New Opportunities in Diagnostics

Your quality source for export testing

With the surge in world trade during the past decades, U.S. agricultural exports have grown more than 15 times since 1971. Exports now account for 31 percent of all of U.S. agriculture's cash receipts, according to Tim Larsen, senior international marketing specialist for the Colorado Department of Agriculture. And Colorado companies are getting in on the trend, now selling more than $1.6 billion worth of products into 99 countries, Larsen reports. Furthermore, the increase is expected to accelerate for state agricultural producers, as Colorado governor John Hickenlooper’s administration has stated its goal is to grow Colorado agricultural exports by 40 percent during the next four years.

As a result, in the past few years, CSU VDL has seen increased requests for export testing of cattle and horses. These requests range from a few or small groups of horses to large numbers of cattle—in some cases more than 2,000 head. The requests may be to establish breeding stock in foreign countries or for the sale of valuable animals to new owners. Requests have come to ship cattle to foreign countries, including Iran, Turkey and Russia. This trend is likely to continue and increase in the future.

With the increasing attention to exports and the need for reliable testing and certification, it's a good opportunity to remind cattle producers and horse owners planning on exporting live animals to be sure to use a laboratory that is USDA approved for the testing protocols. CSU VDL is approved to test for the commonly requested bovine and equine diseases that must be done in a USDA-approved laboratory (see sidebar at left).

Volume discounts may also be arranged. The Fort Collins Laboratory, the Rocky Ford Branch Laboratory and the Western Slope Branch Laboratory can all participate to provide rapid quality results by a USDA approved laboratory. USDA approval ensures quality results, as we are required to pass yearly proficiency tests to ensure that our results are accurate and reliable. Note the “Six Steps” on the following page to help us meet your needs in a timely fashion with quality results.

— Barbara Powers, DVM/PhD/DACVP, CSU VDL Director

SPECIAL ISSUE: FOCUS ON REGULATORY COMPLIANCE TESTING
Six steps to help navigate the regulatory compliance maze

As an AAVLD-accredited lab like CSU Veterinary Diagnostic Laboratories, we are more than a testing laboratory. Our base of expertise can help you understand the unique compliance requirements of testing for issues such as export. Here are six tips from CSU VDL to help navigate that often complicated maze.

1. Obtain the specific test requirements for the importing country, including the specific details of the tests. For example:
   - Does the importing country require virus isolation, or will it accept a PCR test result? In general, virus isolation can take weeks to complete, whereas, PCR tests take only two to five days.
   - What is the requirement for the minimum dilution of serum for serology tests? For example, the vesicular stomatitis virus SN titer is routinely performed at CSU VDL beginning with a serum dilution of 1:8; however, the European Union requires the SVS SN titer be determined beginning with a serum dilution of 1:12. EU will refuse a negative result at 1:8.
   - Is the time frame from sample collection until the test results are reported realistic relative to the date the animal is going to be shipped? For example, if the need for VSV SN titer results is within 10 days from the time of serum collection, and the time it takes to ship the serum to the lab and for the test to be performed and reported is close to 10 days, then you are taking a risk the results will not be reported in time for the animal to be shipped.

2. Call the lab ahead of time to make sure the required tests are offered and that they can be done in a time frame that will work for your client.

3. Call the lab when submitting large numbers of samples so the necessary reagents are available when samples arrive.

4. Indicate the required animal identification for each animal or semen sample on both the sample tube and on the submission form.

5. Discuss with your client what options exist — if any — if the test results are positive. For example, a clinically normal horse may be incidentally positive for WNV IgM antibodies by the ELISA test, which may mean forfeiting the price of the plane ticket.

6. Choose an AAVLD-accredited and USDA-approved laboratory like CSU Veterinary Diagnostic Laboratory.

— Hana Van Campen, DVM, PhD, DACVM, CSU VDL Virology Section Head

Discuss with your client what options exist — if any — if the test results are positive.

The Veterinary Diagnostic Laboratories at Colorado State University are a member lab of the American Association of Veterinary Laboratory Diagnosticians and are AAVLD-accredited. AAVLD Accreditation is based on the internationally recognized ISO/IEC 17025 standard and consistent with the World Organization for Animal Health (OIE) Quality Standard for Veterinary Laboratories. Accreditation is a formal recognition of the competency of laboratories and increases client confidence in diagnostic test results. In order to further demonstrate technical competence between accreditation assessments, personnel from accredited laboratories are also required to participate in relevant proficiency testing programs. Accreditation contributes to continuous improvement and tool that can be used to increase laboratory efficiency, which is critical in times of emergency or limited funding.

Laboratories participating in the USDA’s National Animal Health Laboratory Network may be involved in surveillance for early detection of foreign animal disease, surge testing during an outbreak, and testing samples during the outbreak recovery phase. As such, there must be a high degree of confidence in the quality of the laboratories and associated test results. USDA recognizes the value of quality management systems and requires that all NAHLN laboratories have a functional quality management system. Laboratories that are fully accredited by AAVLD are admitted to the NAHLN without additional requirements related to documentation of a quality management system.
Reportable Disease Update

We can help you sort out EP testing

Five Moffat County horses reported to have tested positive for equine piroplasmosis (EP) in early March has continued to keep this reportable disease in the spotlight for state practitioners and regulatory veterinarians. All of these recently identified horses originated from the same training facility in California, identified through EP trace-out activities from another state. So far we have been notified of no transmission of this disease to any horses in Colorado. All of the EP test-positive horses were quarantined and eventually euthanized. All cohort horses have been tested and confirmed as negative.

The Veterinary Diagnostic Lab at CSU can help you sort out EP testing requirements and options. USDA has authorized CSU VDL to perform the cELISA tests for *Theileria equi* and *Babesia caballi* for the intra- and interstate movement of equids not displaying clinical signs of piroplasmosis. Tests are run each Friday, but can be run stat. Submit 1 ml of serum, and be sure to include the horse’s identification. Samples must be submitted by an accredited veterinarian. Cost is $16 per sample.

