative organisms were found to survive decontamination, and to grow despite antimicrobials in the medium. Culture medium supplementation with nalidixic acid (NAL) well above the standard 18-19 µg/mL is generally effective against problematic contaminants, but not uniformly so with these organisms. This study was undertaken to identify the organisms in question and determine which antimicrobials will suppress their growth in liquid culture with minimal effect on MAP recovery.

Materials and methods. *Caulobacter crescentus* was identified as the gram-negative environmental contaminant from a single mid-western cattle herd by traditional biochemical tests and rDNA sequencing. *Serratia marcescens* was isolated from the 2007 Johne's Fecal Check Test. Ceftriaxone (CTR) and tobramycin (TOB) were selected for testing based on preliminary susceptibility studies. Seven MAP-positive fecal samples from the 2007 Check Test were processed in accordance with BD BACTEC™ MGIT™ 960 Para TB System protocols and inoculated in triplicate into liquid culture medium with the standard amphotericin B and vancomycin concentrations and 1) the standard 18-19 µg/mL NAL, 2) 100 µg/mL NAL, 3) 200 µg/mL NAL, 4) 50 µg/mL NAL + 8 µg/mL CTR, or standard NAL with 5) 16 µg/mL TOB, 6) 8 µg/mL CTR, or 7) 8 µg/mL CTR + 16 µg/mL TOB. Additionally, triplicate cultures of these conditions were inoculated with 10-fold dilutions of 2 of the processed feces, others were seeded with 90-100 cfu of MAP ATCC 19698 or 1,000-10,000 cfu per culture tube of *B. cereus* endospores, *C. crescentus*, or *S. marcescens*; uninoculated cultures were also prepared. MAP detection by the MGIT™ 960 instrument was supported by acid-fast (AFB) stain and mycobactin J dependency results.

Results. Two sets of cultures inoculated with processed feces, a very low shedder (no colonies on solid medium) and 1 of the 10-fold inoculum dilutions, had less than 25% total MAP detections in liquid culture and so were unsuitable for quantitative analyses. The remaining 5 sets of cultures from processed fecals had a total MAP detection rate of 80%. Their results for MAP detection (% P), contaminated cultures (% C), negative cultures (% N), and mean time to detection (TTD) ± standard deviation in days (d) were as follows: 1) 57% P, 43% C, 17.88 ± 3.64d with 18-19 µg/mL NAL, 2) 100% P, 21.43 ± 6.47d with 100 µg/mL NAL, 3) 86% P, 14% N, 20.68 ± 2.61d with 200 µg/mL NAL, 4) 90% P, 5% C, 5% N, 23.35 ± 7.33d with 50 µg/mL NAL + 8 µg/mL CTR, 5) 57% P, 43% C, 26.06 ± 7.89d with 16 µg/mL TOB, 6) 86% P, 9% C, 5% N, 26.34 ± 9.27d with 8 µg/mL CTR, and 7) 86% P, 14% N, 27.15 ± 9.82d with 8 µg/mL CTR + 16 µg/mL TOB. AFB staining of terminal negatives revealed a false negative rate of 3%. All cultures seeded with MAP ATCC 19698 were detected in less than 20 days. *B. cereus* endospores were well-controlled, with growth in only 2 of 21 seeded culture tubes. All seeded *C. crescentus* cultures without TOB had 100% breakthrough in less than 3 days, but no growth if TOB was present. Conversely, all seeded *S. marcescens* cultures without CTR had 100% breakthrough in less than 24 hours, but no growth if CTR was present. No uninoculated tubes were flagged positive by the instrument.

Conclusions. The 100 µg/mL NAL condition yielded the highest MAP detection rate from processed feces (100%), followed by 50 µg/mL NAL + 8 µg/mL CTR (90%), then the 200 µg/mL NAL, 8 µg/mL CTR, and 8 µg/mL CTR + 16 µg/mL TOB conditions (86% each). **While effective against *C. crescentus*, TOB worked poorly against *S. marcescens* and other fecal contaminants. CTR was effective against *S. marcescens* and other fecal contaminants, but not *C. crescentus*, which has been reported to express a chromosomally-encoded metallo-beta-lactamase ortholog.**

High-throughput nucleic acid isolation and detection of Johne's disease in cattle

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Mycobacterium avium subsp. *paratuberculosis* (MAP) is the causative agent of Johne's disease, a contagious bacterial infection of the intestinal tract of ruminants. Johne's disease can result in severe economic losses for dairy and beef industries.

Current detection methods involve culturing MAP from fecal samples or from blood. Culture methods are time consuming (requiring up to 16 weeks), laborious, and costly. To expedite MAP detection, we have developed a rapid, high-throughput sample preparation and nucleic acid purification method using a high-throughput bead-beating HT Homogenizer, the Ambion® MagMAX™ magnetic bead technology, and a highly sensitive quantitative PCR (qPCR) assay. These tools provide a system for the purification and identification of MAP DNA. The fast, simple procedure isolates MAP nucleic acid from difficult sample matrices such as feces, milk, and blood, in approximately 1 hour. MagMAX technology effectively removes PCR inhibitors commonly carried over by other nucleic acid isolation methods, and is easily adaptable for high-throughput processing using commonly available automation platforms such as the KingFisher® Magnetic Bead Processor. The purified nucleic acid can be used directly in the Ambion AgPath-ID™ MAP Reagent Kit, a highly sensitive qPCR assay targeting a unique MAP sequence.

In model experiments, >50% of spiked MAP-like organisms were recovered from fecal, milk, and blood samples (25 µL of cells were spiked into 150 µL of matrix per sample). *M. smegmatis* cells were used because of their similarity to MAP. Using this coupled nucleic acid isolation and qPCR assay on an automated platform, MAP was successfully detected in fecal samples obtained from low-, medium-, and high-shedder naturally infected cattle (detection sensitivity was down to 2 cells/µL)

These results demonstrate a highly effective process for rapid, automated MAP detection.

Peri-parturient bacterial shedding and ELISA testing of a Johne's disease "super-shedder" cow

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An adult Jersey cow from one of the Johne's disease demonstration herds was classified as a super-shedder cow based on fecal cultures. The animal was followed from February 27 to June 22, 2006, when she was euthanized for humane reasons. During this time, fecal samples and serum was collected every other week up until calving on June 5 and then milk samples were collected daily for 1 week, and feces, serum and milk for nearly 2 weeks.

Fecal samples were tested for the presence of *Mycobacterium avium ssp. paratuberculosis* by real-time PCR (Tetracore) and culture (TREK para-JEM), serum samples were tested by ELISA (Biocor and IDEXX), and milk samples tested by ELISA (Antel Bio) and PCR (AnDiaTec). Fecal testing determined that the animal continued to shed 2-5 million cfu/g of feces. ELISA testing was positive for the first 2 weeks and then dropped rapidly to give results well within the negative category around calving and then rose slightly but remained negative. Milk testing was ongoing at the time this abstract was prepared.

The fecal PCR and ELISA testing confirm 2 principles of Johne's disease testing:

1. The immunocompromising effects associated with parturition and/or the end-stage disease state can result in negative serological test results in spite of huge numbers of organisms being shed in the feces.
2. Even in the dry period, 2 months prior to calving, super-shedder cows may be test negative by ELISA.

The test results from this single cow reinforce the fact that the ultimate goal of Johne's disease testing needs to be considered before selecting a method, and that, in some cases such as valuable animals or where the consequences of false-negative results are high, multiple test methods may be warranted.

Species identifications of *Exophiala* from investigations of phaeohyphomycosis in aquaria

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Introduction. Phaeohyphomycosis is a collective term for cutaneous, subcutaneous and systemic infections caused by dark-walled, i.e., melanized or phaeoid, fungi that form filamentous septate hyphae with or without yeasts in tissues. During the period from 2002 to 2007, infections by melanized fungi were identified with greater frequency in a variety of exhibit fish from commercial aquaria, particularly seadragons (*Phyllopteryx taeniolatus* and *Phycodurus eques*) and lumpfish (*Cyclopterus lumpus*), which were submitted to the Connecticut Veterinary Medical Diagnostic Laboratory (CVMDL, University of Connecticut, Storrs, CT). In conjunction with several of these aquaria, a concerted effort was made to characterize the agents associated with pheohyphomycotic lesions in these commercially relevant species.

Materials and methods. Samples from 18 seadragons and 4 lumpfish were received from 3 institutions and included fresh or frozen and formalin-fixed tissues from necropsy and biopsy specimens. Formalin-fixed tissues were processed routinely for histologic examination, sectioned at 4 µm, stained using hematoxylin and eosin, Grocott's methenamine silver, and Fontana-Masson techniques. Fresh and frozen tissues were cultured for fungi on Sabouraud's agar only or both Sabouraud's and inhibitory mold agar with gentamicin at 30ºC at the CVMDL, and isolates were transferred to the Fungus Testing Laboratory (University of Texas Health Science Center, San Antonio, TX) for morphologic identification and sequence analysis of the ITS and D1/D2 domains. Sequences were forwarded to the Centraalbureau voor Schimmelcultures (Utrecht, The Netherlands) for genetic comparison and phylogenetic analysis.

Results. In seadragons, lesions consisted of parenchymal and vascular necrosis with fungal invasion of gill, kidney, and other coelomic viscera with or without cutaneous ulceration, whereas, in lumpfish, cutaneous ulcers were a consistent finding. Fungal cultures yielded velvety, olivaceous or brown molds. **Isolates from seadragons were identified as a novel *Exophiala* species based on nucleotide sequence comparisons and phylogenetic analyses**, in which this novel species appeared as a sister taxon to species isolated from aquatic sources and poikilothermic animals. **Isolates from lumpfish were determined by morphologic and molecular analyses to be *Exophiala angulospora*.**

Discussion. Identification of a novel species of *Exophiala* from lesions in seadragons extends our collective knowledge of pathogenic *Exophiala* species and presents challenges for management and conservation of these threatened and commercially important exhibit animals. Identification of the ubiquitous saprophyte *Exophiala angulospora* from lesions in lumpfish is a novel finding that raises concern regarding the pathogenic potential of this species, particularly in aquatic environments, in that this species has heretofore not been associated with pathology in a vertebrate host.

Conclusion. Species identification of *Exophiala* can provide insights into relationships between these melanized fungi, their hosts and their environments that can be significant to our understanding of their pathogenic potential.

* Graduate student presentation

Unusual sources and isolates of *Salmonella* spp

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Salmonella spp are common bacterial pathogens associated with mammals, poultry and reptiles. Such isolates as *Salmonella enterica* serotype Typhimurium and *S.* Agona (both serogroup B) are commonly found in cattle and equine patients. Recently, *Salmonella* Javiana (serogroup D_1) was recovered from an equine fecal sample, and *S.* Dublin (serogroup D_1) was found in swine tissue.

Salmonella spp can be found in clinical materials when least expected. *S.* Typhimurium has been reported in abscess material from a horse (1). Recently, *Salmonella* Muenchen (serogroup C_2) was recovered from a dog with rhinitis. *Salmonella* Give (serogroup E) was recovered from a feline urine sample that produced a CFU of greater than 100,000/mL. *S*. Enteritidis (serogroup D_1) was recovered from the feces of a cat with diarrhea.

Salmonella spp are usually recovered from clinical materials with the use of tetrathionate enrichment broth in conjunction with the use of selective and differential media such as brilliant green (BG), MacConkey (MAC) and xylose-lysine-desoxycholate (XLD) agar. However, in the case of finding *Salmonella* from a case of rhinitis, blood agar (BA), MAC and thioglycollate enrichment broth were used as *Salmonella* was not expected. In like manner, the case of feline cystitis was subjected to only BA and MAC as *Salmonella* was not expected in this case either.

The media were inoculated, incubated at 35ºC for 18 hours and the plates read. Lactose and non-lactose fermenting colonies were identified with the Trek-Sensititre AP-80® Gram Negative Identification System (932 Keynote Circle, Suite 6, Cleveland, OH 44131). *Salmonella* isolates were serogrouped and forwarded to USDA, APHIS, NVSL, VS, AMES, IA for speciation.

