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COMMENTARY

IN THIS ISSUE

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Department of Veterinary Science

LLOYD'S

THE TECHNOLOGY FOR HARNESSING THE INNATE specificity of nucleic acids for disease diagnosis has advanced considerably in recent years, as discussed in the article entitled *Nucleic Acid Based Tests in Disease Diagnosis* in this issue. The tedious purification techniques of the past have been replaced by automated micro (even single cell) extraction procedures and visualization with sophisticated fluorescent dyes. Nucleic acid sequencing and polymerase chain reaction (PCR) techniques that detect as few as five molecules have largely supplanted relatively crude methods such as hybridization and restriction endonuclease fragment analysis. The hybridization protocols of today are frequently in association with microarrays that permit the expression of thousands of genes that can be analyzed simultaneously with robotic operation.

The technical ability to amplify bits of unique genome material of pathogens has broadened our scope in diagnostics. What are the strengths and weaknesses of such sensitive procedures? When are they most appropriate, and when are they misleading? A few examples follow.

Two of the most powerful applications of PCR have been with pathogens that pose a risk to humans, namely HIV and West Nile encephalitis. By amplifying subgenomic stretches of the pathogen, the work can proceed under lower levels of biosecurity once the nucleic acid is extracted. In HIV, PCR is widely used to monitor viral burden (number of viral RNA copies) in blood of HIV-infected patients through time and to monitor the effectiveness of antiviral drug therapy. In the former, the assays are directed at highly conserved regions of the most conserved gene. In the latter, application of the PCR reaction is followed by sequencing to monitor for drug-resistant mutants. For West Nile virus (WNV), the PCR amplification of bits of the viral genome has permitted human and veterinary diagnostic laboratories to isolate WNV and participate in the surveillance of West Nile virus in North America.

A weakness of the PCR approach to diagnostics is also its strength: it is sensitive enough to theoretically amplify one copy of the pathogen genome. The likelihood of amplifying the genome of inactivated or defective pathogens under those circumstances is high. The incredible sensitivity of PCR-based techniques means there is also a constant threat of cross-contamination with the possibility of generating false-positive results. In any case, the presence of a positive PCR signal must be carefully assessed by "real-time PCR" where a copy number of the pathogen genome can also be addressed. The powerful nucleic acid techniques require expensive equipment, often beyond the reach of small laboratories. Highly trained personnel must be employed to ensure the optimal handling of sample materials to prevent degradation and contamination. Despite these constraints, the potential for such techniques in medical/veterinary diagnostics is exceptionally high.

One of our major fears about the widespread adoption of nucleic acid techniques is that they will lead to a mindset or culture in which isolation, identification, and basic research on pathogens is considered too mundane for funding. A healthy balance must be struck between the contemporary and traditional camps to continue to make progress in the identification of known, emerging, and novel pathogens that leads to their effective control.

CONTACT: Dr. C.J. Issel, (859) 257-1710, cissel@uky.edu; or Dr. R.F. Cook, rfcook1@uky.edu, Maxwell H. Gluck Equine Research Center, University of Kentucky.

INTERNATIONAL

Testing for Equine Viral Arteritis in Japan

THERE IS NO CLINICAL EVIDENCE OF EQUINE VIRAL arteritis (EVA) in Japan, and epidemiological studies indicate that Japan is free from EVA. Over the last 10 years, several thousands of horses have been imported from foreign countries. The Animal Quarantine Service of the Japanese Ministry of Agriculture, Forestry, and Fisheries (JMAFF) undertakes a strict quarantine inspection for EVA. The serum neutralization (SN) test as the prescribed test by the Office International des Epizooties is undertaken whenever any horse is imported into Japan. The SN test is time consuming and laborious and, because it requires live-exotic equine arteritis virus (EAV), it must be performed in designated high biosecurity facilities.