Equine piroplasmosis is a reportable disease and leads to regulatory consequences. A quarantine and subsequent disease-control plan for any test-positive horses is determined by the State Veterinarian of Colorado, USDAAPHIS-VS, along with input from the owners. Currently, there is no vaccine or approved treatment for EP in the United States. The Colorado Department of Agriculture reports that many race tracks across the country are requiring horses that enter their grounds to be negative. Arapahoe Park Racetrack in Aurora has instituted EP testing requirements for all horses entering the track facilities. Horses must be *T. equi*- and *Babesia caballi*-negative within 30 days of admission to Arapahoe Park.

In addition, some states have initiated EP testing as part of their state import requirements. Many countries also require a negative EP test on horses that are imported from the United States. Before writing a health certificate, it is important to research the import requirements of the destination to which your client is transporting the horse, whether it is for racing, import to another state, or for international transport.

Since 2008, EP-infected horses have been found in several states. Horses that test positive for the disease are quarantined, euthanized, enter an experimental treatment program, or exported to a country that will accept EP-positive horses. Any horses that have had contact with infected horses are tested and USDA’s Animal and Plant Health Inspection Service (APHIS) has developed strict guidelines for managing infected and exposed horses.

EP is a blood-borne parasitic disease affecting horses, ponies, donkeys, mules, and zebras. A high percentage of horses that test positive for the infection may not show clinical signs, and horses with persistent EP infections are carriers of the parasites that cause the disease and are potential sources of infection to other horses.

Questions? Contact the State Veterinarian’s Office at (303) 239-4161

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FOCUS ON COMPLIANCE

A reminder also that CSU VDL is one of only 18 laboratories approved by the National Veterinary Services Laboratory (NVSL) to conduct testing of animals for contagious equine metritis (CEM). Following an eight-state outbreak in December 2008 that involved 23 stallions and five mares, the quarter horse industry has shown increased interest in response to animals that have tested positive for the organism *Taylorella equigenitalis*. Testing protocols vary depending on whether the testing is part of a traceback investigation, if the tests are for clients who want testing to assuage fear of exposure, if it is for routine surveillance, or for export. Testing protocols require specific media, timelines and reporting.

From Jan. 1 to May 15, CSU VDL has run a total of 708 tests for EP.

Tests are run each Friday, but can be run stat upon request.

Samples must be submitted by an accredited veterinarian.
 Conjunctivitis and keratitis are associated with a variety of bacterial and viral organisms. It’s important to determine which organism is involved so appropriate treatment can be instituted.

CSU VDL offers diagnostic panels of PCR tests and aerobic bacterial culture for conjunctivitis and keratitis for different species at flat-rate panel prices that are significantly lower than pricing each test separately. Here’s how to get the most out of your pink-eye panels:

- Sample individual animals.
- For PCR tests, swab the inside of the lower eyelid and the third eye lid with a sterile Dacron or cotton swab, and place the swab in a sterile, sealable tube (a red-topped blood collection tube or a “snap-cap” tube) with approximately 0.5 mL of sterile water or saline. The liquid keeps the swab moist and will aid in the retrieval of cells and material from the swab.
- For aerobic bacteriology culture, swab the conjunctivae with a Copan bacteriologic culturette, and place the swab into the media within the culturette tube.
- Please ship the tubes with a cool (blue ice pack) by overnight delivery.

### Differential Diagnostics

**Conjunctivitis panels to pinpoint therapy**

— Jeanette V. Bishop, MS, CSU VDL Molecular Diagnostics Research Associate

<table>
<thead>
<tr>
<th>TESTS INCLUDED</th>
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<tr>
<td><strong>Bovine/Bison</strong></td>
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<tr>
<td>BHV-1 PCR</td>
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<tr>
<td>Chlamyphila PCR</td>
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<td>Mycoplasma PCR</td>
<td></td>
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<tr>
<td>Malignant Catarrhal Fever PCR</td>
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<tr>
<td>Bacterial culture</td>
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<td><strong>Camelid</strong></td>
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<td>Chlamyphila PCR</td>
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<td>Bacterial culture</td>
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<tr>
<td><strong>Canine</strong></td>
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<td>CDV PCR</td>
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<td>Chlamyphila PCR</td>
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<tr>
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<tr>
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<tr>
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<tr>
<td>Feline Herpesvirus PCR</td>
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<td>Mycoplasma PCR</td>
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<tr>
<td><strong>Ovine/Caprine</strong></td>
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<td>Mycoplasma PCR</td>
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<tr>
<td>Bacterial culture</td>
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**LABORATORY PERSONNEL UPDATE**

**Sushan Han** joined the faculty in January as an assistant professor at the CSU Veterinary Diagnostic Laboratory with dual appointment in the Department of Microbiology, Immunology and Pathology. An anatomic ACVP-board certified veterinary pathologist, Dr. Han received her DVM and did her residency training, post-doctoral appointment and graduate work in infectious diseases and immunology at Washington State University in Pullman. She received her bachelor of science degree in wildlife management from University of Idaho. Her special interests encompass wildlife diseases, zoonotic diseases, immunology and teaching. She looks forward to being a part of the outstanding group at the veterinary diagnostic laboratory, and is enthusiastic about the opportunities and challenges of an appointment at CSU. Originally from Boise, Idaho, Dr. Han is passionate about anything involving the outdoors and sunshine.
Choose from Johne’s test options

The Veterinary Diagnostic Laboratory at Colorado State University offers multiple choices when it comes to diagnostic tests for *Mycobacterium avium* ssp. paratuberculosis. Contact us about the options available to best monitor and benchmark this important herd disease:

- **CSU VDL** once again has passed the individual 2010 fecal proficiency panel for *M. avium* ssp. paratuberculosis using ESP liquid media. This allows us to conduct official testing for the National Johne’s Program using this method until the end of 2011. This method uses a liquid based system that allows for faster detection than culture using solid media — an average of 36 days vs. an average of 12 to 16 weeks, respectively. Using this method, we can grow the organism faster and use PCR to determine if it is *M. avium* ssp. paratuberculosis rather than another species of *Mycobacterium*.