In summary, *Salmonella* Muenchen, *S*. Give, *S*. Dublin, *S*. Enteritidis, *S*. Typhimurium, and *S*. Javiana have been recovered from clinical materials when they were not expected.

Reference

1. Blikslager AT, et al. Salmonella typhimurium abscess as a postoperative complication in a horse with colic. J Am Vet Med Assoc. 1991;199:1757-1759.

Validation of a 1.5 hour TSE assay: an ultra-short microtiter-based EIA

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The IDEXX HerdChek™ Bovine Spongiform Encephalopathy (BSE) and IDEXX HerdChek™ Bovine Spongiform Encephalopathy (BSE-Scrapie) Antigen Test Kits are antigen-capture enzyme immunoassays (EIAs) for the detection of the abnormal conformer of PrP^{SC} in postmortem tissues from cattle affected by BSE and small ruminants affected by scrapie. The IDEXX BSE & BSE-Scrapie Test Kits currently have a "short" assay protocol approved in which the EIA portion of the assay is completed in approximately 2.2 h. For laboratories requiring an even faster turnaround time, IDEXX has validated an even shorter assay protocol in which the EIA portion of the test is completed in 1.5 h. This new ultra-short assay protocol utilizes increased temperature (32-37^{0}C) during all assay incubation steps and a slow-speed plate shaker during the sample incubation step to accelerate PrP^{SC} capture from the viscous brain homogenate sample. The enhanced binding kinetics provided by the elevated temperature and plate shaker allow for a significant reduction in the sample incubation and conjugate incubation times as compared to the approved original "standard" protocol (4 h) and the "short" (2.2 h) protocols.

In a study of 2,100 negative bovine brain samples collected in Europe and 2,008 small ruminant samples collected in the US and Europe, the ultra-short assay protocol performed with 100% specificity and compared extremely well with the original standard (4 h) protocol. The population mean OD to cutoff ratio was >15 standard deviations from the test cutoff for the negative bovine samples and >22 standard deviations from the test cutoff for the negative small ruminant samples. Studies conducted on negative bovine and ovine fallen stock samples (n = 208) showed similar results with OD to cutoff ratios of >12 standard deviations from cutoff and 100% specificity.

Diagnostic and analytical sensitivity for the ultra-short assay protocol match those of the original standard assay protocol. For evaluation of diagnostic sensitivity, 20 BSE-positive bovine brain samples and 20 scrapie-positive ovine samples were tested in quadruplicate on the ultra-short and standard protocols, resulting in a diagnostic sensitivity of 100% on both methods. In addition, the OD relative to cutoff values for the positive samples strongly correlated between the two assay protocols with R^2 values of > 0.97 for bovine samples and >0.99 for ovine samples. For evaluation of analytical sensitivity, 20 BSE-positive bovine brain samples and 20 scrapie-positive ovine samples were serially diluted out to >1:1000 and tested on the ultra-short and standard assay protocols. In all cases, the limit of detection for these samples was the same on both assay protocols.

The IDEXX HerdChek™ ultra-short assay protocol delivers the same high level of sensitivity and specificity as seen with the standard and short assay protocols.

A new sensitive assay for the detection of ovine and caprine PrP^{res} TeSeE™ Sheep/Goat

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Scrapie is a slow degenerative disease of the central nervous system of sheep and goats induced by an abnormal prion. A new rapid assay has been developed by Bio-Rad Laboratories for the post-mortem diagnosis of TSEs in small ruminants.

This new assay is based on the same procedure as the current TSE kit with a combination of monoclonal antibodies selected for their high affinity and specificity to sheep and goat PrP^{sc}. The assay involves purification and concentration of PrP^{sc} followed by detection by an immunoenzymatic technique (sandwich format). Internal and external evaluations conducted in nervous tissues (obex, brainstem, spinal cord) and in peripheral tissues (lymph node, tonsil, spleen, ileum) from naturally or experimentally infected sheep and goats clearly demonstrate the very high sensitivity of this new test. In an analytical sensitivity dilution series experiment on various genotypes of sheep and goats, the new assay was able to detect, in most cases, a 1/2,500 dilution of positive tissue. Tissue was standardized in homogenized tissue that tested negative for TSEs. Specificity studies conducted on the semi-automated platform with 838 fresh samples of nervous or peripheral tissues collected from slaughterhouses show that the assay is well adapted for screening large numbers of samples. The semi-automated system allows the user to process 800-1,000 samples per day with full sample traceability. Twenty-eight autolysed samples were tested and the performance of the assay was not affected when tissue was held at 25°C for up to 9 days.

The TeSeE™ Sheep/Goat assay represents a promising tool for determination of the prevalence of scrapie in surveillance and routine testing programs.

Oral fluid sampling as an alternative to serum for population-based diagnostics for PCV-2

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Porcine circovirus 2 (PCV-2) is an important causative agent in many clinical diseases/syndrome that are now referred to as "PCV-associated diseases (PCVAD)". Recently, a severe form of PCVAD emerged and caused significant economic losses to the swine industry in North America. In response, commercial PCV-2 vaccines in various forms have been made available to swine practitioners and producers. Furthermore, many swine operations have launched a monitoring program for early detection of PCV-2 infection. Traditionally, monitoring of viral infections has been based on serology. As serum sampling is rather expensive and laborious, it often hampers large-scale surveillance for pathogens. Recently, our laboratory observed that oral fluid samples may be used to detect PRRSV and anti-PRRSV antibody. Therefore, a field-based longitudinal cohort study was conducted to assess the utility of pen-based oral fluid samples as an alternative to serum for herd PCV-2 testing.

For the study, pens of approximately 25 pigs each were monitored over time for PCV-2 infection using oral fluid and serum samples. Six pens at each of 3 sites were selected for surveillance. Samples were collected when pigs entered the facilities at 3 weeks of age, and subsequently at 5, 8, 12, and 16 weeks of age. All samples were tested for the virus by a real-time PCR and PCV-2 antibodies (IgM, IgA and/or IgG) by IFA and 3 different ELISAs (SERELISA®, ISUVDL indirect ELISA, INGEZIM® ELISA). Sequencing was also conducted to further characterize the virus circulating in each site.

The genome of PCV-2 was detected in oral fluids sporadically until 8 weeks of age, and all pens in each site were positive for PCV-2 DNA at 16 weeks of age. When serum samples were tested, no viremic pigs were detected until 8 weeks of age except for 1 pen at 5 weeks of age, but PCV-2 infection took place in all pens by 16 weeks of age. All of the viruses circulating in all 3 sites were determined by sequencing as typical North American type PCV-2 (a.k.a. PCV-2A). **A relatively good correlation in viral loads between oral fluid and serum was observed (R^2=0.61).**

Most of the pens in all 3 sites were positive for PCV-2 antibody until 5 to 8 weeks of age by various serologic assays (i.e. IFA and ELISAs) performed on both serum and oral fluid, although a lesser number of positive pens were detected using oral fluid samples. Similarly, most of the pens in all of the sites became positive for anti-PCV-2 antibody by testing serum at 16 weeks of age, whereas all of the pens in site 1 and 1 pen in site 2 became positive for PCV-2-specific antibody at the same time when oral fluid was tested. IgA antibody also was detected in the oral fluid samples collected from all of the pens at 16 weeks of age which were also positive for IgG, although neither IgA nor IgM was detected by IFA in serum. **Serology results on oral fluid samples demonstrated some degree of correlation with results on pooled serum samples (R^2=0.29).**

Collectively, the results suggest that oral fluid samples can be an alternative to serum for PCV-2 diagnostics and provide a cost-effective way to monitor virus activity or immune status in a population.

Evaluation of different PCR assays for detection of *Porcine circovirus 2* DNA in semen samples and development of an internal control

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A high mortality syndrome in growing pigs has spread across North America in the last 2 years. Recent reports indicate a similar disease may be occurring in China. Affected farms typically report severe respiratory disease and wasting in connection with a high increase in morbidity and mortality in 10-26 week old pigs. It is generally accepted that *Porcine circovirus 2* (PCV-2) is associated with the syndrome in some way. It remains to be determined if PCV-2 strain virulence differences can fully explain these outbreaks. Little is known about PCV-2 transmission; however, it has been speculated that the widespread use of artificial insemination could have helped disseminate the virus quickly. Nested PCR assays for PCV-2 on semen have been described in the literature and are used to some extent in North American veterinary diagnostic laboratories. In general, PCR on semen is cumbersome due to the presence of inhibitory substances that may interfere with the extraction and PCR process. The objectives of this study were to compare a published nested PCR to a quantitative real-time PCR and to develop an internal control for PCV-2 semen samples.

Known PCV-2 negative and positive semen samples from experimentally infected boars and field samples of unknown status were used to compare published nested PCR assays with a quantitative real-time PCR assay. A total of 208 semen samples from 10 Midwest boar studs were tested. Twenty of the 208 (9.6%) were found to be positive by real-time PCR and 6 of 208 (2.9%) samples were found to be positive by nested PCR. Only 1 sample was positive by all 3 PCR assays. In addition, 97 boar serum samples that were tested by ELISA were positive for antibodies to PCV-2, indicating exposure to PCV-2. PCV-2-DNA was detected in individual boars in 9 of 10 boar studs. **Preliminary results from evaluation and comparison of the 2 PCR assays on semen samples from experimentally PCV-2-infected boars indicate that the real-time PCR assay is more sensitive.**

Additionally, an internal control for PCV-2 was developed. The internal control consisted of PCV-2 DNA in which the probe sequence was altered (mutant PCV-2) so that it was not recognized by the probe used for detection of PCV-2 DNA. The same amount of mutant PCV-2 was added to each sample, and DNA extraction and real-time PCR were performed as usual. The PCR assay was done in the form of a multiplex PCR with 2 different probes: one was specific for the mutant PCV-2 DNA and the other probe was specific for wild-type PCV-2 DNA. Each sample was expected to test positive for the mutant PCV-2-DNA. Samples that were positive for wild-type PCV-2 DNA yielded a second positive signal (specific for wild-type PCV-2 DNA). **Preliminary results indicate that the assay performs well on PCV-2 negative and positive semen samples from experimentally infected boars.**

PCV-2 quantitative PCR (qPCR) test - correlation to IHC and mortality

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Introduction and objectives. A *Porcine circovirus 2* (PCV-2) quantitative PCR (qPCR) testing project was designed to better understand the role of PCV-2 infection dynamics in disease-associated periods of high mortality in commercial swine populations. Studies have shown that clinically affected pigs had significantly higher amounts of virus, usually at least 1.0 x 10^6 genomes per mL in tissues (1) and 1.0 x 10^7 PCV2 genomes per mL of serum (2). Therefore, quantitation of PCV-2 in serum may help in the ante-mortem diagnosis of PCVAD. We examined the correlation between tissue immunohistochemistry (IHC), qPCR, and peak mortality by analyzing samples from necropsied pigs using both IHC and qPCR.

Materials and methods. Fifty nine farms were sampled across the United States. In each case, 5 pigs were humanely euthanized for sampling at: 5-6 weeks prior to the age of historical peak clinical disease and mortality, 3-4 weeks prior, and at the peak. This sampling scheme was chosen to mimic previous challenge models. Tissue and serum samples were collected from each pig. Tissues were analyzed by IHC testing at the Iowa State University Veterinary Diagnostic Lab. The qPCR testing of serum was performed at the BIVI HMC Diagnostic Lab per a published method (2). A TaqMan-based, real-time PCR was used that included a standard curve created from serial dilutions of a plasmid encoding the open reading frame 2 (ORF2) of PCV-2 to reveal the viral load in serum samples.

Results. At 5-6 weeks pre-peak mortality, 68% of the pigs had low PCV-2 viral loads (≤log 4 to 5), whereas 72% of the pigs sampled at peak mortality had high viral loads (≥log 6-9; Table 1). The same pattern was observed with IHC testing (Table 2); 73% of the animals at the age of peak mortality tested positive for PCV-2, whereas 70% of animals at 5-6 wks pre-peak mortality were IHC negative.