An ELISA test for EVA has been developed that does not require live virus. The test utilizes a fusion protein antigen of GL and N proteins of EAV expressed in a recombinant baculovirus system. This permits the test to be performed in several non-high security facilities. In order to evaluate sensitivity and specificity of the ELISA, studies were undertaken using serum samples collected from horses infected experimentally with EAV and from imported horses at the Animal Quarantine Service. In addition, kits of the ELISA test were sent to Dr. Peter Timoney of the University of Kentucky, USA, and Dr. Sandor Belak of the National Veterinary Institute, Sweden, to undertake collaborative studies on sera collected from horses in the field. Comparative studies of ELISA and the existing SN test for EVA undertaken at three laboratories indicated that positive horse sera by the SN test were positive by ELISA, but some negative SN sera showed false positive results with ELISA. From 1998 to 2000 the Expert Committee of the Liaison Council for Control of Equine Infectious Disease (LCCEID), comprising the JMAFF and the Japan Racing Association, evaluated the ELISA test results. They determined the ELISA is a useful and applicable serological test for EVA. The Animal Quarantine Service tested 2,000 imported horses in 2001 using both the ELISA and the SN test. Nine sera collected from vaccinated horses were positive in both tests; the remaining 1,991 were negative in the SN test. Using the ELISA, 1,934 sera were negative, and 66 (3.3%) were positive.

The ELISA test has now been adopted as a screening test for EVA with all positive samples confirmed by the SN test, thereby reducing the number of samples to be SN tested.

CONTACT: Dr. Yoshio Fukunaga, Epizootic Research Station, Equine Research Institute, the Japan Racing Association, yoshiof@epizoo.equinst.go.jp.

Glanders

NO CASES OF GLANDERS WERE REPORTED IN Brazil from 1968 to 1999. At the end of 1999 the disease was diagnosed in the states of Pernambuco and Alagoas. The University Federal Rural of Pernambuco (UFRPE) informed the local office of the Brazilian Agriculture Department in Recife City, Pernambuco. The diagnosis was based on clinical signs, pathological exams, complement fixation tests, and bacterial cultures. Some of the sick animals were working horses and mules from sugar cane mills. Immediately the Office International des Epizooties (OIE) in Paris was informed by the Brazilian Agriculture Department.

Horses and mules were affected, and several mules died. Clinical signs included nasal purulent discharge, fever, and edema (legs and ventral chest). Alteration in the hemograms (high leukocyte numbers, over 25,000/mm^3) and in the plasma fibrinogen levels (over 0.8 g/dl) was frequently observed. Mules that remained alive had long periods with cutaneous clinical signs, swellings, nodules on the medial hock and abdomen, and ulcers, which produced dark honey-colored pus. Lameness was frequently observed.

In horses the disease was more common as latent glanders, but when these animals worked during the sugar crop season, between Sep-

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Editors
Roberta Dwyer
David Powell
Neil Williams

Staff
Diane Furry

Correspondence should be addressed to the editors, Department of Veterinary Science, Maxwell H. Gluck Equine Research Center, University of Kentucky, Lexington, Kentucky USA, 40546-0099
Telephone (859) 257-4757
Fax (859) 257-8542

Internet address:
http://www.uky.edu/Agriculture/VetScience/gluck1.htm

tember and February, they developed the chronic or respiratory form, with cutaneous lesions frequently observed.

Necropsies showed severe pleuropneumonia on numerous occasions accompanied by bilateral sinusitis and necrosis of areas of the nasal cavity (septum and conchae). Bacteriological examination did not always reveal presence of the bacterium that causes glanders (*Burkholderia mallei*). Histopathological findings did suggest the presence of the bacteria, with calcification observed around the skin lesions. Failure to isolate the bacteria was probably due to the long time between specimen collection and bacteriological examination.

While only a small number of animals were treated, it was observed that treatments did not eliminate the bacterium.

During the last two years, an epidemiological survey has been undertaken in several states. Veterinarians collected blood from working horses for complement fixation testing from 11 states. These studies will support a new program to control and eradicate glanders in Brazil.

During 2002 results were evaluated and a national eradication program adopted. Since January 2000 interstate movement of horses has been controlled by the Brazilian Ministry of Agriculture to prevent disease dissemination. A sanitary education program was also developed by the university to inform all horse owners and landowners about this and other equine diseases.