- The CSU VDL also has passed the 2010 individual fecal proficiency panel for *M. avium* ssp. paratuberculosis using solid culture. Solid culture allows us to conduct official testing based on a solid culture system. This diagnostic test allows the differential growth of different species of *Mycobacterium* as well as quantification of the amount of growth seen in the culture. Results for this test typically are available in 12 to 16 weeks.

- In addition, the CSU VDL has passed the 2010 direct fecal PCR for *M. avium* ssp. paratuberculosis. This allows us to conduct official testing for the organism directly from a fecal sample without the delay of enrichment culture. Results for this test typically are available in less than one week after the samples have arrived at the laboratory.

- **CSU VDL** has passed the 2010 serological (ELISA) proficiency tests for *M. avium* ssp. paratuberculosis, as well. This allows us to conduct official testing for a serological response to the organism from blood as well as milk samples. These results typically are available in less than one week after the samples have arrived at the laboratory.

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### CSU VDL ON THE ROAD: UPCOMING CONFERENCES, SYMPOSIA AND OTHER APPEARANCES

**Jim Kennedy**, Director of the CSU VDL Rocky Ford Branch will give a 30-minute presentation on providing an adequate history for diagnostic workups of hypoaanamnesis in multiple species, at the Colorado Veterinary Medical Association 2011 Convention, Sept. 15 through 18, Keystone Resort and Conference Center. For details: myasadler@colovma.org

**Barb Powers**, CSU VDL Director, Kristy Pabilonia, VDL Avian Diagnostics and BSL3 Operations Section Head, and Sushan Han, assistant professor, will also be attending the Colorado Veterinary Medical Association 2011 Convention, Sept. 15 through 18.

**Barb Powers**, CSU VDL Director, and Jim Kennedy, Director of the CSU VDL Rocky Ford Branch, will be available to meet you and answer questions at the Lab’s trade show booth at the 144th annual convention of the Colorado Cattlemen’s Association, June 20 through 22 in Steamboat Springs. See ColoradoCattle.org for details.

**Barb Powers**, CSU VDL Director, will attend the annual convention of the Colorado Livestock Association, June 23 and 24 in Broomfield. Stop by the trade show booth to talk with her. ColoradoLivestock.org for details.

**Barb Powers**, CSU VDL Director, **Kristy Pabilonia**, Avian Diagnostics and BSL3 Operations Section Head, **Lora Ballweber**, VDL Parasitology Section Head, **Doreene Hyatt**, Bacteriology Section Head, and VDL Pathologists **Tawfi k Aboellail** and **Sushan Han** will all be in attendance at the annual meeting of the American Association of Veterinary Laboratory Diagonsticians, Sept, 29 through Oct. 5 in Buffalo, NY. Go to AAVLD.org for details.

**Lora Ballweber**, CSU VDL Parasitology Section Head, will present original research on the comparison of two fecal flotation techniques for the detection of gastrointestinal parasites of dogs and cats at the July 16 through 19 joint meeting of the American Association of Veterinary Parasitologists, Livestock Insect Workers Conference and the International Symposium on Ectoparasites in Saint Louis. See AAVP.org for details.

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### Lab Updates

**FOCUS ON COMPLIANCE**

- **Liquid culture**
  - $25 per sample
  - 30-42 day turnaround
- **Solid culture**
  - $22 per sample
  - 12-16 week turnaround
- **Direct PCR**
  - $30 per sample
  - About 1 week turnaround
- **Submit feces or tissues**
- **ELISA**
  - Submit 1mL serum
  - **Volume discount available**
  - Call us for details

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— Doreene Hyatt, PhD, CSU VDL Bacteriology Section Head
Bovine Testing Options

Pooled testing on three fronts to improve test affordability

--- Jim Kennedy, DVM, MS, Director, CSU VDL Rocky Ford Branch

The CSU Veterinary Diagnostic Laboratory at Rocky Ford offers pooled PCR testing strategies for three bovine diseases, *Mycobacterium avium* subsp. paratuberculosis (Johne’s Disease), BVDv persistent infection (PIs), and *Trichomonas foetus* (*T. foetus*). The protocols and validation for BVDv PIs and for *T. foetus* were formulated and accomplished at the CSU VDL Branch Lab at Rocky Ford. The protocol for Johne’s was published and dispersed to participating laboratories by the National Veterinary Service Lab at Ames, Iowa (NVSL). PCR pooling strategies are the result of applying the high analytical sensitivity of PCR to diagnostic specimens for detecting the etiological agents associated with these diseases.

**POOLED FECAL TESTING FOR JOHNE’S**
The Rocky Ford Laboratory successfully completed the NVSL proficiency test for pooling fecal samples to detect Johne’s and now offers it to our clients as an alternate method of testing for Johne’s disease. Pooled Johne’s testing has not been requested extensively; however, for the purpose of discussing pooled PCR testing strategies, a brief discussion of it use and limitations in detecting Johne’s infected cows is relevant.

Feces contain substances that are known to inhibit the PCR reaction. So, the development of a method of extracting the organism’s nucleic acid has been a challenge in using molecular diagnostics to detect *Mycobacterium avium* subsp. paratuberculosis (MAP). TetraCore® when coupled with VetAlert™ was shown to be effective in extracting and detecting MAP in pools containing up to five fecal samples, with little to no sensitivity loss over individual animal testing.

