Detection of misfolded prion protein in retina samples of sheep and cattle by use of a commercially available enzyme immunoassay

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Objective — To determine the usefulness of retina samples for detection of disease-associated prion protein by use of a commercially available enzyme immunoassay (EIA) intended for rapid identification of sheep and cattle with transmissible spongiform encephalopathies (TSEs).

Samples — Retina, brainstem at the level of the obex, and retropharyngeal lymph node samples obtained from 15 TSE-inoculated sheep (scrapie [n = 13] or transmissible mink encephalopathy passaged through a bovid [2]); retina and brainstem samples obtained from 11 TSE-inoculated cattle (transmissible mink encephalopathy passaged through a bovid [7] or classical BSE [4]); and negative control tissue samples obtained from 2 sheep and 2 cattle that were not inoculated with TSEs.

Procedures — Tissue samples were homogenized and analyzed for detection of abnormally folded disease-associated prion protein with a commercially available EIA and 2 confirmatory assays (western blot analysis or immunohistochemical analysis).

Results — Retina sample EIA results were in agreement with results of brainstem sample EIA or confirmatory assay results for negative control animals and TSE-inoculated animals with clinical signs of disease. However, TSE-inoculated animals with positive confirmatory assay results that did not have clinical signs of disease had negative retina sample EIA results. Retina sample EIA results were in agreement with brainstem sample immunohistochemical results for 4 TSE-inoculated sheep with negative retropharyngeal lymph node EIA results.

Conclusions and Clinical Relevance — Results of this study suggested that retina samples may be useful for rapid EIA screening of animals with neurologic signs to detect TSEs. (Am J Vet Res 2014;75:268–272)

Conventional tests for the diagnosis of TSEs in sheep and cattle include IHC and WB analysis for the detection of misfolded, disease-associated prion protein in tissue samples (typically brain samples). However, those methods can be labor-intensive and are not amenable to high-throughput screening. Given the BSE epizootic in the United Kingdom and the ensuing detection of a new variant of Creutzfeldt-Jakob disease in humans that consumed beef obtained from animals with BSE,1 diagnosis of prion diseases in animals has become a public health and economic concern. Subsequently, rapid screening tests, which rely on the detection of PrPSc, were developed2 for identification of livestock at risk for TSEs. Such tests are most commonly available as EIAs or ELISAs and have been validated for use with samples of brainstem obtained at the level of the obex or, for small ruminants and cervids, samples of certain lymphoid tissues.3–5

Retinas of animals with TSE accumulate PrPSc, but the usefulness of retina samples for detection of PrPSc with EIAs or ELISAs has not been determined, to the

ABBREVIATIONS

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
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<tr>
<td>BSE</td>
<td>Bovine spongiform encephalopathy</td>
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<td>cBSE</td>
<td>Classical bovine spongiform encephalopathy</td>
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<td>EIA</td>
<td>Enzyme immunoassay</td>
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<td>IHC</td>
<td>Immunohistochemical analysis</td>
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<td>OD</td>
<td>Optical density</td>
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<td>PRNP</td>
<td>The gene encoding prion protein</td>
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<td>PrPSc</td>
<td>Disease-associated prion protein</td>
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<td>RPLN</td>
<td>Retropharyngeal lymph node</td>
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<td>TME</td>
<td>Transmissible mink encephalopathy</td>
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<td>TSE</td>
<td>Transmissible spongiform encephalopathy</td>
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<td>WB</td>
<td>Western blot</td>
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authors’ knowledge. Because eyes are easily obtained and their contents are contained, retina samples may be useful and hygienic for performance of rapid diagnostic tests for the detection of PrPSc. Accumulation of PrPSc has been detected in the retina of various natural host species of TSEs, including sheep naturally or experimentally infected with scrapie and cattle experimentally infected with H-type or L-type BSE. Retina samples obtained from cattle naturally infected with BSE can transmit TSE to other animals. Addition- ally, cattle experimentally infected with the agent that causes TME (a prion disease of mink) have alterations in retinal function before clinical signs are detectable and have corresponding retinal accumulation of PrPSc when examined at necropsy after the onset of clinical signs. Clinopathologic findings for cattle infected with the TME agent are similar to those for cattle with BSE. Retinas are an extension of the CNS; therefore, retinas may be readily accessible tissues that accumulate PrPSc in ruminants, at least during late stages of disease. The objective of the study reported here was to determine the usefulness of retina samples for detection of PrPSc by use of a commercially available EIA intended for rapid identification of sheep and cattle with TSEs.