Table1. Viral load values obtained by qPCR

Age group	Negative* (<log 4)	Log 4-5	Log 6-7	Log 8-9	Log 10-11	Number tested
5-6 wks pre-peak mortality	**34%**	**34%**	21%	9%	1%	233
3-4 wks pre-peak mortality	16%	25%	30%	32%	5%	240
Peak mortality	3%	19%	**50%**	**22%**	5%	268

Chi-square = 114.2270, 6 DF, $p < 0.0001$; Likelihood ratio chi-square = 117.9210, 6 DF, $p < 0.0001$

* "Negative" mean either virus was present below the lower detection limit of the test (10^4 or lower) or was absent altogether.

Table 2. Percent of positive animals by age group, according to IHC results

Age group	IHC negative	IHC positive	Number tested
5-6 wks pre-peak mortality	**70%**	30%	232
3-4 wks pre-peak mortality	33%	67%	239
Peak mortality	27%	**73%**	267

Conclusions. Virus load measured by qPCR correlated with peak mortality and gave us a good understanding of the PCV-2 dynamics under field conditions in the production line. Results for qPCR were also consistent with the findings from IHC testing.

References:

1. Krakowka S, et al. J Vet Diagn Invest 2005;17:213-222.
2. Brunborg IM, et al. J Virol Methods 2004;122:171-179.

PCV-2 farm infection dynamics based on cross-sectional sampling using a quantitative PCR (qPCR) test

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Introduction and objectives. *Porcine circovirus 2* (PCV-2) has emerged as a major contributor to disease and mortality in swine. Diagnosis of PCV-2-associated disease (PCVAD) on a herd basis relies on the best estimate of the contribution of PCV-2 (within the context of the contributions by other endemic and epidemic agents) to overall clinical morbidity and mortality in the herd. Herd diagnosis becomes important because of the range of interventions and economic resources that can be applied. One of the main objectives of this project was to better define the role of PCV-2 and understand the dynamics of PCV-2 viremia in clinical situations.

Materials and methods. The diagnostic protocol consisted of either cross-sectional or longitudinal serum sampling, and targeted post-mortem examinations. Serum was collected from the breeding herd (20 animals) and growing herd (50 animals, 10 per age group at 3-7, 8-12, 12-16, 16-20, and 20-24 wks of age), with sample size based on detecting at least 1 positive sample when the estimated prevalence of disease is at least 10%. Serum samples were tested by a quantitative PCR method to determine the load of PCV-2, in addition to being tested by serological assays to identify the presence of different PCV-2 infectious cofactors (data not shown). DNA was extracted and PCR was performed per a published method (1). Serial dilutions of a plasmid standard were included in every run to create a standard curve that allowed determination of the amount of PCV-2 present in each sample in viral genomic equivalents/mL.

Results. The breeding herd (gilts and adult sows) remained negative (Table 1). High-level viremia (≥log 6-9) started mainly around 8 to 12 wks of age through 16 to 20 wks of age within the systems sampled.

Table 1. Distribution of viral load from cross-sectional samples using qPCR in serum

Stage	Negative* (<log 4)	Log 4-5	Log 6-7	Log 8-9	Log 10-11	Number tested
Sows P0	**93%**	7%	0%	0%	0%	164
Sows P1	**98%**	2%	0%	0%	0%	186
3 – 7 wks	88%	11%	1%	0%	0%	171
8 – 12 wks	52%	**21%**	**24%**	**3%**	0%	139
12 – 16 wks	25%	**41%**	**28%**	**5%**	0%	151
16 – 20 wks	28%	**49%**	**21%**	**1%**	1%	149
20 – 24 wks	52%	42%	6%	0%	0%	139

* "Negative" mean either virus was present below the lower detection limit of the test (10^4 or lower) or was absent altogether.

Conclusions. PCV-2 is clearly present in animals close to the age of peak mortality (from 8 - 20 wks of age). The sow herd remained largely negative* with only a few highly viremic animals. **Viral load values obtained by qPCR illustrated the PCV-2 virus infection dynamics within PCVAD-affected herds.**

Reference:

1. Brunborg IM, et al. J Virol Methods 2004;122:171-179.

Shedding of *Porcine circovirus 2* by boars and the role of semen in PCV-2 transmission

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PCV-2 associated disease (PCVAD) is still on the rise in North America. In previous studies, it has been determined that PCV-2 is present in semen, and detection of PCV-2 in semen has been accomplished using nested PCR techniques and virus isolation. The quantity of PCV-2 DNA shed in semen is generally low, and little is known about shedding and transmission of PCV-2 via semen. The objectives of this study were 1) to determine if there are differences in semen shedding of genetically-distinct PCV-2 isolates (PCV-2a and PCV-2b), and 2) to determine if PCR positive semen is infectious.

Seventeen 12-15-day-old segregated early-weaned purebred Landrace boars were raised at Iowa State University. At approximately 7 months of age, the boars were randomly allocated to 3 different groups and trained to mount a collection dummy. Three boars served as negative controls, 7 boars were inoculated with a PCV-2b (European-like) infectious DNA clone, and 7 boars were inoculated with a PCV-2a (North American–like) infectious DNA clone. Semen and serum samples were collected prior to PCV-2 inoculation and 20 times after inoculation until termination of the study at 90 days post-inoculation (DPI). Boars from each treatment group were euthanized at 3 different time points during the study to determine the distribution and amount of PCV-2 in tissues and associated histological lesions.

Results of the study indicate that both inoculated groups became viremic, seroconverted and shed PCV-2 viral DNA in semen in low amounts as detected by quantitative PCR. Boars inoculated with PCV-2b remained viremic up to DPI 90. In contrast, boars inoculated with PCV-2a were viremic up to DPI 45. To assess whether viral DNA detected in semen was infectious, PCV-2 positive semen was used to breed PCV-2 negative gilts and in a swine bioassay model.

Preliminary results indicate that the PCV-2 in semen remains infectious in the swine bioassay but is not transmissible to naïve gilts via artificial insemination.

* Graduate student presentation

Quantification of specific antibodies to ORF2 of PCV-2 using a blocking ELISA

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Introduction and objectives. The objective of the study was to develop a quantitative serum antibody test for *Porcine circovirus 2* (PCV-2) in swine with a commercially available test (SERELISA® PCV2 Ab Mono Blocking, Synbiotics Corp.). This test is based on a blocking enzyme-linked immunosorbent assay (ELISA) that allows specific detection of antibodies to open reading frame 2 (ORF2) of PCV-2.

Material and methods. Results of the test are expressed as sample to negative control optical density (OD) ratio corrected by the positive control OD and referred to as s/n ratio. The linear range of the bELISA was determined by conducting the assay with the s/n ratio of a positive reference sample at different dilutions starting at 1:10 (increasing by 2 and 10 dilution factors) and 1:50 (increasing by 10 dilution factors) in order to produce a panel ranging from strong positive to weak positive. Determination coefficients (r^2) were calculated for different models with variable transformations for s/n ratio and the dilution of titer. Transformations were analyzed for the relationship between titer (T), 1/T, and log T and s/n ratio (sn), 1/sn, log sn and logit sn. To achieve a valid quantification method from negative to highly positive samples, different dilutions were selected and interpolation was calculated between results obtained from different wells. Coefficients and regression equations, and ANOVA for the robustness study, were calculated using R version 2.4.1.

Results. Linear s/n ratio values ranging from 0.11 to 0.93 were determined using the reference PCV-2 positive serum sample at different dilutions. The best model was achieved utilizing the log of titer and the logit of s/n ratio. This model was linear with an r^2 of 0.988, a slope of β=-0.703 and an intersection of α=2.652. This linear model covered at least a range of a $\log_{10}$ of titer. In order to have a quantitative method valid from negative status to high positive titer, an interpolation using different wells is needed. Inside the linear range, titers range from 100 to 1,000 for s/n ratio respectively ranging from 0.895 to 0.242. An arbitrary decision was made to apply this result to the 1/1,000 final dilution in the well. Derived from this decision, interpolation was calculated for the next 2 dilutions of 1:100 and 1:10,000. A correction factor (multiply by 10 or divide by 10) was applied to each of the titer results obtained within these wells and therefore linearity is respected across the 3 dilution wells. The final model was not limited on the lower boundary of the 1:100 well and an arbitrary limit was fixed on the upper boundary of the 1:10,000 well. This limit corresponds to the limit of linearity of the regression model (s/n=0.107). Titers obtained are expressed in ELISA units (EU).

Discussion and conclusions. **This method for a blocking PCV-2 ELISA allows the quantitative detection of a specific antibody subpopulation to ORF2 of PCV-2.** Linearity and robustness were proven to be effective using 3 wells in a blocking ELISA. This method is independent of the seroneutralizing properties of the targeted antibody subpopulation and is therefore a nice alternative to serum neutralization tests. This tool that may lead to better understanding of PCVD epidemiology, and may be used to assess PCV-2 control measures such as vaccination.

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Efficacy of *Classical swine fever virus* vaccine (LOM strain) in pigs infected with *Porcine reproductive and respiratory syndrome virus*

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Introduction. Porcine reproductive and respiratory syndrome (PRRS) is an important swine disease, characterized by reproductive disorders in sows and gilts, increased pre-weaning piglet mortality, and pneumonia in young growing pigs. Some studies have reported that PRRS virus (PRRSV) may suppress host immune responses. However, controversial results associated with PRRSV infection and secondary infection or vaccination efficacy, have been demonstrated. With regard to the effect of PRRSV infection on vaccination against *Classical swine fever virus* (CSFV) using a Chinese (C) strain vaccine, PRRSV reduced the antibody response against CSFV vaccine. In Korea, the live attenuated LOM-strain CSFV vaccine has been used for the control of CSFV, except for Jeju Island. Therefore, this study was carried out to investigate the effects of PRRSV infection on the efficacy and safety of LOM-strain CSFV vaccine.

Material and methods. Twenty-eight 45-day-old crossbred pigs from a CSFV-free, PRRSV-free farm on Jeju Island were randomly divided into 4 groups of 7 each, including an untreated control group. The LOM group (LOMV) was given 2 mL of LOM CSFV vaccine intramuscularly. The other 2 groups were infected intranasally with $2 \times 10^{6.0}$ $TCID_{50}$/mL of PRRSV (LMY strain) either 7 days before administration of LOMV (PRRSV/LOMV) or 7 days after administration of LOMV (LOMV/PRRSV). Serum samples for neutralizing antibody titration were collected every week until the end of the experiment (7 wk after inoculation). Clinical signs were observed throughout the experiment, and 2-3 head per group were euthanized by exsanguination from the axillary artery under deep anesthesia at the end of weeks 5, 6, and 7. After necropsy, histopathology and immunohistochemistry were performed.

Results. CSFV antibodies were detected in the sera from PRRSV/LOMV, LOMV/PRRSV, and LOMV groups at 2 wk after inoculation. The serum level of CSFV antibody was elevated during the experiment. However, no significant difference in the antibody titers was observed among the 3 groups. Grossly, non-collapsing, firm, rubbery lungs, enlarged or red lymph nodes, and kidneys with petechial hemorrhages were observed in the 3 groups. Histologically, interstitial pneumonia, nonsuppurative meningitis, nonsuppurative encephalitis, focal blood resorption, and lymphoid depletion were seen in these 3 groups. Immunohistochemically, LOMV antigen was not detected in the tissues of any of the groups injected with LOMV. However, PRRSV antigen was detected in affected tissues of PRRSV-infected groups.

Conclusions. The humoral immune responses to CSFV were elevated in spite of PRRSV infection. This finding is consistent with a previous study that suggested that PRRSV had no effect on immune responses of pig injected with Pseudorabies virus vaccine. **These findings indicate that PRRSV infection does not affect the efficacy of live attenuated LOM-strain CSFV vaccine.**

Molecular characterization of vaccine-related Infectious laryngotracheitis virus isolates using PCR/RFLP and sequencing

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Infectious laryngotracheitis virus (ILTV, *Gallid herpesvirus 1*), a member of the family Herpesviridae, causes an acute respiratory disease of chickens.