**BVD PI POOLED SCREENING**
The original development and validation of pooled BVD PI PCR testing began in 2005, with the initial article published in the *Journal of Veterinary Diagnostic Investigation* in 2006. A second article appeared in the *Journal of the American Veterinary Medical Association* in November 2006 which expanded the data set and further validated the test method. Since then, the continued pooling of ear notches and PCR testing has been monitored. The table below reflects the data observed in the past five fiscal years. The pooled PCR is used as a screen and the individuals are identified using AC-ELISA.

<table>
<thead>
<tr>
<th>Fiscal year</th>
<th>BVD PCR pools</th>
<th>BVD PCR positive pools</th>
<th>Pool size</th>
<th>Number tested in pools</th>
<th>BVD PI suspects</th>
</tr>
</thead>
<tbody>
<tr>
<td>05-06</td>
<td>1,375</td>
<td>7.20%</td>
<td>65</td>
<td>89,851</td>
<td>0.19%</td>
</tr>
<tr>
<td>06-07</td>
<td>1,179</td>
<td>3.56%</td>
<td>59</td>
<td>69,043</td>
<td>0.22%</td>
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<tr>
<td>07-08</td>
<td>1,371</td>
<td>3.87%</td>
<td>48</td>
<td>66,373</td>
<td>0.66%</td>
</tr>
<tr>
<td>08-09</td>
<td>1,872</td>
<td>5.61%</td>
<td>39</td>
<td>73,078</td>
<td>0.49%</td>
</tr>
<tr>
<td>09-10</td>
<td>1,316</td>
<td>3.57%</td>
<td>39</td>
<td>51,375</td>
<td>0.21%</td>
</tr>
</tbody>
</table>

This technology is a significant advance in detecting MAP and classification of Johne’s herds. The methodology of pooling fecal samples, extracting MAP nucleic acid and detecting the organism’s presence by real-time PCR are outlined in the SOP from NVSL, and an annual proficiency test is available to participating laboratories. Pooling fecal samples for Johne’s testing may allow for detection before other testing methods. By pooling, costs are kept in mind.
at 0.12 percent. The NAHMS study reported results on 44,150 ear notches and utilized AC-ELISA as the test method. A comparison between the herd incidence by pooling and the herd incidence by individual testing showed no significant difference in the two methods.

Pooling helps lower the cost of maintaining a BVD surveillance program. The Rocky Ford lab charges $75 per pool, with a maximum of 50 samples per pool and no charge for identifying an AC-ELISA positive within positive pools, while a fee of $5 is charged per sample for AC-ELISA if greater than 50 are submitted. If we apply those costs to the NAHMS study, the pooled testing strategy would have cost $66,225 vs. an individual testing strategy cost of $220,750.

POOLED PCR FOR *T. FOETUS* TESTING

The protocol and laboratory validation of pooled *T. foetus* testing was described in the January 2008 issue of the *Journal of Veterinary Diagnostic Investigation*. Since then, more than 8,800 pools formed from more than 38,000 bulls have been tested using the pooled testing strategy. *T. foetus* has been detected in 290 pools, and from those positive pools, 732 bulls infected with *T. foetus* have been identified. The concept of pooled *T. foetus* testing has not been well accepted in states outside of Colorado, and other state laboratories have elected not to develop the pooled testing strategy. The reason for the hesitance may be attributed to the potential revenue loss comparing pooled testing to individual testing and the inability to successfully complete an intra-laboratory validation. One of the potential sources for the failure to validate pooled testing lies in not selecting an optimum DNA extraction method. The original studies and the current protocol at the Rocky Ford lab utilize a commercial extraction kit that provides a clean extract and minimizes the presence of inhibitors that may interfere with PCR. Other labs may have elected to use heat to extract *T. foetus* DNA, which produces a less optimum extract containing numerous inhibitors that may cause the PCR reaction to fail and result in a false negative diagnostic test. PCR is a highly analytically sensitive test, but only when a quality nucleic acid starter is available from an optimum extraction process. Less than optimum extraction increases the risk of test failure. As has been shown, the most critical factor in a successful test by any method is a quality sample attained by an accredited veterinarian.

Pooled testing is filling a producer request to provide affordable diagnostic tests while maintaining a high degree of accuracy. Pooling does not fit every disease, but it should be considered as part of every production health program, especially when pretest probability of infection is unknown or low. Although PCR has high analytical sensitivity it should not be expected to forgive improper sampling or any factors that may fail to provide an adequate quantity of quality nucleic acid starting material.
Validation of *Brucella ovis* ELISA serology method using field data

The *Brucella ovis* ELISA has been used to evaluate sera from sheep for antibodies to *Brucella ovis* since the 1980s. In 2006 the National Veterinary Services Laboratory, along with participating laboratories—including CSU’s Western Slope Veterinary Diagnostic Laboratory at Grand Junction—developed and evaluated a modified ELISA procedure named “NVSL SeroPro1054.” It used a new internationally accepted *Brucella* antigen (REO198) and several different reagents and ELISA plates.

We began using this procedure in June 2006 and reported our experience at the U.S. Animal Health Association meeting in October 2006, including comparative data on banked sera evaluated with both the old and new ELISA procedure. At that same meeting, scientists from NVSL presented a validation study on this new procedure involving 179 sera. They reported a test sensitivity of 100 percent, a test specificity of 98.5 percent, a positive predictive value of 99.1 percent and a negative predictive value of 100 percent.

Because of difficulties obtaining reagents, the “NVSL SeroPro1054” protocol was slightly modified. In October 2008 we and other labs began using the new standard procedure, called “NVSL SeroSOP1061.”

We have now been performing this version of the *Brucella ovis* ELISA serology method at the CSU WSVDL for more than 2.5 years and have tested more than 23,000 sheep sera. The test has performed well and is a stable and reproducible laboratory method. There have been occasional problems, as with the older *B. ovis* serologic methods, with positive or indeterminate serology results in a very low percentage of virgin rams or other rams where exposure to *Brucella ovis* seems highly unlikely. In clearly infected flocks, the current cutoff values have been useful in identifying potentially infected rams for culling.