Materials and Methods

Animals—Study protocols involving animals were performed in accordance with guidelines of the Institute for Laboratory Animal Resources of the National Academy of Sciences and were approved by the National Animal Disease Center Animal Care and Use Committee. Retina, RPLN, and brainstem (collected at the level of the obex) samples that had been obtained from 12 Suffolk sheep in another study were used in the present study. These sheep had been inoculated orally (n = 11) or intracranially (1) with the National Animal Disease Center 13-7 isolate of the scrapie agent and were euthanized at 17 to 20 months after inoculation and 2 without clinical signs euthanized at 23 months after inoculation, at termination of the experiment.7 Cattle inoculated intracranially with a TME isolate that had been passaged through a bovid (5 with clinical signs euthanized at 17 to 20 months after inoculation and 2 without clinical signs euthanized at 23 months after inoculation), and 2 negative control steers (these animals were maintained in a separate facility). Intracranial inoculations were performed as described for sheep with injection of 1 mL of 10% brain sample suspension. All cattle were euthanized with an overdose of barbiturate.

Tissue sample collection—Retina samples were collected from eyes of animals that had been removed in toto; these samples were collected from eyes at the time of necropsies or from eyes that had been stored frozen at –80°C. For collection of retina samples from eyes that had been stored frozen, globes were bisected and allowed to thaw completely. Vitreous humor was carefully removed, and retinas were gently collected from choroid with forceps, and placing retina samples in a tissue homogenization tube that had been weighed. Retina samples were stored at –80°C. For these samples, buffer provided with the EIA kit was added to the tubes (30% [wt/vol] solution). Retina samples collected at the time of necropsies were collected by bisecting globes, carefully removing vitreous humor, separating retinas from choroid with forceps, and placing retina samples in a tissue homogenization tube that had been weighed. Retina samples were stored at –80°C. For these samples, buffer provided with the EIA kit was added to the tubes (30% [wt/vol] solution) on the day of the assay. Bovine retina samples were collected by means of a similar method at the time of necropsy or from eyes that had been stored frozen. For retina samples collected from eyes of 2 cattle, PBS solution was added instead of homogenization buffer provided with assay kits (20% [wt/vol] solution). Brainstem homogenates were prepared with PBS solution or EIA kit buffer (20% and 30% [wt/vol] solutions, respectively), and EIA results for each preparation were compared for these 2 samples. There were no differences in results among these preparations. All other tissue samples collected from cattle were prepared in homogenization buffer provided with assay kits (30% [wt/vol] solution). All tissue samples were homogenized prior to performance of EIAs.

EIA—Homogenates of retina, brainstem, and RPLN samples obtained from sheep and retina and brainstem samples obtained from cattle were assayed with a commercially available EIA kit approved for BSE and scrapie testing in the United States. Assays were performed in accordance with the manufacturer’s instructions. The EIA kit instructions indicated 3 protocols (standard, short, and ultrashort). The short protocol was used for testing of tissue samples in the present study. Each tis-
Sue sample homogenate was assayed in duplicate on 2 microtiter plates with manufacturer-provided negative and positive control samples and a retina sample homogenate obtained from at least 1 uninoculated negative control animal. Two conjugate concentrate products were included with the kit: a conjugate concentrate intended for use with brain samples obtained from small ruminants and a conjugate concentrate intended for use with brain samples obtained from cattle or lymph node or spleen samples obtained from small ruminants. The conjugate concentrate intended for use with small ruminant brain tissue samples was used for testing of retina samples obtained from sheep, and the other conjugate concentrate product was used for testing of retina samples obtained from cattle. Other types of tissue samples were tested with the conjugate intended for use with such samples as recommended by the manufacturer. Absorbance was measured at 450 nm with a reference wavelength of 620 nm. Cutoff values were established for each run in accordance with kit instructions whereby a value of 0.120 was added to the mean negative control sample value. Samples were interpreted as having positive results if their absorbance value at 450 nm minus the reference value at 620 nm was higher than the established cutoff value. Brainstem samples obtained from sheep and cattle and RPLN samples obtained from sheep were also assayed by means of IHC or WB analysis for detection of PrPSc as previously described. Briefly, tissues for histologic evaluation and IHC were immersion-fixed in neutral-buffered 10% formalin, embedded in paraffin, processed with standard histologic methods, and sectioned (thickness, 5 µm). For IHC, tissue samples were deparaffinized and rehydrated, autoclaved for 30 minutes at 121°C in antigen retrieval solution, and stained by use of an indirect avidin-biotin system or horseradish peroxidase polymer system with primary antibodies against PrPSc (99/97.6.1 [5 µg/mL] or 6H4 [0.1 µg/mL]). For WB analysis, tissue samples were digested with proteinase K (final concentration, 0.08 mg/mL) for 40 minutes at 48°C. Tissue samples were dissolved in SDS-PAGE sample buffer and assayed by use of standard WB procedures; PrPSc was detected with a monoclonal antibody (6H4 [0.1 µg/mL]) and an indirect avidin-biotin system with a chemiluminescent detection kit.