Since 1998, we have used PCR for the detection of ILTV directly from tissue, and as confirmation of virus isolation using chicken embryos and/or chicken kidney cell culture. The GE genes of 94 ILTV isolates obtained from 1998-2006 from broiler flocks in the southeastern United States were analyzed by PCR and restriction fragment length polymorphism (RFLP). All isolates belonged to chicken embryo origin (CEO) vaccine pattern A (CEO/A). The thymidine kinase (TK) gene of 30 isolates and 3 different vaccine strains were sequenced and compared with the GenBank entries. Compared with UK strains Thorne and 216 in GenBank, all 33 strains had an amino acid deletion at 302, and different amino acids at 301 and 303, respectively. The 33 total sequences could be assigned to 3 groups: 3 vaccines belonged to group 1; 26 isolates belonged to group 2; and 4 isolates in group 3. Other differences among the 33 TK gene analyses occurred at amino acids 97 and 306.

These data are suggestive of mild diversity among vaccine-related ILTVs and merit further study.

A novel sandwich ELISA for the measurement of insulin in canine serum and plasma

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Background. Diabetes mellitus is a common endocrine disorder in dogs, with clinical features resembling human type 1 diabetes, such as polydipsia, polyuria and weight loss. Diabetes typically occurs in dogs between 5 and 12 years of age and is diagnosed in dogs with persistent hyperglycemia and glucosuria. Furthermore, certain breeds of dog appear to be predisposed to diabetes, and female dogs are more likely to develop the disease. The mechanisms underlying the development of insulin resistance are currently unclear and further investigations are warranted. The objective of this study was to evaluate a specific and sensitive ELISA, optimized for quantitative measurements of canine insulin. The ELISA assay has recently been developed at Mercodia AB, Uppsala, Sweden.

Methods and results. Monoclonal antibodies directed against different antigenic determinants were evaluated and chosen with care for the canine insulin molecule. Optimal immune reaction was established where the samples and calibrators were allowed to react simultaneously with the capture antibody and the HRP-conjugated detection antibody. A highly purified porcine insulin preparation was used as calibrator material. A representative set of samples from 42 healthy dogs was analyzed for the purpose of determining the appropriate measuring range. The established ELISA was validated for accuracy, precision, sensitivity and specificity. The assay has a dynamic range of 0.02-1.5 µg/L. Intra-assay and inter-assay variation for 6 canine samples measured on 7 different occasions, were 4.5% and 6.5%, respectively. Statistical analysis was conducted using one-way ANOVA. Capability of detection was 0.01 µg/L according to the methodology described in ISO 11843. Recovery upon dilution was calculated as 92-112% (mean 99%), and recovery upon addition was calculated as 99-128% (mean 111%). Cross reactivity with porcine c-peptide and proinsulin was found to be less than 0.001% and 0.2% respectively.

Conclusion. This novel insulin ELISA provides analytical performance, precision and reliability equal to methods currently used in human clinical research. This method enables the performance of additional mechanistic investigations, which may contribute to a more complete understanding of the development of insulin resistance and the essential link between canine diabetes and the metabolic syndrome.

Sample preparation methods for high quality nucleic acid isolation from a variety of veterinary samples

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Effective and reproducible isolation and purification of nucleic acids is one of the key success factors for amplification and detection of nucleic acids of pathogens by molecular methods. Diagnostic tests using PCR (e.g., for Johne's disease in cattle) are replacing traditional ELISA or culture-based methods due to higher sensitivity and speed.

An easy and standardized method for nucleic acid isolation that gives high-quality nucleic acids is required. The major challenge when working with such diverse material is to develop optimized pretreatments for all sample types. Dependent on sample type, content of inhibitors, and content of nucleic acids, the samples demand different pretreatment conditions such as mechanical disruption, enzymatic digestion, and incubation times.

Various animal organ tissues, fresh and dried pig ears, horse hair, ruminant feces and ticks were prepared by using manual and automated sample preparation. Manual preparation comprised different methods depending on the starting material (e.g., DNeasy® Blood & Tissue Kit, QIAamp® DNA Stool Mini Kit). To compare manual to automated extraction methods, 1 manual and 2 automated protocols were evaluated, including the new QIAcube automated extraction system for single spin column processing. Quality of the isolated DNA was confirmed by gel electrophoresis, enzymatic digestion, and standard as well as real-time PCR as downstream applications.

DNA isolation from a variety of different samples and animals was successful. All types of pig ear samples, including frozen, lyophilized, and dried tissue, gave good yields and were used successfully in PCR analysis. Detection of *Borrelia* DNA with the *artus*® Borrelia LC PCR Kit subsequent to a DNA isolation from ticks was performed: the lowest positive curve in the real-time PCR corresponded to 5 copies.

DNeasy Blood & Tissue Kit, QIAcube, BioRobot EZ1, *artus* Borrelia LC PCR Kit: For Research Use Only. Not for use in diagnostics procedures. No claim or representation is intended to provide information for the diagnosis, prevention, or treatment of a disease.
The QIAamp DNA Stool Mini Kit is intended for general laboratory use. No claim or representation is intended to provide information for the diagnosis, prevention, or treatment of a disease.

Brodifacoum poisoning in backyard chickens

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Introduction. In order to overcome the problem of controlling rodents that become genetically resistant to warfarin, second-generation anticoagulant rodenticides were developed. These rodenticides are much more potent as they have a longer biological half-life/faster action so only low levels are needed to kill rodents after the ingestion of a single lethal dose. Unfortunately, non-target species - mammals (dogs, cats, livestock, wildlife) and birds (domestic poultry/captive wild birds) - can also be affected by these toxic compounds. Chickens can ingest these chemicals accidentally via commercial bait formulations, by malicious intent, or by pecking poisoned rodents; accumulations in their livers may pose a risk to human health. These pesticides block the synthesis of functional vitamin K-dependent clotting factors by affecting the vitamin K enzyme complex in the liver. In birds, depletion of these factors prolongs the extrinsic and common coagulation pathways preventing blood clotting and causing widespread hemorrhage and death due to severe blood loss/hypovolemic shock.

Material and methods/results. In mid-March 2007, one of the chickens from a small backyard flock of 10 birds (fighting cock roosters and layer hens) was brought to our diagnostic laboratory for necropsy. Six birds had died within 2 weeks. Hematemesis was observed in most of the affected birds and, when moribund, whole body shaking was also noted; some were just found dead. Deaths occurred within 24-48 h after birds were noticed sick. Oral activated charcoal was given to the affected birds but none recovered. Necropsy findings included clotted blood on the beak and nares and in the oral cavity and tracheal lumen, dark tarry contents in the esophagus/crop/proventriculus/ gizzard, locally extensive pulmonary hemorrhage, paleness of the internal organs, petechiae around the sciatic nerves, multiple ecchymoses in the thymus, bloody contents in the ceca, and presence of a blood clot in the coelomic cavity on the dorsal lobe of the pancreas. Based on the gross lesions, anticoagulant rodenticide poisoning was suspected. Anticoagulant screening on the liver and gizzard contents included testing for warfarin, bromodiolone, coumachlor, brodifacoum, diphacinone, chlorophacinone, and difethialone. Brodifacoum was detected in the liver but not in the gizzard contents. Histopathology confirmed hemorrhage in different organs and tissues. Results of other laboratory tests were either negative or non-significant, except for the presence of a moderate amount of coccidia in the cecal contents. The source of brodifacoum was found to be a nearby dump/storage area where neighbors put anticoagulant baits to get rid of rats. The chickens had free, unlimited access to the area and thus the poison. Deaths ceased right after the access of these chickens to this area was denied.

Discussion/conclusion. Anticoagulant rodenticide poisoning was suspected based on the clinical picture/necropsy findings consistent with a coagulopathy, and was confirmed by the detection of brodifacoum in the liver. No cases of accidental poisoning by brodifacoum or by any other second-generation anticoagulant rodenticide have been documented in chickens previously. Diseases to be considered in the differential diagnosis of anticoagulant rodenticide poisoning in chickens are those characterized by sudden onset, peracute-acute clinical course, high mortality, and in which generalized hemorrhage (petechiation and ecchymoses affecting a multitude of organs and tissues) is observed, e.g., viscerotropic velogenic Newcastle disease, highly pathogenic avian influenza, septicemias, and hemorrhagic syndrome induced by *Chicken anemia virus*.

* Graduate student presentation

Analytical method for melamine and related compounds in swine urine by GC/MS

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Melamine in the form of resins is used to produce plastics in a wide variety of products. Melamine is also a metabolite and/or degradation product of cyromazine, an insect growth regulator fed as a larvicide to control flies on animals or as a spray to control insects on plants. Most recently, melamine and related compounds have been implicated in the cause of renal toxicosis in cats and dogs fed contaminated pet food. Also pigs fed salvaged contaminated pet food were shown to have melamine in their urine.

Recently at the ISU-VDL, we have conducted a dosing study in pigs of melamine and cyanuric acid. Groups of 4 pigs were dosed with 200, 400, or 1000 mg/kg melamine, 400 mg/kg cyanuric acid, and a combination of 400 mg/kg melamine/cyanuric acid, plus a control group of 2 pigs. To support this study, we developed an analytical method for melamine and cyanuric acid in urine.

A method for the determination of melamine in muscle by LC/MS obtained from the University of California-Davis was adapted for the determination of melamine, cyanuric acid, ammelide, and ammeline in urine. Ammeline, ammelide and cyanuric acid are by-products and/or degradation products in the production of melamine. A 5mL aliquot of urine was used; additional samples were spiked at 50, 100, 200, 500 and 1000 ppb melamine, cyanuric acid, ammelide, and ammeline. Urine samples were extracted using an ion exchange solid phase extraction technique. Melamine and related compounds were eluted from the solid phase with 5% ammonium hydroxide in methanol. The extract was concentrated to dryness and resolvated in 50:50, acetonitrile:water, v:v. A portion of this extract was concentrated and derivatized with BSTFA + 1% TMCS and sent to GC/MS for determination. The TMS derivatized compounds were separated using a 30 meter DB5 capillary column. Retention times were as follows: cyanuric acid 9.2 min., ammelide 10.1 min., ammeline 10.8 min. and melamine 11.3 min. **The limit of quantification (LOQ) in urine of this method for melamine, cyanuric acid, ammeline and ammelide was 200 ppb. The limit of detection (LOD) was 0.5 ng**. Matrix effects from the urine made calculations of spike recoveries difficult. The standard curve within the matrix was linear with a correlation factors ranging from 0.99 to 0.93. Further investigation is ongoing.

Establishing a normal sodium range in the equine brain using atomic absorption spectroscopy

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Introduction. Water deprivation/sodium ion toxicosis is well documented in certain species. A normal sodium range as well as a toxic range has been previously established for cattle, poultry and swine. Diagnostic laboratories often receive requests for brain sodium levels to use in conjunction with histological findings for diagnosis of water deprivation/sodium ion toxicosis. No reference values for equine brain sodium levels have been found in the literature.

Materials and methods. Brain tissue was harvested from 122 horses submitted to the Oklahoma Animal Disease Diagnostic Laboratory and frozen until time of testing. Signalment and history were noted on each animal when given. Cause of death was sorted into four categories: trauma, colic, infection, and other. Also noted were cases in which the animal was euthanized. Clinical specimens were not harvested from animals that had been presented with neurological problems. Samples weighing approximately 1 g were harvested from the cerebral frontal lobe and cerebellum. Identification number, harvest site and weights were noted on each sample and digested in 10 mL 1:1 nitric acid and reduced to approximately 3 mL volume using heat. Tissue digest was filtered and brought up to 1,000 mL final volume with distilled water. Samples were tested in 2 batches using a Perkin Elmer 3110 flame furnace. Standard concentrations of sodium were created to calibrate the flame furnace in 1 ppm, 2 ppm, and 5 ppm solutions. The AA flame furnace lamp was set at 589 nm wavelength, 0.4 nm slit with an acetylene-air flame.

Discussion. Atomic absorption readings showed levels of sodium from the frontal lobe ranged from 890 ppm to 2,453 ppm sodium, mean 1,250 ppm, SD 229. Readings from the cerebellum ranged from 637 ppm to 4,934 ppm, mean 1,420 ppm, SD 427.