**CURRENT TEST INTERPRETATION**

Based on our experience with the serologic test, we have interpreted the potential for an animal to be infected based on its serologic response to be as follows:

**POSITIVE.** There is a very high likelihood that an animal with a positive serologic reaction to this *Brucella ovis* ELISA test is currently infected. If a positive animal is from a flock or situation where exposure to *Brucella ovis* seems extremely unlikely—especially if this is a valuable breeding ram—retesting the animal four to six weeks later with negative or indeterminate results, combined with a physical and semen examination can provide reasonable evidence of a noninfected status. This is especially true if the S/P ratio in this animal is hovering near the cutoff value.

**INDETERMINATE.** Animals with an indeterminate serologic reaction from an otherwise serologically negative flock should be separated from the flock and, if retained, retested four to six weeks later. Upon retesting, if these animals remain serologically indeterminate or test negative, they should be regarded as noninfected animals. If there is no evidence of infection determined by testicular palpation, semen evaluation, semen culture or other appropriate methods, these animals should be considered noninfected.

If, however, indeterminate animals are identified...
in flocks where there are positives, the indeterminates should be considered as possible culls, separated from the flock and reevaluated later. We have found that approximately 50 percent of indeterminate animals in infected flocks will move into the positive range in four to six weeks, providing evidence of infection. Again, physical examination and semen evaluation are valuable to determine the true status of indeterminates.

EVALUATION OF FIELD DATA
To evaluate the ELISA method’s rate of false positives from field data, we selected from our WSVDL database a group of animals that in all likelihood were not infected. We chose the 1,679 sera from sheep located east of Colorado, including North and South Dakota, Nebraska, Kansas, Oklahoma and all states further east, excluding Texas. If the assumption is correct that these eastern flocks are free of Brucella ovis, then any positive sera from these flocks would be “false positives” and this analysis would give us valid test specificity information. If the assumption that the animals are not infected is incorrect, then the resultant test specificity values would only be higher, not lower.

Chart 1 shows the results. Of 1,679 sera, seven were positive, for a rate of 0.42 percent, and 24 were indeterminate, for a rate of 1.43 percent. These results suggest the test specificity is above 99.5 percent, and that all the positive S/P ratios were fairly low—below 1.5. This low rate of false positives is even lower than the 1 percent we observed with past ELISAs and represents a significant improvement.

Next, we evaluated serology results from the 21,360 sheep sera collected from western U.S. flocks that had been examined by the WSVDL since late 2008. Because we wanted to rigorously test for false positives, we classified flocks as “non-infected” only if they showed less than one positive serum per 50 animals tested; flocks with more than one positive per 50 tests were considered “infected.” If this classification is correct, any positive sera from the noninfected flocks would be false positives. In reality, several flocks classified noninfected were, in fact, infected flocks that had been successfully cleaned up. However, if some of the flocks classified as noninfected had a few infected animals remaining, then the test specificity would only be higher, not lower.

Chart 2 shows the results of this wider validation. Of 9,979 sera from western flocks classified as noninfected, 25 were positive, for a 0.25 percent rate, and 111 were indeterminate, or 1.11 percent. These results again suggest false positives are extremely low and the test specificity is more than 99 percent—99.75 percent from the data. The large number of flocks that could be classified as noninfected also suggests efforts to clear flocks of Brucella ovis over the past 30 years have succeeded.

Finally, we evaluated the serologic results from the Western flocks classified as “infected,” to determine the relative incidence of positive and indeterminate reactors in these flocks and to view the spread of the S/P ratios. Chart 3 shows those results. Of 11,381 “infected” sera from western flocks, 1,959 were positive, for a rate of 17.2 percent within a range of 2 percent to 51 percent. A total of 365 were indeterminate, for a rate of 3.2 percent.

The 2 percent to 51 percent spread demonstrates the high number of serologically positive animals in many infected flocks. The ratio of positive to indeterminate animals in infected flocks is about 5.4:1, slightly lower than the ratio reported with the NVSL SeroPro1054 protocol, at 7.1.

Looking at the spread of S/P ratios in infected flocks in chart 3 makes apparent that the S/P values cluster in a low range—which we can presume are noninfected animals—and then rise in almost a continuum making it difficult to clearly distinguish between infected and noninfected animals based solely on serologic data.
Cryptococcus gattii has emerged as a zoonotic pathogen that causes an air-borne, potentially fatal infection in both immunocompromised and immunocompetent individuals. After being first recognized on Vancouver Island, British Columbia, in 1999, the organism infected more than 60 people since 2004 in Washington, Oregon and California. Fifteen people have died as a result. All infected humans and animals either lived within or traveled from and to the areas where C. gattii has been established as an endemic pathogen (British Columbia and the adjoining Pacific Northwest).

Symptoms include runny nose, persistent cough and sharp chest pain associated with shortness of breath, nausea, headache, skin eruptions, meningitis or pneumonia. The spore-forming yeast can cause symptoms in people and animals two weeks to several months after exposure.

**THE ORGANISM**

_Cryptococcus gattii_ (formerly _C. neoformans_ var _gattii_) is basidomycotic yeast that is typically found in the tropics and subtropics. It has been found to be endemic to Australia and New Zealand, South and Southeast Asia, parts of Latin America, and certain parts of Europe. The origin of how _C. gattii_ strains were introduced into the Pacific Northwest and the factors that encouraged their establishment as endemic pathogens remains a mystery.

_C. gattii_ appears to differ from other cryptococcal pathogens in phenotype, natural habitat, epidemiology, clinical disease and response to antifungal treatment. Pathogenic cryptococci have been divided into four serotypes based on their polysaccharide determinants. Capsular serotypes A, B, C, and D comprise pathogenic cryptococci, but serotype B seems to be the predominant _C. gattii_ responsible for cryptococcal disease in most parts of the World and in the Pacific Northwest.

