

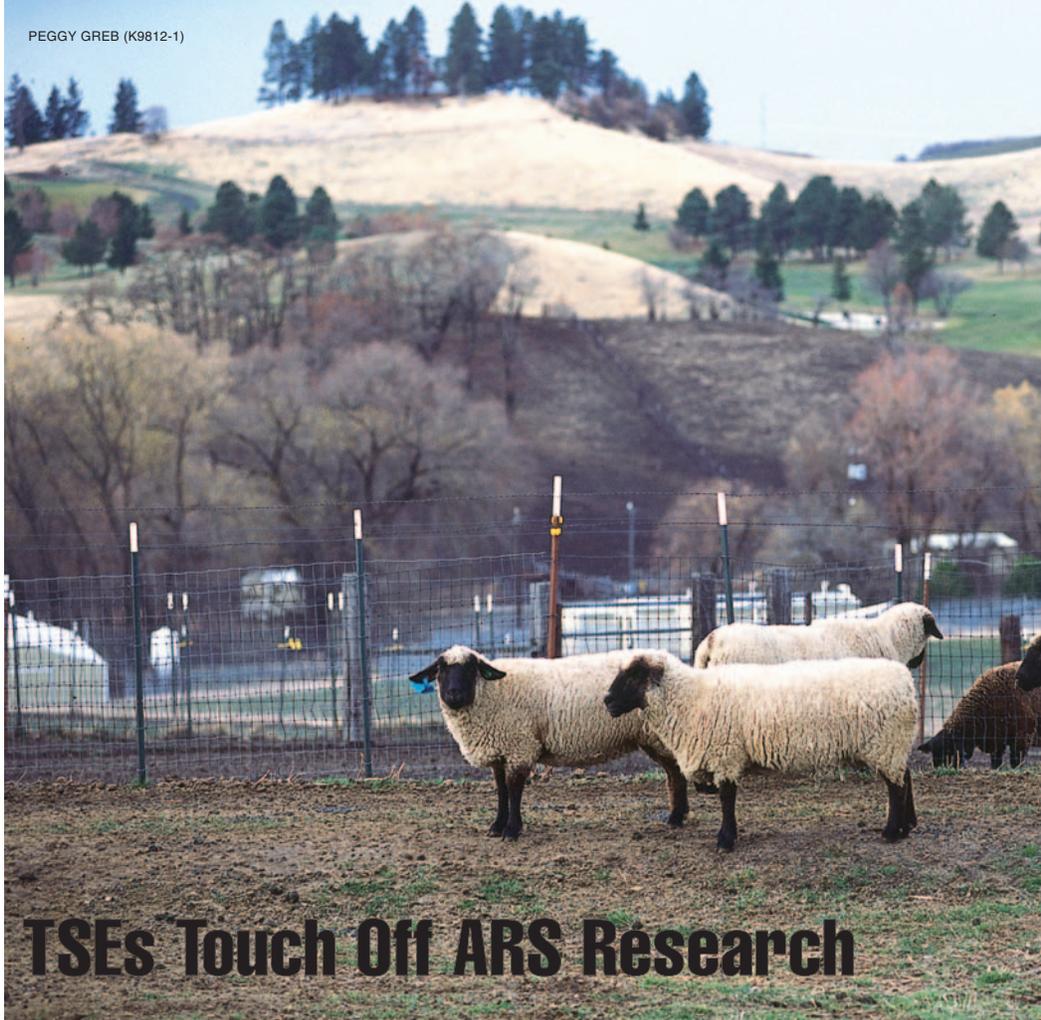
**A** year ago this month, a group of ARS scientists and technicians gave up their Christmas time off and even delayed family vacations to provide characterization of the first case of bovine spongiform encephalopathy (BSE)—commonly called mad cow disease—to be found in the United States.

On December 23, 2003, a Canadian cow shipped to slaughter from a farm in Mabton, Washington, had come up presumptively positive for BSE in testing by USDA's Animal and Plant Health Inspection Service (APHIS), which has diagnostic responsibility and regulatory oversight for BSE issues. APHIS had already used the "gold standard" diagnostic immunohistochemistry test, which was originally developed by ARS. But for the first U.S. case of BSE, APHIS wanted additional scientific information that could be provided by the Western blot test.

PEGGY GREB (K11644-1)



Disease-causing prions accumulate in the brains of host animals. Here, veterinary medical officers Robert Kunkle (left) and Amir Hamir examine prion distribution in brain tissue of TSE-affected animals.