Conclusion. The sodium levels found suggest that an average normal sodium concentration for horses to be 1,250 ppm in the frontal lobe and 1,420 ppm in the cerebellum. These levels are slightly higher than those considered normal in cattle, and close to the levels considered toxic in the pig. Due to the increased public awareness of cruelty/neglect cases, these values will allow for more accurate diagnosis of water deprivation/sodium ion toxicosis in horses.

* Graduate student presentation

Correlations between normalized difference vegetation index data patterns and hazardous nitrate accumulation in forage plants

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Nitrate poisoning is a common and economically important problem in ruminant livestock production. Nitrate accumulation in plants is linked to climatic conditions that lead to plant stress. We hypothesized that satellite-derived normalized difference vegetation index (NDVI) data can be used to predict plant stress and nitrate accumulation risk.

Kansas State University Veterinary Diagnostic Laboratory forage nitrate assay records from zip code 66427 during 2001-2005 were analyzed in comparison to NDVI and precipitation data. **Increased frequency of excessive nitrate accumulation events in forage plants followed declining NDVI during the summer growing season, which was correlated with summer drought.**

These results suggest that risk models for nitrate poisoning in livestock can be developed based on remotely sensed environmental data such as NDVI. Such models have the advantages of not being dependent on interpolation of environmental parameter values between weather stations, and of providing complete coverage of the land surface area at resolutions relevant to individual animal production units.

Method development for the analysis of algal toxins microcystin and nodularin by LC/MS/MS

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Microcystin (MC) and nodularin are cyanobacterial metabolites found worldwide in freshwater and in brackish environments. Exposure to algae-contaminated water has been a cause of acute toxicosis in both animals and humans. However, there are limited studies that directly link exposure to the toxins and observed toxicological lesions.

The objective of the study was to establish a direct link of exposure to microcystin or nodularin with compatible liver lesions and measurable levels of toxin in the plasma and tissues. This is accomplished by:

- Dosing channel catfish with microcystin or nodularin either intraperitoneally (IP) or by gavage.
- Comparison of histological liver lesions between the treated versus untreated control fish.
- Measurement of microcystin and nodularin concentration levels by LC/MS/MS.

Hepatocytes and their nuclei were enlarged in microcystin-treated liver, sometimes separated from adjacent hepatocytes. The chromatin of a few hepatocyte nuclei were peripheralized leaving a clear central space. Blood vessels within the liver were mildly or moderately distended by red blood cells. The method detection limit for MC and nodularin in plasma is 50 ppb and the relationship was found to be linear from 50 ppb to 10 ppm.

This work demonstrates the direct relationship between algal toxin exposure, histological lesions, and the presence of toxin in the plasma.

Diagnostic PCR assays for detection of BVDV in pooled ear notch samples to characterize herd BVDV status: influence of inhibition, sample condition, pool size and BVDV from different herds on assay results

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Diagnostic laboratory tests commonly used to detect persistently infected (PI) animals in beef or dairy cattle herds include: virus isolation (VI), immunohistochemical (IHC), antigen capture ELISA (ACE), and PCR assays. Pooling of samples collected from multiple animals for PCR testing is done to reduce costs in herd surveillance testing. Inhibition of nucleic acid amplification by inhibitors present in biological samples has been implicated as a cause of reduced sensitivity of diagnostic PCR assays when applied to pooled samples to determine herd BVDV status. The objectives of the present study were to characterize the influence of inhibition, sample condition, pool size and BVDV from field origin ear notch samples used to prepare eluate pools for real-time and gel-based RT-PCR assays.

Ear notches collected from 9992 calves, representing 32 Nebraska herds, individually tested by ACE and IHC assays, were tested in pools of 50 (n = 170) and 100 (n = 70). To test for the presence of inhibitors in each pool, an aliquot of each pool was spiked with eluate of a skin notch obtained from a confirmed PI calf and tested in tandem with a non-spiked aliquot of each pool. It was determined that 29 herds were BVDV-free. One herd of 661 calves and a second herd of 895 calves each had one PI calf. A third herd of 94 calves had 5 PI calves. All of the PI calves in all 3 herds were detected by ACE. Both of the single PI calves in the first and second herd, and 4 of the 5 calves in the third herd were detected by IHC assays. Real-time RT-PCR detected PI animals in pooled samples comprised of the 5 PI calves in the third herd while the single PI calves in the first and second herd were not detected. Gel-based RT-PCR assay of pools detected the PI calf in the second herd and the multiple PI calves in the third herd, while the PI calf in the first herd was not detected. Based on test results of spiked pools, the presence of endogenous inhibitors did not appreciably reduce the sensitivity of pooled sample PCR assays.

To determine the effects of sample condition on detection of BVDV by real-time RT-PCR, ear notches from a confirmed PI calf were treated as follows: 1) 2 mL PBS, 2) a sealed red-top serum tube, or 3) an open petri dish. Samples were stored at 4^{o}C, 25^{o}C or 37^{o}C for 1, 3 or 8 days. Samples were then soaked in 2 mL PBS overnight and eluate was diluted 1:100 and tested. Eluate from the notch placed in PBS provided the lowest Ct value when evaluated by real-time RT-PCR compared to the eluates from the notches stored in the serum tube or petri dish. A decrease in Ct as storage time increased was shown for all eluates, at all temperatures, with the exception of the eluate from the notch stored in the serum tube at 37^{o}C (lowest Ct value after storage for 1 day) and the eluate from the notch stored in the petri dish at 4^{o}C (lowest Ct value at 3 days). The treatment resulting in the lowest Ct value was the eluate from the notch placed into 2 mL PBS and stored at 37^{o}C for 3 days.

The effect of pool size on detection of BVDV from ear notches was evaluated by making pools of 10, 25, 50 and 100 samples spiked using notches collected from 9 different PI calves from 7 herds. Five of 9 positive 100-sample pools were detected, 6 of 9 positive 50-sample pools were detected, 8 of 9 positive 25-sample pools, and all 9 positive 10-sample pools were detected.

In summary, under these conditions, diagnostic PCR assays for detection of BVDV in pooled ear notch samples did not appear to be influenced by inhibition, sample condition nor by BVDV isolate variability, whereas pool size did influence the ability to detect BVDV.

Detection and genotyping of *Bovine viral diarrhea virus* from the white-tailed deer population in Indiana

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Bovine viral diarrhea is one of the economically important diseases of cattle. For many years, different types of vaccines have been commercially available, yet this disease is hard to control in high density population areas. Detection and isolation of *Bovine viral diarrhea virus* (BVDV) from any potential reservoir is very important, especially when considering virus eradication from a herd or locale. One potential source is wild ruminants.

Ear notches and lymph nodes were colleted from the wild population of white-tailed deer (*Odocoileus virginianus)* during the fall 2006 deer-hunting season in Indiana state-wide and tested for BVDV using a commercial BVDV antigen capture ELISA. Two samples out of 745 samples collected were positive for BVDV by cELISA test. BVDV was isolated from 1 ear notch and 1 lymph node and characterized. These isolates were genotyped as type 1a and 1b based on sequence analysis of the 5' UTR region.

The results of this study indicate that the prevalence of BVDV in the white-tailed deer population of Indiana is about 0.3 %. Wild ruminants infected with BVDV should be taken into consideration during an eradication program of BVDV from the livestock population.

Failure of BVDV detection with antigen capture ELISA using serum

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Bovine viral diarrhea virus (BVDV) causes arguably the most costly viral disease for cattle producers in the United States and one is one of the most difficult infectious agents to eradicate from a herd. Control of BVDV infection is achieved by good management practices, including identifying and removing persistently infected (PI) animals thus preventing spread to naive herdmates or perpetuation of the cyclic spread of BVDV through generation of additional PI calves each year. Presently 4 detection methods are used in the US; IDEXX HerdChek®, antigen-capture ELISA (AgELISA), immunohistochemistry (IHC), virus isolation (VI), and reverse transcription polymerase chain reaction. The IDEXX HerdChek® has been validated for use with ear notches in PBS on any postnatal animal and serum on pre-colostral calves and calves >3 months of age. Beginning in 2006, it became apparent at the Wyoming State Veterinary Laboratory (WSVL) that the AgELISA was not consistently detecting BVDV in serum (and plasma) from calves that were positive for BVDV by ear notch AgELISA or IHC testing when submitted concurrently.

Ten PI calves, ages 5-7 months upon arrival, were held together at the WSVL and sampled weekly (blood and nasal swabs) and monthly (ear notches) over a 3-month period. Detection of BVDV antigen in serum, plasma and ear notches in the AgELISA was compared. Immunohistochemistry on ear notches and VI on blood and nasal swabs were used to validate these AgELISA results. Duplicate serum and ear notch samples were tested at IDEXX Laboratories with the IDEXX HerdChek® kit and with a European AgELISA kit, manufactured by IDEXX Laboratories and marketed in Europe only. IDEXX HerdChek®, the AgELISA commercially available in the US, uses the BVDV monoclonal antibody 15.c.5, which recognizes an epitope within the E^{rns} (E0, gp48) viral glycoprotein. This monoclonal antibody is also used to detect BVDV antigen by IHC in ear notches at the WSVL. BVDV antigen is detected in the European kit by a pool of 3 monoclonals, all of which are directed against the E^{rns}.

Of the 77 serum and 80 plasma samples tested by AgELISA at the WSVL, 34% and 55% were determined to be falsely negative, respectively, when compared with virus isolation results on lymphocyte preparations from samples collected simultaneously. BVDV was isolated from every blood sample taken on every date. **There were no false negative ear notches in the AgELISA when compared with IHC; both procedures detected BVDV in all ear notch samples on all sampling dates**. Of the 74 serum samples and 38 ear notch samples tested at IDEXX Laboratories, the European AgELISA test kit detected 97% of positive serum samples (3% false negative) when compared to VI in lymphocyte preparations and 100% of positive ear notch samples.

In summary, while the IDEXX HerdChek® BVDV AgELISA detected 100% of skin samples in PBS from PI calves sampled over a 3-month period, it failed to detect over one-third of the serum samples from the same group of calves. **Using serum in this AgELISA may no longer be an effective method for identifying cattle infected with BVDV.**

Prevalence of *Bovine viral diarrhea virus* infections in US alpacas

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Bovine viral diarrhea virus (BVDV) infections of pregnant alpacas may lead to reproductive failure or to persistent infections of crias. Persistently infected (PI) crias are persistently viremic, shed virus continuously, and are the primary reservoirs of viral exposure within and among herds.

The objective of this study was to determine the current prevalence of BVDV-infected alpaca herds in the United States. This was done by testing crias less than 6 months old from a sample of herds for BVDV neutralizing antibodies and for BVDV. Sixty two breeders, representing 27 states, participated in the study by submitting blood samples from crias during a 10-month period extending from May, 2006 to March, 2007. Seventeen of the herds (27%) had crias with BVDV neutralizing antibody titers, while in 45 herds, all crias tested were BVDV seronegative. PCR and virus isolation assays showed that in 61 herds, all crias tested were BVDV-free; 1 herd had a PI cria. However, herd histories subsequently provided by each of the 17 breeders with seropositive herds revealed that 3 additional herds recently had laboratory-confirmed PI crias. Hence, **a total of 4 herds in the study (6.5%) had PI crias.** Infections in 3 of the 4 infected herds were linked, since herd histories showed that 2 of the infected herds acquired BVDV infections by exposure to 1 of the other infected herds. In addition to exposure of dams to PI crias within herds, BVDV antibody titers in crias in BVDV-free herds were associated with ingestion of bovine colostrum provided at birth, as well as with colostrum from dams previously exposed to BVDV in other herds.

These findings confirm the importance of BVDV infections in US alpaca herds and underscore the merit of adhering to sound herd biosecurity practices to avoid exposure to PI crias.

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Increased detection in baitfish of a novel positive-sense RNA virus belonging to the order Nidovirales

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Background. In 1997, a novel virus was isolated from moribund fathead minnows (*Pimephales promelas*) exhibiting hemorrhages in their eyes and skin. Classical virus characterization including morphology, pH tolerance, solvent sensitivity, and analysis of proteins suggested that this virus was similar to members of the Rhabdoviridae family. Recent genetic analysis of this isolate revealed a positive-sense virus more similar to the Nidovirales order, but formal classification of this virus is not complete.