Multiple genotypes have caused infections in the Pacific Northwest, but the major strain VGIIa seems to be the predominant molecular genotype from most human, veterinary, and environmental _C. gattii_ isolates.

The organisms are usually found in the soil and on trees. They are spread through the air, fresh water and sea water. Freezing, luckily, can kill the organisms, and climatic change may be helping the spread of the fungus into biogeoclimatic zones characterized by warm, dry summer and wet winters. Colorado weather may limit the establishment of _C. gattii_ as an endemic pathogen.

**PACIFIC NORTHWEST VETERINARY CASES**

Many wild and domestic animal species contracted the infection before humans, a situation that reiterates the value of sentinel animal surveillance programs for detecting emerging diseases. To date, dogs, cats, ferrets, porpoises, camelids (llamas and alpacas), small ruminants (sheep and goat), birds, elk and horses are among the species reported to have contracted natural infection.

Identified risk factors include, but are not limited to, disturbance of soil or vegetation caused by hiking, digging, logging and construction. All might increase aerial dispersion of the organisms.

**COLORADO CASE**

A six-year-old, black male alpaca had traveled to Northern Colorado from Oregon several months prior to the onset of clinical disease, which started as skin eruptions on the lips. Papules soon ulcerated forming multifocal and coalescing ulcerative dermatitis that did not respond to antibiotic treatment for more than two months.

Progressive respiratory disease ended up in complete obliteration of the left lung, which made the referring DVM suspect a neoplastic disease. Four months into the clinical disease, the animal succumbed to worsening respiratory distress with pericardial and pleural fluid associated with chronic weight loss. The animal was submitted to the CSU VDL in November 2010 for necropsy. On necropsy, the left lung was diffusely consolidated with a large, blood-filled cystic cavity present in the middle of the cardiac lobe that was firmly adhered to the costal pleura.
Tracheobronchial and mediastinal lymph nodes were markedly enlarged and on cut surface were diffusely gelatinous with taut capsules. Also, discrete lymph nodes were visible along the ventral surface of the thoracic aorta.

Histological examination of the lungs, aforementioned lymph nodes and skin revealed mats of yeast-like organisms organized as soap bubble replacing most of the parenchyma in affected organs, including the skin. Little to no inflammation is associated with these fungal mats.

**DIAGNOSIS**

Histological examination is the gold standard for diagnosing fungal infections, especially in animals with a history of protracted clinical disease that did not respond to conventional treatment like this case. The lungs were submitted to CDC for genotyping, which confirmed the identity of the fungal organisms to be VGIIa, the common isolate from the Pacific Northwest.

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**CSU VETERINARY DIAGNOSTIC LAB CATCHES EQUINE HERPESVIRUS-1 OUTBREAK**

CSU VDL was the lab that first diagnosed infection by Equine Herpesvirus 1 associated with the May outbreak that has led to multiple quarantines in Colorado and has spread to several western states. As of presstime, horses in Arizona, Nevada, New Mexico, Oregon, Oklahoma, Texas, Idaho, Utah, Colorado, California, Washington and Canada had been infected with the highly contagious disease. The Associated Press reported the infected horses had attended the National Cutting Horse Association Western National Championships in Ogden, Utah, in early May. Owners of horses that attended the competition are being warned to monitor animals for symptoms and to have horses tested if they suspect the disease. Colorado now requires permits for any horses being brought into the state.

Although the outbreak has been tragic, it demonstrates how well the system works, says VDL Director Barb Powers. “This started with a single show, and now it’s all across the west coast, postponing shows and interfering with a good deal of horse movement in general,” Dr. Powers says. “But it’s important to remember it could have been much, much worse. The teachable moment here is that a quick, accurate diagnosis made possible by an astute practitioner working in partnership with a quality laboratory along with rapid response by the state veterinarian made all the difference between an outbreak and a wider spread epidemic affecting even more horses.”
A roundup of VDL faculty research


CSU VDL Bacteriology Section Head Doreene Hyatt contributed to a prospective study that collected fecal samples from large commercial pens of western U.S. feedlot cattle raised either with normal antimicrobial exposures or under “natural” systems, with no exposure to antimicrobials, hormonal implants or anthelmintics. Samples were cultured for non-specific Escherichia coli and Salmonella enterica, which were then evaluated for resistance against a panel of antimicrobials.

The study results suggest conventional cattle feedlot methods, including parenteral and feed use of antimicrobials, do not predictably nor uniformly increase the prevalence of antimicrobial resistance when compared with methods that don’t expose cattle to antimicrobials. Although some differences in resistance prevalence were found between the groups and over time, they were not reliably associated with recorded antimicrobial exposure, supporting the hypothesis that these relationships are complex.


VDL Avian Diagnostics and BSL3 Operations Section Head Kristy Pabilonia developed and coordinated a study that examined human-bird interactions in the upland gamebird industry. Noting that humans who have regular contact with poultry or wild birds may be at greater risk of infection with highly pathogenic avian influenza and other zoonotic avian diseases, the study surveyed upland gamebird permit holders for information on human-bird contact, biosecurity practices, facility management practices, flock/release environment and bird health. Their results suggest that some upland gamebird facilities provide an environment for extensive and intimate human-bird interaction that could increase the risk for zoonotic disease transmission.

Nemeth NM, Thomesen BV, Spraker TR, Benson JM, Bosco-Lauth AM, Oesterle PT, Bright JM, Muth JP, Campbell TW, Gidlewski TL, Bowen RA. Clinical and Pathologic Responses of American Crows (Corvus Brachyrhynchos) and Fish Crows (C ossifragus) to Experimental West Nile Virus Infection. Vet Pathol. 2011 In Press.