In Pullman, Washington, ARS researchers developed the first practical live-animal test for scrapie, the TSE that afflicts sheep.

So APHIS put in a high-priority call to veterinary medical officer Juergen Richt and his colleagues at the Virus and Prion Diseases of Livestock Laboratory, which is part of ARS's National Animal Disease Center (NADC) in Ames, Iowa.

"We had experience with the Western blot test and we had all the reagents on hand," explains Richt. "So we put our holiday plans on hold and got everything ready so that APHIS would have verification of the results from the immunohistochemistry test."

On Christmas Eve, Richt and lab technicians Semakaleng Lebepe-Mazur and Deborah Clouser provided APHIS with a report, 22 long hours after the samples arrived in Ames. ARS veterinary medical officers Robert Kunkle and David Alt and technician Dennis Orcutt provided additional DNA sequence information, confirming that the tissue samples actually came from a cow and not a sheep, deer, or other animal.

Then on December 27, APHIS contacted Will Laegreid, animal health research leader at ARS's U.S. Meat Animal Research Center (MARC) in Clay Center, Nebraska, to orchestrate DNA testing and analysis to trace the origin of the BSE-positive cow. His group had previously developed bovine DNA markers for identifying animals that could be used for epidemiological traceback. MARC teams worked around the clock preparing DNA samples. Late on New Year's Eve, after the last critical tissues arrived, the processed samples were driven to the first of two independent, certified laboratories for genotyping. Within days, MARC analysis of DNA evidence confirmed the positive cow was of Canadian origin.

### A Mysterious Enemy

Conducting such urgent testing is not a usual part of ARS's work, but very little is usual when it comes to the enigmatic



had not been believed to be infectious on their own.

BSE itself is a fairly new disease; it was first diagnosed in 1986 in Great Britain. The disease has cost the European Union livestock industry at least \$107 billion as of this writing. USDA has maintained an aggressive import exclusion and surveillance program since 1986 to minimize the spread of BSE. As of this date, only one imported BSE case has been found in the United States.

Three other animal prion diseases are known today: Scrapie, which affects sheep and goats, was first recognized in Great Britain more than 250 years ago. The disease did not appear in the United States until 1947, when it was found in a Michigan flock. Transmissible mink encephalopathy (TME) is a rare illness that affects mink. It too was first detected in the United States in 1947, on a mink

ranch in Wisconsin, and on ranches in Minnesota and Idaho in the 1960s. Epidemiologic data from these outbreaks trace the cases to one common purchased food source. Since then, TME outbreaks have also been reported in Canada, Finland, Germany, and the republics of the former Soviet Union.

Chronic wasting disease (CWD) is a TSE of deer and elk. CWD has been reported in free-ranging mule deer, white-tailed deer, and Rocky Mountain elk in Colorado, Wyoming, South Dakota, New Mexico, Utah, Wisconsin, Nebraska, and Illinois; and in game-raised elk in South Dakota, Kansas, Montana, Oklahoma, Colorado, Nebraska, Minnesota, and Wisconsin. The disease has also been found in game-raised elk and a few free-ranging deer in Canada.

ARS has one of the world's most comprehensive research programs investigating TSEs. It is the only organization

class of animal diseases called transmissible spongiform encephalopathies (TSEs). These diseases are caused by abnormal prions.

Normal cellular prion proteins occur naturally in many tissues, including brain and other nerve tissue, but their functions are not well understood. These normal prion proteins can change and aggregate to form disease-causing prions.

The prevailing theory is that prions change their shape and fold into an abnormal form that accumulates in the brain and causes lesions. If the abnormal prions are transmitted from an afflicted animal to a new host, they may cause the new host's prions to begin folding abnormally.

Discovery of these prion traits has altered the accepted scientific ground rules for what can cause disease. Prions do not contain DNA or RNA as do fungi, bacteria, viruses, viroids, or any other previously known infectious entities. They are simply proteins, and proteins

PEGGY GREB (K11639-1)



**A Western blot analysis done by technicians Deborah Clouser (sitting) and Semakaleng Lebepe-Mazur was crucial in tracking the first U.S. BSE case.**

PEGGY GREB (K11646-1)



**Histotechnologist Jean Donald prepares 5-micrometer-thick sections of tissue collected from TSE-affected animals. The sections are then mounted on glass slides, stained, and examined by pathologists.**

studying all four TSEs in animals. ARS is taking a very integrated approach to TSE research, with collaborative projects involving many disciplines and scientists. While each TSE is unique in many respects, there is so much to learn about prion diseases that what researchers learn about one TSE may give insight into another.