No additional cases of this virus were reported until recently. The occurrence of *Viral hemorrhagic septicemia virus* (VHSV) in the Great Lakes has resulted in increased awareness of the role that wild baitfish might play in spreading the disease to other areas of the country. A Federal order was issued in November 2006 by the USDA APHIS for mandatory VHSV testing of finfish, including baitfish species originating from the Great Lakes basin and intended for interstate movement. The resulting increase in testing for finfish health certification, and the investigation of clinical signs observed in baitfish species, has produced additional *Nidovirus*-like isolates.

Detection methods. Tissue homogenates made from pools of 5 fish were processed for virus isolation. Fluids from cell cultures demonstrating cytopathic effects (CPE) were evaluated for the presence of virus by electron microscopy. RNA was extracted from CPE-positive cultures and evaluated with primers designed from the sequence of the 1997 isolate. PCR amplicons were sequenced and compared to the original isolate. The virus isolation work on the cases described below was conducted in independent laboratories, but the virus identification work for these cases was completed at the National Veterinary Services Laboratories.

Recent cases. Case A: Virus was isolated from fathead minnows from 2 sources located in different states. These fish were being screened for use as feed for fish used to raise the glochida stage of freshwater mussels. **Case B**: Hemorrhaging of the eyes and skin was observed in fathead minnows found in a bait shop. Remaining fish were euthanized and shipped on ice overnight to the diagnostic laboratory. No virus was isolated in cell culture, but RT-PCR conducted on RNA extracted from the tissue homogenates produced cDNAs with sequences identical to the 1997 isolate. **Case C**: Virus was isolated in cell culture from dead fish that were collected daily and frozen in a chest freezer in the bait shop. Fish were stored for approximately 2 weeks prior to submitting to the diagnostic laboratory. Fish exhibited hemorrhage on the ventral surface and fins. **Case D**: Virus was isolated in cell culture from apparently healthy fish that were collected from a bait shop, euthanized and shipped on ice overnight to the diagnostic laboratory for fish health inspection.

Conclusions. It is not known if the increase in detection of this novel virus is a direct result of increased testing or increased prevalence. **However, the increase in virus detection suggests that baitfish can harbor undetected viruses that could be spread by baitfish movement.**

Comparison of 4 serological methods for the diagnosis of eastern equine encephalomyelitis

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Eastern equine encephalitis virus (EEEV) is an arthropod-borne virus of the family Togaviridae, genus *Alphavirus.* The virus is transmitted by several species of mosquitoes of the genera *Aedes, Coquillettidia and Culiseta.* EEEV infects birds as well as humans, horses and a variety of other mammalian species. The virus is maintained in an enzootic cycle by mosquitoes and birds; mammals are considered to be a dead-end host. In naïve horses, the disease is normally acute with mortality rates as high as 90%. Since the disease in horses progresses rapidly, laboratory samples submitted for antemortem diagnosis usually consist of a single serum sample collected a short time following the onset of clinical signs.

Standard laboratory tests consist of hemagglutination inhibition (HI), plaque reduction neutralization test (PRNT), complement fixation (CF), and immunoglobulin M capture enzyme-linked immunosorbent assays (IgM ELISA or MAC-ELISA). Since both the HI and PRNT are known to detect titers produced by vaccination, neither has been considered to be suitable for making a diagnostic determination based on only a single serum sample. Complement-fixing antibodies are reported to appear later in the course of infection so, where survival of the host is unlikely, this method is less useful.

During the 2005 transmission season, a large number of EEE cases were reported throughout the eastern section of the United States. More than 900 samples were submitted to the Diagnostic Virology Laboratory (DVL) of the National Veterinary Services Laboratories (NVSL) Ames, Iowa for EEE diagnostic evaluation. Of those, more than 100 tested positive on the EEEV IgM ELISA.

A project was undertaken to compare the serological results of 88 serum samples submitted for EEE diagnosis by testing samples by IgM ELISA, PRNT, CF and HI methods. If tissues were submitted, polymerase chain reaction tests were also conducted.

The outcome of the project indicates that the IgM ELISA is a very sensitive tool for identifying acute EEEV infections from a single serum sample. The PRNT was nearly as sensitive but presents problems for many laboratories because it requires the use of live virus. The HI and CF tests were much less likely to accurately support the diagnosis of an EEEV infection from a single serum sample.

Herpesvirus infections in rock hyraxes (*Procavia capensis)*

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Seven juveniles and 3 adults from a closed group of 19 rock hyraxes (*Procavia capensis)* housed in a zoo's indoor rock exhibit died or were euthanized after developing blepharoconjunctivitis, ulcers in periorbital and perioral skin, and ulcers in their tongues over a 2-week period. Histologic examination of skin and tongues revealed necrosis of scattered epithelial cells and amphophilic to basophilic intranuclear inclusion bodies in epithelial cells bordering ulcers. Epithelial cells with inclusion bodies were often characterized by cytomegaly and karyomegaly, and syncytia with intranuclear inclusion bodies were also seen. Examination of inclusion bodies in tongue epithelium by transmission electron microscopy revealed icosahedral nucleocapsids, approximately 80-95 nm in diameter, with morphologic features consistent with herpesvirus.

Cytopathic effect (CPE) typical of alpha-herpesvirus infection was seen in bovine turbinate cell, equine dermal cell, and Vero cell monolayers after inoculation with homogenates of the skin lesions, but was not seen after inoculation onto Madin-Darby canine kidney or swine testicle cell monolayers. PCR analysis using degenerate primers that targeted a portion of the herpesvirus polymerase gene generated a product of approximately 227 base pairs, and the product was cloned, sequenced and then analyzed using the Basic Local Alignment Search Tool program of the National Center for Biotechnology Information. **Results indicated 77%, 77% and 76% similarity of the hyrax virus to its nearest neighbors, *Human herpesvirus 1, Human herpesvirus 2* and *Cercopithecine herpesvirus 2*, respectively.**

Herpesvirus infections in rock hyraxes have not been characterized. The data presented here indicate that **a novel alpha-herpesvirus caused the lesions seen in these rock hyraxes.** The source of the infections is currently being investigated. Reactivation of latent infections remains a possibility.

All affected hyraxes were treated initially with antibiotics. They were then treated with antibiotics and acyclovir once herpesvirus infection was suspected. Although 1 juvenile and 2 adult hyraxes survived, acyclovir therapy was perceived to have had no positive clinical effect. Six hyraxes from this closed group remained clinically normal throughout the 2-week period.

Identification by PCR of a virus with similarities to a newly described genogroup of bovine enteric caliciviruses

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The family *Caliciviridae* is divided into 4 genera, *Sapovirus (*SLV*), Norovirus (*NLV*), Vesivirus,* and *Lagovirus*. Animal enteric caliciviruses are mainly assigned to the last 2 genera, although some enteropathogenic bovine enteric caliciviruses have also been identified with morphologic similarities to human noroviruses, but with sequence homologies to SLV and lagoviruses. The prototype strain of these newly identified bovine enteric caliciviruses is the NB strain (AY082891).

Since 2000, only 2 cases of Bovine enteric calicivirus (BEC) infection have been identified by electron microscopy at the Wyoming State Veterinary Laboratory (WSVL). In January of 2007, a 10-day-old calf was submitted to the WSVL with diarrhea. Calicivirus was observed by negative contrast electron microscopy in a fecal sample. The virus could not be cultured by standard virus culture techniques. The diagnosis of calicivirus was confirmed by reverse transcription-PCR done on RNA extracted from the fecal sample utilizing primers that target a region of the RNA dependent-RNA polymerase (primers P289/290). **Sequence analysis of the translated PCR product showed homologies to the NB strain of BEC and the recently described Newbury agent 1. However, phylogenetic analysis also indicates this strain roots apart from NB-Newbury agent 1, clustering closer to other prototype members of the *Lagovirus* genera.**

Canine distemper virus infection of anteaters (*Myrmecophaga tridactyla*)

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Three giant anteaters (*Myrmecophaga tridactyla*) - a 17-year-old male, a 6-year-old female, and their 10-month-old female offspring - located at the Little Rock Zoo, became ill over a 3-day period with discharge from the nose and mouth. Their condition continued to deteriorate with observed pulmonary congestion, nasal discharge, lethargy and anorexia. The anteaters either died or were euthanized and were brought to the Arkansas Veterinary Diagnostic Laboratory for necropsy.

Analysis of multiple tissues, including lung, stomach, intestine, bladder, brain, and skin, yielded positive *Canine distemper virus* (CDV) results by a direct fluorescent antibody assay. Confirmation of these results was done using the polymerase chain reaction on RNA extracted from brain and lung tissue from the affected animals. Sequence analysis at the University of Missouri of the phosphoprotein gene of the virus from all 3 anteaters and partial sequence analysis of the viral fusion and nucleoprotein genes indicate the CDV found in the anteaters was distinct from the CDV vaccines typically used at the zoo indicating that **the CDV infecting the anteaters did not come from vaccine virus**. Because of a recurring wild raccoon problem at the zoo, **the most likely source of infection of the anteaters was from contact with wild raccoons**.

As far as we could determine, **this is the first description of *Canine distemper virus* infection in anteaters** and has implications for other zoos around the country for vaccinating anteaters against CDV.

Comparison of different cell types for the growth of *Avian metapneumovirus* type C

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This study was conducted to evaluate 17 different cell types for the growth of a Minnesota strain of *Avian metapneumovirus* (AMPV) type C. As reference, Vero cells were also used since they are commonly used for the propagation of AMPV. The virus was inoculated into these cell types and virus growth was monitored by the development of cytopathic effects (cpe) followed by indirect immunofluorescence.

Virus growth was obtained in 6 of 17 cell types tested, with the highest virus titers being observed in BGM (baby grivet monkey kidney) and DF-1 (continuous cell line of chicken embryo fibroblast) cells. Flow cytometric analysis of cells at 72 h post inoculation indicated the highest number of infected cells in BGM cells followed by QT-35 cells (Japanese quail fibrosarcoma). At 48 h post inoculation, DF-1 and BGM cells showed the highest number of infected cells.

T**hese results suggest that, in addition to Vero cells, BGM, QT-35, and DF-1 cells can be used for the propagation of AMPV-C.**

Bluetongue in sheep in Colorado: a case report

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Bluetongue (BT) is caused by *Bluetongue virus* (BTV), family Reoviridae, genus *Orbivirus*, transmitted by *Culicoides* spp. gnats. Since the first isolation of BTV in the USA in California sheep in 1953, 24 serotypes have been identified worldwide of which 4 (serotypes 10, 13, 11, 17) are commonly isolated in the United States. Bluetongue causes significant morbidity in sheep. Clinical signs typically include weight loss during the acute infection, decreased wool production due to "wool break", lost income from ram sales due to interstate trade restrictions on BTV test positive animals, and decreased reproductive rates due to consequences of in utero infections and transient ram infertility.

Field investigations performed during the fall of 2006, revealed several BTV-positive bluetongue flocks along the South Platte River in Northeastern Colorado. One flock that had yearly cases of BT was examined over a 4-month period for evidence of BT infection. Two bottles of MLV BTV vaccine containing serotype 10, 11 and 17 had been administered by the owner 2 months prior to the observation of clinical signs. The purpose of this study was to provide a diagnosis.

Serum and whole blood (EDTA) samples were collected from 64 lambs, 5 ewes, and 2 rams on September 1, 2006. The serum samples were tested for antibodies to BTV by AGID. Viremia was assessed by the detection of viral RNA in whole blood samples by RT-PCR. Five RT-PCR test positive samples were tested utilizing virus isolation in embryonated chicken eggs. The BTV vaccine was tested for the presence of viral RNA by RT-PCR.