CSU VDL Pathologist Terry Spraker co-authored a study attempting to shed light on the underlying reasons for differences in susceptibility to West Nile virus among different bird taxa, particularly why the American crow is relatively highly susceptible. The study captured and then infected three American crows and three fish crows with West Nile, and then tracked behavioral and clinical observations, fecal and urate analysis, neurology, blood chemistry, hematology, virus isolation, pathology and immunohistochemistry. They found the American crow’s susceptibility owes to a cascade of events, including marked and widespread viral replication and delayed or depressed humoral immune responses, leading to cellular injury within the kidney, intestine, and other tissues and finally multiorgan malfunction.


Dr. Hyatt also took part in a study to better understand the impact starlings have on disease transmission to cattle. The study sampled feed, water and feces on a large cattle feeding operation for Salmonella enterica before and after starling baiting, compared to a comparable reference feedyard not controlling starlings. Results suggest that although starling control shouldn’t be relied upon
as the sole tool to reduce S. enterica infections, control does show promise to help manage the disease. Within the starling-controlled CAFO, S. enterica contamination disappeared from feed bunks and substantially declined within water troughs following starling control.

VDL Director Barb Powers contributed to a review of discoveries made and limitations encountered by studies evaluating the prognostic and predictive factors for soft tissue sarcomas in dogs. The review of 56 studies concludes that although valuable prognostic information may be gleaned from histologic grading, mitotic index and completeness of surgical margins, further research is needed to determine more precise estimates for recurrence rates and metastatic potential. Other potential factors such as markers of cellular proliferation, tumor dimension, tumor location, histologic type, invasiveness, and cytogenetic profiles require additional investigation.

Canine Bacteriology Case Study

Bile culture in gall bladder disease

A n 11 year-old female spayed Sheltie presented to the Veterinary Teaching Hospital with a four-to-five-day history of anorexia and vomiting. On presentation, she was dehydrated with evidence of diarrhea. Blood chemistry findings included increased ALP, ALT, GGT, cholesterol and BUN. Ultrasound findings were compatible with a ruptured gallbladder, possibly a ruptured mucocele, with secondary local peritonitis. The patient was taken to surgery, where the gallbladder rupture was confirmed. It was resected along with part of the liver. Free bile was present in the abdominal cavity, and a swab was taken for culture. Liver and gallbladder tissues along with the swab of free bile were submitted to the CSU VDL for histopathology and aerobic and anaerobic culture and sensitivity.

Histopathology results showed necrotizing and suppurrative cholecystitis with transmural necrosis and hemorrhage of the gallbladder wall. No mucocele was identified in the sample of gallbladder wall submitted for microscopic examination, but at the time of surgery a large amount of mucus and bile was identified outside of the ruptured gall bladder. Liver changes included chronic portal hepatitis with lobular atrophy and fibrosis as well as nodular hyperplasia. Within the hyperplastic focus, multifocal random hepatocellular necrosis was found and suspected secondary to showering of bacteria from the gall bladder. Culture results of free bile from the abdomen yielded moderate growth of Enterococcus spp. which were resistant to beta lactomes but variably susceptible to fluoroquinolones.

Exact pathogenesis of bacterial cholecystitis, while not fully understood, is thought to be caused by reflux of intestinal bacteria or by hematogenous spread from hepatic circulation. Associated conditions include causes of biliary stasis — especially cholelithiasis — gall bladder mucoceles and non-hepatobiliary conditions, including hypoadrenocorticism and sepsis. Enteric bacteria have also been cultured from dogs with gall bladder rupture secondary to gallbladder infarction.

Enterococci are Gram positive bacteria, which are part of the flora in the gastrointestinal tract of mammals. Enterococci species have been associated with bacterial hepatobiliary disease in humans, dogs and cats. One recent report, looking specifically at cases of canine gall bladder disease and rupture, reported 25 percent of bile culture results came back positive with E. coli, Streptococcus and Enterococcus, all common in gastrointestinal flora. This report included positive bacterial culture results in dogs with evidence of gallbladder infarction without concurrent inflammation. Another report in cats and dogs showed similar results and suggested bacterial cultures from bile samples may be more likely to yield positive results than liver samples. Enterococci are commonly involved in human bacterial cholangitis, and vancomycin-resistant Enterococci have been reported to increase incidence of serious, but usually rare, complications. Unfortunately, septic bile peritonitis carries a poor prognosis in dogs undergoing surgery for gall bladder rupture. Timely bacterial culture and sensitivity of bile may provide valuable information for management.

— Deanna Dailey, DVM, CSU Microbiology, Immunology and Pathology Veterinary Resident; and EJ Ehrtart, DVM, DACVPhD, CSU VDL Pathologist and CSU Microbiology, Immunology and Pathology Associate Professor

1 Maxie (ed) Pathology of Domestic Animals: Inflammatory Diseases of the liver and biliary tract.
5 Scott, Bacteria and disease of the biliary tract Gut 12 1971.
The U.S. Centers for Disease Control and Prevention says Salmonella spp. bacteria are the most common cause of foodborne illness in the United States, accounting for 11 percent of all cases of foodborne illness between 2000 and 2008. Salmonella bacteria were also responsible for the highest proportion of hospitalizations, at 35 percent, and deaths, at 28 percent, among all foodborne illnesses.

Although several Salmonella serotypes have been connected to outbreaks and sporadic illness in humans associated with consumption of eggs, including Salmonella Typhimurium and Salmonella Heidelberg, Salmonella Enteritidis (SE) is among the most common serotypes of Salmonella reported around the world as an important cause of illness in humans. The most common source of infection has been attributed to raw or undercooked eggs. The most recently recognized nationwide outbreak of SE from shell eggs, between May and December 2010, involved more than 1,900 confirmed human cases in multiple states, as well as a nationwide egg recall. While attributed to a number of large-scale outbreaks, SE contamination is still considered a rare event in the U.S. commercial egg industry, with only one contaminated egg per 20,000.