### Diagnostics

Now-retired ARS veterinarian Janice Miller developed the first immunohistochemistry method for diagnosis of scrapie in sheep in 1993. This test was much more specific and less burdensome than any other at that time. In 1998, ARS microbiologist Katherine I. O'Rourke at the Animal Disease Research Unit in Pullman, Washington, further increased the test's specificity and ease of use by incorporating monoclonal antibodies. Use of these monoclonal antibody reagents was then broadened to be able to diagnose the other TSEs.

Later, O'Rourke had a real breakthrough when she discovered that prions collect in pockets of lymphoid tissue in a sheep's nictitating membrane, or third eyelid. A veterinarian can take a sample of the tissue with only a local anesthetic, which meant that there was finally a practical, live-animal test for scrapie. This live-animal test is now an approved diagnostic test for scrapie in the United States.

A very rapid, ultra-sensitive test that could be used before animals show any symptoms, especially with BSE in cattle and CWD in deer and elk, is still a major research goal.

One approach being taken today by ARS chemist Bruce C. Onisko at the Foodborne Contaminants Research Unit in Albany, California, is use of mass spectrometry to identify extremely low levels of prions. Mass spectrometry reveals structural information from biological compounds by ionizing a molecule of interest, fragmenting it by collisions with an inert gas, and then applying mass analysis to the fragmentation products.

PEGGY GREB (K11654-1)



At the ARS National Animal Disease Center in Ames, Iowa, animal caretaker Gary Hansen tends to two jersey steers. The steers are used as controls in a CWD cross-species transmission experiment in which cattle were inoculated intracerebrally with CWD-infected brain tissue.

PEGGY GREB (K11628-1)



Chemist Chris Silva (left) and research leader J. Mark Carter load samples for analysis via nanospray liquid chromatography coupled to mass spectrometry. This state-of-the-art technology characterizes BSE prions with unprecedented precision.

“Antibodies only let us find prions in amounts greater than 1 picomole. For live-animal testing we need to be able to reliably and quantitatively detect concentrations 3 to 4 orders of magnitude less from easily obtainable tissues,” explains Onisko. “And we need to be sure we are looking at only the abnormally configured prion protein.”

Such a sensitive test would help diagnose animals with abnormal prions before they start showing clinical symptoms. ARS has now applied for a patent for a new diagnostic test based on this technology.

ARS chemist Christopher J. Silva, also at the Foodborne Contaminants Research Unit, is using mass spectrometry to develop a way to test feeds for the presence of animal materials.

“A test for the presence of prions in animal feed is problematic. Epidemiologists in the United Kingdom showed that prions are not evenly distributed in animal feed, so an analytical sample might not be representative of the whole feed lot. Furthermore, could such a test be sensitive enough to detect rendered prions?”

Instead, Silva’s work on detecting the presence of prohibited animal materials in animal feeds would serve as an important indirect test for prions. BSE is transmitted to cows through feed containing animal parts from prion-infected cows. Using prohibited animal materials in cattle feed has been outlawed to prevent BSE transmission. “But it would be nice to have a way to double-check that feed is free of prohibited animal materials (and prions), should contamination ever be suspected,” Silva says.

### Transmission

Another major question that ARS is studying is whether and how TSEs spread between animals, either of the same species or different species. BSE is not communicable from animal to animal except through the recycling of bovine protein, which is now banned. Transmission from cows to humans appears to

require contact with specific infected tissues. Routes of transmission have not all been firmly established, but the oral route is most likely.

Meat from BSE-infected cows has not been shown to be infectious or associated with transmission. Exposure in people is most likely through consumption of meat products contaminated with central nervous system tissue. Since 1990, 157 people worldwide are believed to have contracted the abnormal prion-related disease called variant Creutzfeldt-Jakob disease from consumption of BSE-contaminated food.