Forty-two percent of the lambs, 61% of the ewe lambs and 24% of the ram lambs were clinically affected. Clinical signs were observed in 2 yearling rams, but none were observed in the adult ewes or rams. Clinical signs in the lambs and rams included fever, coronitis, oral ulcerations, edematous ears, wool loss, and esophagitis. Sixty-four percent of animals <1 year and 71% of animals >1 year of age had antibodies to BTV. Fifty-seven percent of lambs had BTV in their blood as detected by RT-PCR at the time of the first blood collection. Virus was not isolated from any samples. The bottle of BTV vaccine administered to the ewe lambs was positive for viral RNA by RT-PCR; whereas, the vaccine administered to the ram lambs was negative.

Most of the clinically affected and viremic animals were lambs. This result was expected as lambs would be infected with BTV for the first time and had little or no immunity to the virus, despite the use of vaccines. The higher seroprevalence in adults reflects yearly BTV infection and acquired immunity. The use of MLV BTV is controversial. As found in this study where the vaccine had been stored and used as labeled, the quality of the vaccines may be poor and needs to be monitored. There is little known about the prevalence, epidemiology, and economic impact of bluetongue on Colorado's sheep industry; therefore, rational control measures for the disease are difficult to develop or recommend.

* Graduate student presentation

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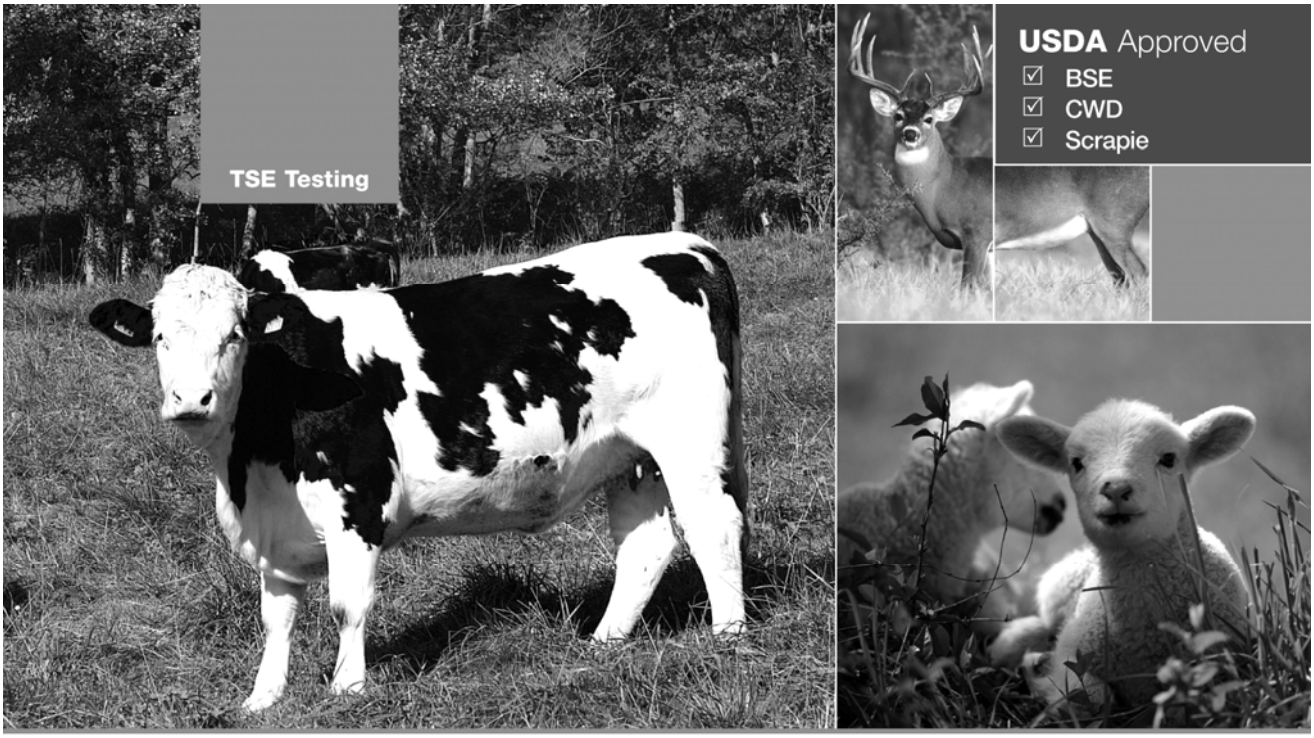

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BioSAFE Engineering LLC is a consortium of design and engineering licensed professionals whose recent technical developments were derived through years of manufacturing, installing, and servicing the majority of state-of-the-art equipment presently in operation today worldwide.

BioSAFE Engineering areas of expertise include high containment pass-through Biological Tissue Disposal Systems and Sterilizers, total Laboratory Effluent Management, Red-Bag Hospital Waste Disposal, rendered Specified Risk Materials (SRM), and human cadaver alternative processing.

BioTrove

Booth **116**

12 Gill Street #4000
Woburn, MA 01801
www.biotrove.com
Contact: Annita Dennis
781-721-3623
adennis@biotrove.com

BioTrove provides micro- and nano-scale platforms for the advancement of life science and drug discovery research. The OpenArray™ Platform provides a nano-fluidic solution for massively parallel, low-volume reactions. OpenArray is available for SNP Genotyping, Endpoint Analysis and Real-Time qPCR.

Calbiotech, Inc.

Booth **113,115**

10461 Austin Dr.
Spring Valley, CA 91978
www.calbiotech.com
Contact: David Barka
619-660-6162
dbarka@calbiotech.com

Calbiotech provides ELISA, Chemilumenescent ELISA (CELISA) and RIA kits for testing of different animal species. Products categories include Thyroid, Fertility, Cancer, and Steroid markers. Calbiotech can custom design products to meet customer needs. Our FT4 dialysis by RIA is used by many reference labs. We also offer ELISA kit for Horse Cortisol, Sheep ACTH, Sheep Cortisol and others.

Centaur, Inc.

Booth **217**

P. O. Box 25667
Overland Park, KS 66225-5667
www.centaurunavet.com
Contact: Mark Metrokotsas
913-390-6184
centaurunavet@aol.com

Centaur sells diagnostic products converted from human healthcare to equine applications. Examples include our 45-minute equine infectious anemia test, our 10-minute influenza test, a test for IgG in foals, and tests for two different blood reagents.

Centaur's newest pharmaceutical products include three different strengths of iodine formulations, two formulations of isopropyl alcohol, three different topical sprays, bulk liquids to treat metabolic disorders, serve as laxatives, preserve tissues and to serve as delivery vehicles for custom-compounded medicinals. Centaur also has creams to treat inflamed, abraded tissue and solutions and stains to help identify parasites.

Cepheid

Booth **214**

904 Caribbean Drive
Sunnyvale, CA 94089
www.cepheid.com
Contact: Barbara Colletta
408-400-8456 408-718-4425
barbara.colletta@cepheid.com

Cepheid develops, manufactures, and markets fully integrated systems for genetic analysis including DNA or RNA analysis, in the clinical, life sciences and biothreat markets. Our systems enable rapid, sophisticated genetic testing by automating otherwise complex manual procedures.

Corbett Robotics, Inc.

Booth **209**

185 Barry Street Suite 5200
San Francisco , CA 94107
www.corbettlifescience.com
Contact: Dale Levitzke
415-348-1166
dale_levitzke@corbettrobotics.com

Corbett Robotics provides innovative instrumentation for the Life Sciences. Our product line is comprised of Automated Extraction Robotics, Automated PCR Workstations, and the Roter-Gene Real-Time Analyzer. We recently received the 2006 Frost & Sullivan Technology Innovation award for our Roter-Gene 6000 product line including High Resolution Melting (HRM).

Crawford Industrial Group

Booth **210**

9101 Parkers Landing
Orlando, FL 32824
www.animal-cremation.com
Contact: Brian Gamage
407-851-0993
gamage@crawfordequipment.com

Since 1974, Crawford Industrial Group has equipped thousands of animal care, research, and control facilities with incineration/cremation systems which meet their unique operational demands and professional standards. Every one of our incineration systems meets the most stringent Federal EPA and State environmental requirements. Underwriters Laboratories (UL) has thoroughly tested and approved many of our products for your peace of mind and safety. We offer both “random feed” and “batch loading” incineration systems, ideally designed to perform large carcass and communal disposals as well as individual cremations. Visit our booth and speak with our representative to receive your free brochure and product literature.

Global Vet Link

Booth **314**

2625 N. Loop Dr. #2130
Ames, IA 50010
www.blobalvetlink.com
Contact: Sharon Eddie
515-296-0860
seddie@globalvetlink.com

Company Representatives: Sharon Eddie, Kevin Maher.

GlobalVetLink is the nation's innovator of moving a paper-based regulatory process to real-time, online technology since 1999. We have improved the efficiency of the regulatory work-flow and business process!

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Fast, secure, online.....we make paperwork, paperless.

Harley Ellis Devereaux

Booth **117**

26913 Northwestern Hwy. #200
Southfield, MI 48034-3476
www.harleyellisdevereaux.com
Contact: Nicholas F. Raab, PE
248-262-1500
nfraab@hedev.com
Other Offices: Chicago, IL; Los Angeles, Riverside & San Diego, CA

Value-Added/Value-Enhanced

Complex design solutions require contributions from multiple areas of technical expertise. Harley Ellis Devereaux’s depth of full- service experience offers clients single-source accountability. Being both a big "A" and big "E" firm, Harley Ellis Devereaux has devoted considerable resources to developing both our architecture and engineering capabilities for science and research facilities. As a result, the solutions we develop are more comprehensive and have the appropriate amount of design and engineering required for this complex facility type. Each Harley Ellis Devereaux client receives the technical leadership and innovation expected from a national Architecture and Engineering firm.

Harley Ellis Devereaux has been involved with more than 12,000,000 sf of complex lab space in the last 10 years. This includes a focus on AHDL and large animal containment facilities. In addition to the direct project experience we are also involved with a number of national technical committees including:

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- NFPA Fire Safety for Laboratories
- NSF International - Testing & Certification of Biosafety Cabinetry

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Our team understands the economies associated with interdisciplinary discovery and leveraging new knowledge across departmental lines. Our approach, rooted heavily in customer/user and designer interaction, recognizes that we must bring technical and economical points of view to the design process.
Harley Ellis Devereaux is registered to ISO 9001.
Please visit www.harleyellisdevereaux.com for recent experience and additional information.

Hydrol-Pro Technologies, Inc.

Booth **106**

37842 Avalon Drive
Zephyrhills, FL 33541
www.hydrolpro.com
Contact: Kim Etherington
813-780-6753
kime@pharmamedsci.com

Booth personnel Kevin Morris, Kim Etherington, Mark Muth.

Hydrol-Pro Technologies featuring the BioMEER system of advanced alkaline hydrolysis for carcass processing for destruction of TSE's and recovery of energy from carcass waste. Dry or wet output versions available. A low-cost, effective alternative to incineration. New Bio-effluent De-con systems are also now offered.

IDEXX Laboratories

Booth **203,205**

One Idexx Dr.
Westbrook, ME 04092
www.idexx.com/production
Contact: Contact: Mary Spear
207-856-8059 207-838-0792
mary-spear@idexx.com

The Production Animal Services (PAS) Division of IDEXX Laboratories, Inc. develops, manufactures and sells over 60 different ELISA, and PCFIA-based detection systems for animal health and quality assurance applications. Laboratories around the world use IDEXX PAS products for the detection and monitoring of diseases affecting livestock and poultry, and our xChek* data management software simplifies and organizes test results. One of the latest additions to the PAS product portfolio is the IDEXX HerdChek* BSE-Scrapie Antigen EIA Test Kit. This second-generation test is USDA and European Commission approved for use in Europe. *HerdChek and xChek are trademarks or registered trademarks of IDEXX Laboratories, Inc. in the United States and/or other countries.

Immuno-Mycologics, Inc.

Booth **318**

2700 Technology Place
Norman, OK 73071
www.immy.com
Contact: Scott Bauman
405-360-4669
scbauman@immy.com

Since 1979, Immuno-Mycologics, Inc. (IMMY) has become "The Fungal Diagnostic Specialist" providing superior quality and reliable service. IMMY's products utilize conventional serological methods (Immunodiffusion, Latex Agglutination and Complement Fixation) for the antibody and/or antigen.