Results

Results of the EIA indicated PrPSc was detectable in retina samples obtained from sheep and cattle. Of 8 sheep with negative IHC or WB results for PrPSc in brainstem or RPLN samples, all had EIA OD values for retina samples that were less than the established cutoff values (0.20 and 0.22 for the EIA replicates). Six sheep with scrapie had clinical signs of disease at the time of euthanasia; these animals had positive WB or IHC results for detection of PrPSc in brainstem or RPLN samples. Retina samples obtained from those sheep had positive EIA results for detection of PrPSc; the OD values for these animals were high (> 3.90). One sheep inoculated with the scrapie agent that did not have clinical signs of disease at the time of euthanasia had positive IHC results for brainstem and RPLN samples. Brainstem and RPLN samples obtained from this sheep had positive EIA results, but results for retina samples obtained from this animal were negative for detection of PrPSc. Two sheep with scrapie that had positive EIA results for retina samples and positive IHC or WB test results for brainstem or RPLN samples had an RPLN sample EIA OD that was less than the predetermined cutoff values for detection of PrPSc. The 2 sheep that had been inoculated with the TME agent had clinical signs of disease; these animals had positive EIA results for brainstem and retina samples but negative EIA results for RPLN samples.

Results for retina samples obtained from cattle were similar to those determined for such samples obtained from sheep. Of 5 cattle with negative IHC or WB test results for detection of PrPSc in brainstem samples (including PRNP-knockout cattle inoculated with the TME agent), all had EIA OD values for retina samples less than the established cutoff values (0.14 and 0.16 for the EIA replicates). Tissue samples obtained from 4 cattle inoculated with the cBSE agent were assayed. Two of these cattle had clinical signs of disease at the time of euthanasia and positive WB test results for detection of PrPSc in brainstem samples; retina samples obtained from these animals had positive EIA test results (OD, 3.95 and 3.91). The other 2 cBSE-inoculated cattle did not have neurologic signs at the time of euthanasia. One of these cattle had negative WB analysis results for brainstem samples and negative EIA results for brainstem and retina samples. The other cBSE-inoculated animal without neurologic signs had positive WB analysis and EIA results for brainstem samples, but negative EIA results for retina samples (OD for brainstem and retina samples, 1.17 and 0.05, respectively). All 5 PRNP wild-type cattle inoculated with the TME agent had clinical signs of disease at the time of euthanasia; these animals had positive IHC or WB test results for detection of PrPSc in brainstem samples. Retina samples obtained from those cattle had positive EIA results with high OD values (> 3.80).