Scrapie, on the other hand, has never been found to cross from sheep to

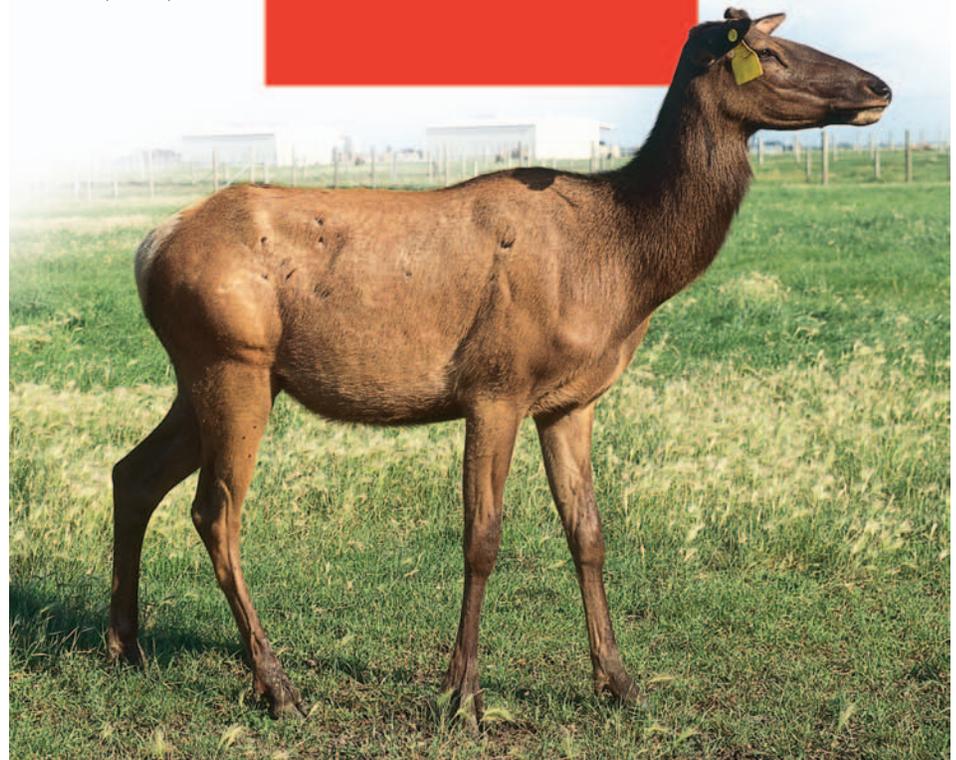
humans, according to Donald P. Knowles, Jr., research leader at the ARS Animal Disease Research Unit, in Pullman. ARS has found that scrapie from North American sheep, when transmitted to cows by intracranial injection, induced a spongiform encephalopathy with subtle microscopic lesions that didn’t mimic

BSE and the accumulation of protease-resistant prions. But oral inoculation of cattle with scrapie of North American origin didn’t result in any detectable lesions or prion accumulation.

Now, Knowles, Janet Alverson, an ARS veterinary medical officer in Pullman, and Robert D. Harrington, a veterinarian and

**C**attle, deer, sheep, raccoons, mice, and elk, like the one shown below, are being used in ARS studies on transmissible spongiform encephalopathies.

PEGGY GREB (K11659-1)



clinical instructor at the University of Washington Medical School in Seattle, are seeing whether CWD can infect mink. Mink were challenged 1 year ago both orally and intracranially and are under observation for clinical signs.

Most cross-species infection studies begin with intracranial injections of infected material, which is, of course, not a transmission route that could occur naturally. “But we do intracranial inoculations as a positive control for infectivity. If you can’t establish a TSE in a species by intracranial injection, you aren’t likely to see it spread any other way,” says Knowles.

Scientists at NADC, including Kunkle and ARS veterinary medical officer Amirali N. Hamir, are involved with studies that will eventually check for possible cross-species transmission with all three U.S. indigenous TSEs—scrapie, CWD, and TME—in a wide assortment of species. (BSE is not considered indigenous in the United States since the positive cow came from Canada.) NADC has the only biocontainment facilities for working with large animals in which TSEs can take a decade to incubate.

“With these cross-species studies,” explains Kunkle, “we learn whether a particular TSE can infect a different species and what it would look like clinically and pathologically if it did, and we build a bank of tissue material to call on for future research.”

Intracranial injections have been found to be able to transmit CWD to cattle. “But it doesn’t produce the same clinical signs as BSE,” Kunkle says. “With the clinical and pathological information we’re developing, if CWD can be transmitted to cattle in a natural fashion, we’ll have a better ability to recognize it.”