CONTACT: Dr. Helio Manso, equivet@globo.com, University Federal Rural of Pernambuco, Brazil.

Third Quarter 2002

THE INTERNATIONAL COLLATING CENTRE, Newmarket, reported the following disease outbreaks.

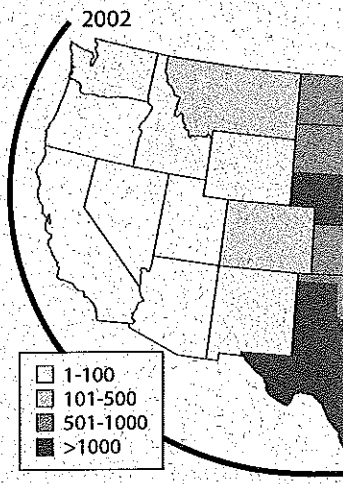
Ten cases of equine herpes virus abortion (EHV-1) on as many premises were reported from New South Wales, Australia. Extensive movement of mares in the later stages of pregnancy is considered to have contributed to the problem. The respiratory form of equine herpes virus was reported extensively in France among several breeds of horses and among a group of foals on a farm in Argentina.

Equine influenza was reported extensively in France and among Thoroughbred performance horses in Sweden. During July, equine influenza type-2 (H3N8) was isolated on several occasions from horses in Kentucky. Among vaccinated horses the clinical signs were generally mild and of short duration.

Clinical cases of equine piroplasmosis were reported in Switzerland, including a horse that had been recently imported from Spain. Rotavirus vaccination of pregnant mares in Argentina at approximately 60 and 30 days prior to parturition has drastically reduced the incidence of rotavirus diarrhea among foals born to vaccinated mares.

The most frequently reported disease worldwide continues to be strangles, with numerous outbreaks reported from Sweden and Switzerland and cases reported from Argentina, the United Arab Emirates, and the United Kingdom.

West Nile Virus Equine Cases
as reported by USDA as of Dece



NATIONAL

Advanced Imaging for Horses

ULTRASOUND HAS BECOME A ROUTINE AND indispensable part of equine practice. Introduced in the early 1980s, it is used for reproductive, gastrointestinal, renal, respiratory, and cardiac diagnosis and intervention. However, other advanced imaging modalities have now become available for the examination of horses.

Nuclear scintigraphy, introduced in the last two decades, utilizes radioisotopes and a crystal detector to identify areas of increased isotope uptake as an indicator of inflammation and increased bone turnover in musculoskeletal diseases.

Computed tomography (CT) and magnetic resonance imaging (MRI) represent imaging techniques that far surpass the abilities of traditional radiographs and ultrasound to diagnose some disease conditions. While accessibility to

these imaging capabilities remains limited, more facilities are becoming available that accommodate large equine patients. Currently, only one MRI facility (at Washington State University's College of Veterinary Medicine) is available to provide imaging for adult horses. Computed tomography facilities that can accommodate adult horses are available at several universities, including Cornell, The Ohio State University, and Colorado State University.

Both CT and MRI are noninvasive, multiplanar, two-dimensional, cross-sectional imaging techniques that provide superior resolution of organs and tissue, requiring computer reconstruction of raw data. Computed tomography uses ionizing radiation similar to X-rays, while MRI uses magnetic fields and radiofrequency pulses. With CT, x-radiation is applied with circumferential rotation delivery around the area of interest and is received by detectors. Sequential, collimated slices are recorded from the patient, and the intensity of transmittance is reconstructed by computer analysis to cross-sectional images. In general, CT is most often used for visualization of bony structures, such as the skull, brain, cervical vertebra, and limbs. It is also effectively used to visualize soft tissue structures within the thoracic and abdominal cavities in horses weighing less than approximately 400 pounds. In small animals, commonly-imaged structures include the paranasal sinuses and nasal cavity, brain, ear canal, lungs, and adrenal glands.

Magnetic resonance imaging is superior for visualization of soft tissue structures, such as brain, spinal cord, soft tissue masses within soft tissue, joints, and tendons. The exception is soft tissue structures within the lungs and intestinal tract, as respiratory and gastrointestinal movement will cause artifacts. Visualization of these structures is more amenable to CT imaging.