Discussion

The objective of the present study was to determine the usefulness of a commercially available rapid screening test for the diagnosis of scrapie in sheep and BSE in cattle by use of retina samples. Our hypothesis was that results of that test would be similar to those of IHC or WB analyses performed with brainstem samples (cattle and sheep) or RPLN samples (sheep) obtained from animals with late-stage disease. The EIA results were negative for all retina samples with negative IHC or WB test results (8 sheep and 5 cattle) in this study. Retina sample EIA results were positive for all sheep that had neurologic signs at the time of euthanasia; brainstem or RPLN sample IHC or WB test results for detection of PrPSc were positive for these animals (6 sheep with scrapie and 2 with TME). Results were similar for retina samples obtained from cattle with cBSE (n = 2) or TME (5). However, retina sample EIA results were not in agreement with brainstem sample EIA results or brainstem or RPLN IHC or WB test results for sheep (n = 1) or cattle (1) without clinical signs of disease. This finding suggested that retina samples were only useful for detection of PrPSc in animals with clinical signs of dis-
ease. For one of the sheep, negative retina sample EIA results may have been attributable to a collection error because that retina sample was obtained from a globe that had been archived frozen; separation of the retina and vitreous humor was difficult for such globes. However, that negative result may have correctly indicated absence of detectable PrPSc in the retina sample because PrPSc may spread from brain to retina tissue during disease progression. Similarly, the negative retina sample EIA results for one of the cattle with BSE that did not have neurologic signs was likely attributable to the pattern of PrPSc accumulation in tissues during disease progression. Such findings supported the conclusion that retina samples may only be useful for detection of PrPSc in animals with clinical signs of disease.

Interestingly, RPLN samples obtained from 2 sheep with scrapie had negative EIA results despite having positive results for the IHC. Brainstem samples from these animals were not available for EIA testing because collected tissues had been consumed in prior assays, but EIA results for retina samples obtained from those sheep were positive. Collection of RPLN samples was performed carefully to maximize the number of lymphoid follicles in such samples and, in accordance with recommendations of the EIA kit manufacturer, tissue samples were minced prior to homogenization to facilitate tissue disruption. Nevertheless, improper sample collection or preparation methods may have resulted in negative EIA results. The finding that retina sample EIA results were positive with high OD values for those sheep suggested that such samples may yield more reliable results than RPLN samples tested with this assay (for animals with clinical signs of disease). Similar EIA results were determined for RPLN (negative result) and retina (positive result) samples obtained from the 2 sheep with clinical signs of disease that had been inoculated with the TME agent. Results for these animals differed from those for the 2 sheep with scrapie in that confirmatory WB test results for RPLN samples were also negative, suggesting there may have been a difference in the pathogenesis of disease between these 2 types of TSE in the sheep. Although large amounts of PrPSc accumulate in lymphoid tissues of sheep with scrapie, large amounts of PrPSc may not accumulate in sheep with TME. Similar disease characteristics have been identified between TME in cattle and L-type BSE in mice.

Results of a study indicate PrPSc does not accumulate in lymphoid tissues of sheep with L-type BSE. Additionally, retina samples from sheep in that study had positive IHC and WB test results for detection of PrPSc. Therefore, retina samples may be better than RPLN samples for rapid testing and identification of sheep with L-type BSE.

Results of the present study suggested that retina samples may be useful for rapid screening of sheep and cattle with TSEs, but only for animals with clinical signs of disease. Because some animals tested for scrapie and BSE in the United States do not have clinical signs of disease, testing of retina samples by means of EIAs could complement, but not replace, the methods currently used for such surveillance programs. Advantages of the use of retina samples for testing include ease of accessibility and collection and possible detection of PrPSc in such samples obtained from sheep with atypical TSE characteristics for which lymphoid tissues have negative test results (because retina is a nervous tissue in which PrPSc may accumulate). In addition, biosafety for personnel may be improved with testing of retina samples before collection and testing of CNS tissue samples because use of retina samples may avoid unnecessary exposure of personnel to TSE agents in CNS tissues and limit contamination of necropsy facilities and equipment. To the authors’ knowledge, PrPSc has not been detected in ocular tissues other than the retina; however, appropriate biosafety and biocontainment procedures should be followed when handling ocular tissue samples of animals with suspected TSEs. Prions are resistant to methods effective for inactivation of microorganisms, such as moderate heating, UV irradiation, and formalin exposure. Prion decontamination methods recommended by the World Health Organization include autoclaving at 134°C for up to 1 hour, prolonged exposure to sodium hydroxide (1N), or prolonged exposure to sodium hypochlorite (≥20,000 μg/mL). These methods are not typically used after routine necropsy in the United States. Because all animals included in the present study were experimentally inoculated, the findings should be confirmed during studies of animals naturally infected with scrapie and BSE. In addition, other EIAs and ELISAs should be evaluated because the EIA used in the present study included a proprietary capture ligand that did not require proteinase K digestion of tissue samples for PrPSc detection, which is typically a required step in other assays.

References
7. Head MW, Northcott V, Rennison K, et al. Prion protein accumu-