With CWD, the situation is especially complicated. Researchers are not even sure yet how the disease spreads from deer to deer or elk to elk. “We know you can put deer in a paddock that infected deer have been kept in and then removed,

and the new deer can become infected,” says Kunkle. “But we don’t yet know whether the prions are shed in urine or feces or something else.”

This is obviously different from BSE and TME, but then “prion diseases are a very loosely knit family,” he adds.

Another NADC project is to see whether reindeer and fallow deer can

PEGGY GREB (K11632-1)



**A sensitive new technique to detect animal products in feed will help formulators and livestock owners identify feed containing only vegetable ingredients. The test’s inventor, chemist Chris Silva, weighs potential feed materials before testing.**

become infected with CWD. APHIS is interested in this because these animals may be co-located with farm-raised elk. The study with fallow deer is already under way. Kunkle is also checking to see whether scrapie can be transmitted to pigs by intracranial injection. “It’s the same type of basic and precautionary research,” he says.

## Faster Models

One of the biggest complications in TSE research is the incubation period—the length of time it takes from exposure until the animal shows symptoms, proving that they have actually developed the disease. Cattle, deer, and elk can take several years or more to show signs of a TSE. To make research more practical, ARS is developing several promising animal models that will have shorter incubation times.

One such model is a collaborative project ARS has begun with Harrington and Knowles. They are developing a line of genetically engineered mice that have an added elk or deer prion gene, making them more susceptible to CWD. The team recently received a National Institutes of Health grant supporting this work for the next 3 years.

“Since such mice may develop CWD in just 1 to 2 years, they would be a powerful research tool,” Harrington says. “It will let us validate new diagnostic tests, hopefully give us clues to how CWD is transmitted, and provide an alternative model to study molecular mechanisms of the disease.”

## Genetic Resistance

With so little known about exactly why and how prions become abnormal, right now the best hope for controlling TSEs appears to be breeding animals that are simply naturally resistant. In Suffolk and other U.S. sheep breeds, O’Rourke has already found sheep with certain genotypes that have very limited susceptibility to scrapie. And she’s shown that if an infected ewe is bred to a resistant ram, the lamb will have a resistant genotype and be born free of scrapie.

Selective breeding for genetic resistance to the disease is a valuable tool for the National Scrapie Eradication Program. Genotyping allows infected flocks to be cleaned up while sparing 60 percent of the sheep. The program also encourages producers to select for resistance and to use scrapie-resistant

rams in flocks that have risk factors for scrapie. Genetic testing and selection, national sheep and goat identification, regulatory slaughter surveillance of mature sheep, investigation of exposed flocks by use of genetics and the third-eyelid test, cleanup of infected flocks, and the Scrapie Flock Certification Program provide an integrated strategy to eradicate scrapie from U.S. sheep and goat populations.

ARS researchers, including O'Rourke, have also been conducting genetic surveys to determine whether there is variation in the prion genes of other species that might identify susceptible and resistant animals. The makeup of a single amino acid sequence appears to be the difference between an animal that's likely to have its prions altered and one that isn't.

When O'Rourke and Alverson began examining deer and elk for genetic susceptibility or resistance to CWD, they discovered an unusual situation. They found that deer may have four copies of the prion gene rather than the two that would be expected.

"Virtually every mule deer we examined had the gene in duplicate, although only 15 percent of the white-tailed deer have the extra set," O'Rourke explains. "The extra set of prion genes is nonfunctional, but it complicates genetic testing to identify what a susceptible or resistant genotype might be."

So far, it is unclear whether there is any natural resistance in deer or elk populations.

ARS scientist Michael P. Heaton and his co-workers at MARC have recently identified extensive nucleotide variation in the prion genes of U.S. cattle, sheep, and deer.

"This information provides new DNA markers for researchers interested in genetic epidemiological studies of prion diseases. For example, if susceptibility alleles are identified in other populations of cattle, we will immediately know the proportion of U.S. cattle that is most

genetically vulnerable to prion disease," Heaton says.—By **J. Kim Kaplan**, ARS.

*This research is part of Animal Health, an ARS National Program (#103) described on the World Wide Web at [www.nps.ars.usda.gov](http://www.nps.ars.usda.gov).*

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PEGGY GREB (K11635-1)

**Biologist Larry Stanker (standing) and chemist David Brandon review results of a rapid immunoassay. They are developing new technology for sensitive detection of BSE, surrogate markers, and risk factors.**