Inverness Medical Professional Diagnostics

Booth **110**

10 Southgate Rd.
Scarborough, ME 04074
www.invernessmedicalpd.com
Contact: Sandra Rodgers
207-730-5723
sandra.rodgers@invmed.com

Inverness Medical Professional Diagnostics is committed to being the global leader in developing and marketing diagnostic tests that provide a foundation for diagnosis and treatment decisions. BinaxNOW® products include the BinaxNOW® Influenza A & B Test, a rapid *in vitro* immunochromatographic membrane assay for the qualitative detection of influenza A and B nucleoprotein antigens.
This product is not available in all areas. Email: AnimalHealth@invmed.com Tele: +1 207 730 5722

Laboratory Automation Solutions, Inc. (LAS)

Booth **207**

613 Rustic Trail
Beavercreek, OH 45434
www.labsutomation.com
Contact: Beverly Storch
937-431-9257
bev@labautomation.com

Laboratory Automation Solutions, Inc. (LAS) specializes exclusively in the laboratory automation market and is one of very few vendor-independent Laboratory Information Management System (LIMS) consulting companies in the US. LAS has performed requirements analyses, developed RFPs, assisted in vendor source selection activities and supported LIMS implementation products for Federal veterinary, clinical, and EPA laboratories, State and Local Clinical, Environmental, and Forensic Laboratories, and a myriad of commercial testing facilities. In addition, LAS works daily with laboratories across the country and abroad, solving problems, implementing quality assurance and business process improvement plans, and assisting in certification and accreditation endeavors including the AAVLD *Essential Requirements*, ISO 17025, and NELAC, etc.

Midland BioProducts Corp.

Booth **403**

P. O. Box 309
Boone, IA 50036
www.midlandbio.com
Contact: Richard Harrington
515-432-7799
sales@midlandbio.com

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National Institute for Animal Agriculture (NIAA)

Booth **108**

1910 Lyda Ave.
Bowling Green, KY 42104
www.animalagriclture.org
Contact: Michele Vice-Brown
270-782-9798
mvisebrown@animalagriculture.org

Company Representatives: *Michele Vise-Brown, Gale Johnson.*

The National Institute for Animal Agriculture (NIAA) is a forum for building consensus and advancing solutions for animal agriculture and to provide continuing education and communications linkages to animal agriculture professionals. Our mission truly reflects NIAA - where it's been, where it is, and where it is going. NIAA is the only forum where producers/owners (cattle, equine, poultry, small ruminants and swine), scientists (corporate, academia and government), veterinarians (private practice, industrial and government), regulators (state and federal), and business executives (corporate and association) meet in a common effort to deal with shared issues and opportunities. Visit our website at www.animalagriculture.org

Prionics USA, Inc.

Booth **400**

9810 Hupp Drive
LaVista, NE 68128
www.prionics.com
Conact: Fabio Rupp
888-205-0807
fabio.rupp@prionics.com

Based in Zurich, Switzerland, Prionics is one of the world's leading providers of farm animal diagnostic solutions and is a recognized center of expertise in BSE and prion diseases. Founded in 1997 as a spin-off from Zurich University, Prionics researches and markets innovative diagnostic solutions for major farm animal diseases, thereby making a major contribution to the protection of consumer health.

In 2005, Prionics acquired Pfizer Animal Health's diagnostic portfolio and, in 2006, entered into a strategic partnership with the Animal Science Group of the University of Wageningen (Netherlands). Winner of the Swiss Economic Award for "Company of the year" (2002) and the European Biotech Award for "Excellence in Biotech Business" (2004), in 2006 Prionics was nominated as the world's best animal health company.

The Company operates Research and Development facilities in Switzerland and the Netherlands and has regional hubs in key markets such as Argentina, Germany, Italy, the Netherlands, and the USA. Prionics is also represented by distribution partners around the world.

Qiagen, Inc.

Booth **304,306**

19300 Germantown Road
Germantown, MD 20874
www.qiagen.com
Contact: Pam Daniels
240-686-7688
pam.daniels@qiagen.com

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Quality Systems Integrators

Booth **206**

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Contact: Marti Turocy
610-458-0539
martiturocy@qsi-inc.com

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Booth **404**

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Roche Applied Science supplies innovative reagents and instrumentation for genomics research, including the LightCycler Instrument for qPCR and mutation detection; MagNA Pure LC and MagNA Pure Compact Instruments for automated nucleic-acid purification; Genome Sequencer 20 Instrument with new kits for amplicon sequencing; and the Universal ProbeLibrary for qPCR.

Saunders/Mosby/Churchill

Booth **114**

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Roseville, CA 95747
www.us.elsevierhealth.com/rep/dculp
Contact: David Culp
916-787-1041
d.culp@elsevier.com

Saunders/Mosby has the latest in laboratory and pathology references, including the new edition of Jubb, Kennedy and Palmer's *Pathology of Domestic Animals.*

Synbiotics Corporation

Booth **316**

600 Broadway #490
Kansas City, MO 64105
www.synbiotics.com
Contact: John Donovin
816-460-3424
customerservice1@synbiotics.com

Synbiotics Corporation is a leading provider of veterinary diagnostics and reproductive products and services. Learn about the DiroCHEK Heartworm, ViraCHEK Product Line, OVUCHECK Premate and our innovative TiterCHEK CDV/CVP. We look forward to discussing the easy and accurate Witness Heartworm test, along with our whole Witness product line. Make sure to ask us about the Synbiotics Repro Network and Freeze Center services, training, and products including Status-LH (Luteinizing Hormone diagnostic) and D-Tec CB (canine brucellosis) tests.

Tetracore, Inc.

Booth **401**

9901 Belward Campus Dr. #300
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www.tetracore.com
Contact: Marcie Newman
240-268-5400
mnewman@tetracore.com

Company Representatives: Dr. William Nelson, Dr. Beverly Mangold, Dr. Johnny Callahan, Tracy Fecteau, John Kelly, Marcie Newman.

Providing Advanced Molecular and Immunological Detection.

Tetracore is a leading biotechnology company providing innovative diagnostic assays and reagents for infectious diseases. The Tetracore VetAlert™ product line features real-time Polymerase Chain Reaction (PCR) test kits and reagents for rapid and sensitive detection of animal pathogens. USDA licensed test kits are available for Johne's disease and *Classical swine fever virus* (CSFV), in addition to specific detection reagents for *Porcine reproductive and respiratory syndrome virus* (PRRSV), *Foot-and-mouth disease virus* (FMDV), *West Nile virus*, *African swine fever virus* (ASFV) and more.

Trek Diagnostic Systems

Booth **105,107**

982 Keynote Circle #6
Cleveland, OH 44131
www.trekds.com
Contact: Carlene Simmons
216-351-8735 x109
csimmons@trekds.com

TREK Diagnostic Systems is a dedicated microbiology company, providing innovative, automated products to the microbiology laboratory. TREK's products provide cost-effective improvements in workflow efficiencies, ergonomics, and test result performance.

USDA National Animal Health Emergency Response Corps (NAHERC)

Booth **213**

700 N. Fairfax Street, Ste 500
Alexandria, VA 22314
www.aphis.usda.gov/vs/ep/naherc/
Contact: Jennifer Norris
703-838-9201
jnorris@excaliburassociates.com

In 2001, the USDA's Animal and Plant Inspection Service (APHIS) established the National Animal Health Emergency Response Corps (NAHERC) to respond to exotic disease outbreaks and other disasters which affect livestock, poultry, companion animals, and wildlife. More volunteers are urgently needed to assure a decisive response to any potential health crisis.
]

Ventana Medical Systems, Inc.
Booth **208**

1910 Innovation Park Dr.
Tucson, AZ 85737
www.ventantamed.com
Contact: Terry Haikara
800-227-2155 x 3867 520-906-2952
thaikara@ventanamed.com

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Ventana Medical is one of the world's leading developers and manufacturers of medical diagnostic instrument and reagent systems providing leading-edge automation technology for use in slide-based diagnosis of cancer and infectious disease. Our products are found in hospital-based histology laboratories, independent reference laboratories, and the drug discovery laboratories of some of the world's largest pharmaceutical and biotechnology companies, government labs, veterinary labs, and medical research centers. We serve our customers through wholly owned subsidiaries in the USA, Europe, Japan and Australia, and our instruments are installed in no fewer than 55 countries.

Veterinary Laboratories Agency
Booth **109**

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VMRD, Inc.
Booth **118**

4641 Pullman-Albion Rd.
Pullman, WA 99163
www.vmrd.com
Contact: Luke Brown
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luke@vmrd.com

VMRD, Inc., a family business that was started in Pullman, Washington in 1980, develops and manufactures high-quality, USDA-licensed diagnostic test kits for infectious disease agents such as Anaplasma, BLV, BTV, CAEV, EIAV, MCFV, and Neospora; research reagents such as FA conjugates, IFA slides and controls, as well as a wide selection of monoclonal and polyclonal antibodies against infectious agents, immunoglobulins, CD markers, MHC antigens, cytokines, and hormones. VMRD also produces an array of RID kits for quantitative analysis of immuno-globulin concentrations in many species, and other immunological assays such as Coombs' and FPT kits.

VMRD's commitment to high-quality products, friendly customer service, and excellent technical support has contributed to our continual growth. Visit www.vmrd.com for more information.

VMRD, Inc., is proud to announce acquisition of American BioResearch (ABR, Inc.). ABR conducts 9 CFR and EMEA extraneous virus testing on cell lines, virus seeds, cell culture reagents and supplements, and other virology related services. ABR and VMRD have worked together for many years and we are dedicated to continuing to refine ABR's services. Visit www.ambio.net for more information.

Whatman, Inc.
Booth **218**

200 Park Ave., Suite 210
Florham Park, NJ 07932
www.whatman.com
Contact: Carol Mueller
973-245-8300
carol.mueller@whatman.com

Whatman FTA® Technology is a quick and easy way to collect DNA samples at room temperature. Blood, buccal or tissue samples are applied to FTA or FTA Elute cards in the field and sent to testing labs by mail. The DNA is purified for analysis in less than 30 minutes. FTA is a cost effective way to collect valuable genetic data for livestock marketing, breeding decisions and molecular diagnostics.

Upcoming AAVLD/USAHA meetings:

2008: October 23-29 The Sheraton
Greensboro, North Carolina

2009 October 7-14, Town and Country
San Diego, California

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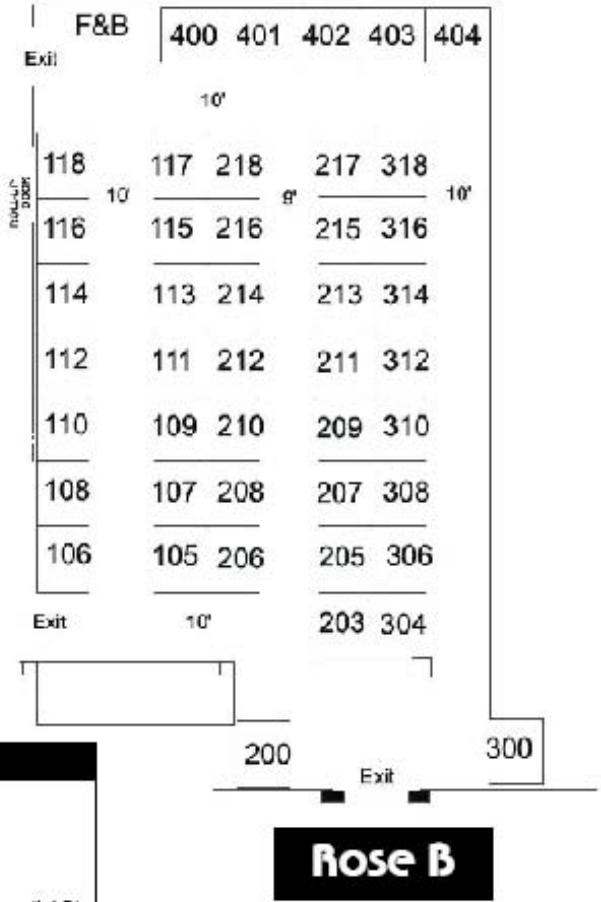

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