While most universities and a growing number of private imaging facilities may accommodate CT (and less commonly MRI) for foals, there remains a paucity of available sites for adult horses. However, as the technology becomes more affordable and the benefits recognized, facilities will become available to accommodate adult horses. In Central Kentucky, an animal-dedicated CT and MRI facility has performed CT or MRI on four foals. The scans were performed to assess spinal cord and vertebral abnormalities associated with paralysis or deformity.

Advanced imaging techniques provide "the next step" when traditional diagnostic tools and imaging technologies fail to establish a definitive diagnosis—the ultimate goal for veterinarians in order to provide appropriate therapy, prognosis, and long-term recommendations.

CONTACT: Dr. Janyce Seahorn or Dr. Richard Park, (859) 277-3336, VetScan, 1725 Harrodsburg Road, Lexington, Kentucky, 40504.

West Nile 2002

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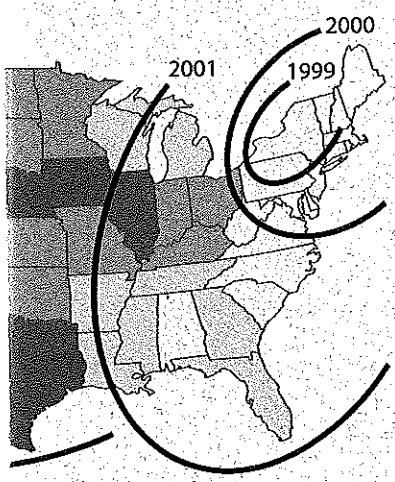

THE YEAR 2002 HAS BROUGHT A DRAMATIC spread of West Nile virus across the United States, reaching the West Coast states of Washington and California. **Figure 1** depicts the spread of the virus since its initial recognition in New York during the summer of 1999. Currently, only Alaska, Arizona, Hawaii, Nevada, Oregon, and Utah are considered free of the disease, which is now officially recognized as endemic in the United States. West Nile has also become widespread in Canada, affecting five provinces from Saskatchewan to Nova Scotia.

As of Dec. 3, 2002, the Centers for Disease Control (CDC) reported 3,775 human cases in 39 states plus the District of Columbia, of which 216 were fatal. The median age of cases was 56, with 78 as the median age for fatalities. In each of the following—Illinois, Michigan, Ohio, and Louisiana—over 300 human cases have been diagnosed.

The USDA reported 14,358 equine cases in 40 states during 2002 as of Dec. 1. It is estimated that between 20% and 30% have died or been euthanized as a consequence of West Nile infection. The distribution of equine cases by state is shown in Figure 1, with four states—Illinois, Iowa, Nebraska, and Texas—each reporting over 1,000 cases. The distribution indicates fewer reported cases in states in the East compared to central and western states. Possible reasons include a combination of mosquito control measures, vaccination of horses, and natural exposure since 1999.

In addition to the equine and human cases, 14,000 dead birds have been reported, which probably represents a considerable underestimation of the number of birds that have died from West Nile. The disease has also been implicated in the deaths of other mammalian species, including deer, dogs, sheep, and goats

plus marine mammals such as seals and most recently, reptiles, including alligators.

Rusty Ford of the Kentucky Department of Agriculture has produced figures relating to the prevalence of the disease in Kentucky during 2002. Toward the end of November he reported over 500 cases in 78 western and central counties involving 27 different breeds of horses plus a donkey and three mules. The majority of cases occurred among horses in an age range of 1 to 20. Mortality amounted to 25%.

Over 175,000 doses of West Nile vaccine were approved for use by Dr. D. Notter, Kentucky State Veterinarian. Of 129 horses that died or were euthanized because of West Nile disease, three had been vaccinated as recommended. One-hundred fourteen received no vaccine, and 12 had been vaccinated but had insufficient time to develop an immunity prior to field challenge. While this data may be interpreted that vaccination is of value in reducing losses due to West Nile, definitive interpretation of vaccine efficacy requires comparison of disease incidence or mortality between the number of vaccinated and unvaccinated mares.