CSU VDL HELPS EGG PRODUCERS MONITOR
The U.S. egg industry has worked hard over the past few decades to reduce the incidence of Salmonella in commercial egg flocks. The U.S. Food and Drug Administration developed its rule, Prevention of Salmonella Enteritidis in Shell Eggs During Production, Storage, and Transportation, also known as the 2009 Egg Safety Rule, to target known risk factors for introduction of SE into commercial flocks and eggs. The goal of the rule is to prevent SE in eggs through environmental controls, breeding practices and regular surveillance testing and documentation in commercial laying flocks of 3,000 or more birds. It requires egg production facilities to conduct environmental testing for Salmonella. If positive environmental samples are found, egg testing must be performed. If egg samples are found to be contaminated with SE, they are diverted to pasteurization or discarded.

The CSU VDL is authorized to conduct Salmonella environmental and egg testing for the commercial poultry industry. We are a USDA National Poultry Improvement Plan Approved Laboratory and we pass Salmonella proficiency tests annually. Salmonella cultures take between five and 14 days and cost $15. Salmonella surveillance is conducted through collection of environmental drag swab samples, a highly sensitive method of testing an entire flock for Salmonella.

In addition to commercial poultry testing, we also provide Salmonella testing services to small-scale semi-commercial and backyard flock owners. In recent years there has been an increase in popularity of egg sales through local and regional sources such as farmer’s markets, local co-op stores, farm share programs and distribution direct to the buyer, making Salmonella testing within this industry increasingly important. Our Colorado Avian Disease Surveillance Program staff are available to visit Colorado flocks and train owners on proper collection and submission of environmental and egg samples for Salmonella testing. We recommend quarterly testing of two to five environmental samples per flock, based on flock size and structure.
Veterinary Community Outreach

Honoring Cappy through advances

For more than two years, Mary Lou Lane worked tirelessly to save her horse Cappy’s life—not after a life-threatening bout of colic, or a traumatic injury or encephalitis. Instead, Cappy had only a minor scrape on his front fetlock that, despite the best care, eventually cost him his life.

To honor Cappy and his fight, Lane established Cappy’s Equine Dermatology Research Fund at the Colorado State University Veterinary Diagnostic Laboratory to help support research related to diagnosing equine dermatology problems.

“The special bond we shared with Cappy is one that is understood by all those who love their horses,” wrote Lane in a tribute to Cappy. “We never want them to know pain and suffering. Yet, despite their great physical strength, they remain fragile in many ways. With all the enormous advances in veterinary medicine, there are still so many conditions science is unable to conquer.”

Lane first met Cappy in 1986 and “adopted” him when he was 9 years old. In 2007, Cappy got a seemingly minor scrap on his front fetlock. Despite prompt and constant care, he developed alopecia and lesions that extended from the fetlock down around the coronary band. Eventually, his foot was compromised and he developed laminitis. His heel, frog and hoof slowly stopped growing and the coffin bone began to drop. Despite all the best efforts of Lane, her veterinarians and farrier, Cappy eventually reached the point where he had to be euthanized.

His lower leg and foot were sent to the Veterinary Diagnostic Laboratory in hopes the veterinary pathologists there would be able to determine what organisms caused the dermatitis that led to the infection, and why it resisted treatment. Pathologists are still trying to determine the underlying cause of the infection, and why it proved so difficult to treat.

“Despite all her efforts, Mary Lou was unable to save Cappy, but wanted to do something to help other horses and their owners,” said Dr. Patricia Schultheiss, CSU VDL pathologist. “What we hope to be able to do through this fund is to improve our equine dermatology diagnostics so that we can determine what a problem is, in order to help veterinarians develop better treatment plans.”

Schultheiss notes that in the equine world, most research is directed at big problems including lameness and colic. Dermatology has not been a focus. Cappy’s Fund will help researchers further develop the field of equine dermatology by helping to improve diagnostic tools and treatment planning.

“The goal is to provide financial support...for research that will identify the origins of specific equine dermatologic conditions and their possible connection to laminitis,” wrote Lane. “Veterinarians will be able to prescribe appropriate treatments which will stimulate healing to stave off dermatitis. Donations to this fund will ensure resources will continually be available to assist in the fight against the debilitating and sometimes fatal consequences of dermatitis and laminitis, which can strike any equine at any time.”

To help support the work of Cappy’s Fund, contact Paul Maffey, Director of Development for the College of Veterinary Medicine and Biomedical Sciences, at (970) 491-3932, or visit advancing.colostate.edu/cappysfund to make a secure online donation.

CAPPY’S FUND RESULTS

To promote research related to the diagnosis, treatment and prognosis of equine dermatologic lesions, a database of equine biopsies was created from samples submitted within the past ten years to both CSU and the Prairie Diagnostic Services in Saskatchewan, Canada. CSU VDL is working on identifying trends within this population of central North American horses, such as relationships between inflammatory or neoplastic conditions and season, geographic location, and other factors like age and breed. In addition to identifying broad trends, the samples in this database will provide an invaluable resource for veterinarians and researchers, allowing them to identify specific cases for follow up or ancillary testing. We are confident that working through this database will help advance the field of equine dermatology and promote equine dermatologic health.
Welcome to the Spring/Summer LabLines. As I write this in mid-May, we have yet to see sustained warm weather; the high today was only 35°.

This issue focuses on export and regulatory testing, an area where we are seeing increased requests. This also highlights the necessity of a quality assurance program and AA VLD accreditation. We are thrilled to welcome our new pathologist, Dr. Han, to our laboratory; she has jumped into the mix and is already busy. In January, we had another successful meeting with our External Advisory Committee, listed below. We greatly appreciate their advice and assistance as we strive to always expand and improve our services. Soon to be available is our 2010 Annual Report with disease statistics. We are also in the process of updating our website to improve its usability and appearance. We look forward to seeing many of you at the September Colorado Veterinary Medical Association meeting at Keystone; and October’s annual American Association of Veterinary Laboratory Diagnostics meeting in Buffalo.

Barbara E. Powers

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DIRECTOR