Nucleic Acid Based Tests in Disease Diagnosis

THE LAST DECADE HAS USHERED IN NEW TECHnologies in molecular science. Techniques that were recently only research tools are becoming more widely used. The trend is to apply nucleic acid based tests toward disease diagnostics. Unknown organisms can be identified; closely related organisms can be differentiated; highly sensitive assays can be developed to detect small numbers of pathogens in complex samples; very fast results can be obtained in tightly controlled assays. These tests exploit the chemistry of natural DNA replication *in vitro,* allowing investigators to identify very specific sequences of a pathogenic genome.

Advances in nucleic acid sequencing technology and the computer-assisted comparisons of sequence data are allowing molecular biologists to have a greater impact on equine disease diagnostics. Sequencing a single microbial gene was once a project that would involve months of effort in highly specialized labs. The amount of work necessary to sequence all the genes on a microbial chromosome (genome) was nearly impossible to imagine 10 years ago. As a reference of magnitude, the genome of the bacteria *Escherichia coli* is composed of 4,377 genes on a double-stranded DNA molecule with a very specific sequence of 4,639,221 base pairs. Today, genes can often be sequenced within a week, and even microbial genome sequencing is becoming more commonplace. Technological advances are occurring that may soon allow microbial genome sequencing within a few hours.

The classification of bacteria is based in part on sequence comparisons of certain genes. The most commonly used is the gene encoding the 16S rRNA molecule, a sequence of about 1,400 bases in length. Databases of these sequences are now available that allow researchers to identify both conserved and unique regions of the sequence. Taxonomists can use this information to group bacteria in hereditary units and develop phylogenic trees. If a bacteria is isolated and its identification is difficult, the 16S gene can be readily sequenced and compared to the sequences of known organisms. A good example applying this directly to disease diagnostics is the bacterium causing most cases of nocardioform placentitis. The bacterium isolated had less than 97% homology to all known bacteria. Nucleic acid based tests indicated it was in the genus *Crossiella,* and it was named *Crossiella equi.* As a general rule if the unknown sequence is 98% identical or higher to the sequence of a known organism, the unknown is probably within the same species as the known organism. As more genes and complete genomes are sequenced, more complete and detailed information will become available.

A sequence that is unique to a pathogenic organism or gene (like a toxin gene) often becomes the target of a diagnostic nucleic acid test. These sequences are targeted because they can allow specific detection of that particular sequence in mixtures containing various other sequences. The most commonly used diagnostic nucleic acid test is the polymerase chain reaction test (PCR). The basic test process flows from isolation to amplification and ends with detection. Nucleic acids from a sample thought to contain a specific organism are first isolated and placed in a tube with primers that target the unique sequence. The chemicals responsible for nucleic acid synthesis (DNA polymerase, nucleotides, salts) are added, and the

sample is placed in a machine that cycles temperature (thermocycler) and enables a chain reaction of DNA synthesis to occur. This amplification step is quick and dramatic. In as little as an hour a single molecule can be amplified over a billion times. Detection is qualitative (positive/negative) and usually achieved by observation of a band of the amplified DNA on an agarose gel. Positive amplification infers the target (pathogenic organism or gene) was present in the sample. Most nucleic acid tests are variations on this theme. Enhanced sensitivity and specificity can be attempted using a re-amplification step called "nested PCR" or by using a labeled probe to detect the amplified DNA. More than one target can be detected in a single reaction using multiplex PCR. RNA targets can be detected using RT-PCR that includes an initial reverse transcription step added before PCR amplification. Real-time PCR combines the amplification and detection steps and can be used for faster and even quantitative results. The ability to detect specific pathogens will continue to be enhanced by the advances in nucleic acid testing.

CONTACT: Dr. Stephen Sells or Dr. Mike Donahue, (859) 253-0571, sfsells1@uky.edu, Livestock Disease Diagnostic Center, University of Kentucky.

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Department of Veterinary Science
Maxwell H. Gluck Equine Research Center
University of Kentucky
Lexington, Kentucky 40546-0099

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