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Use of capillary electrophoresis and fluorescent labeled peptides to detect the abnormal prion protein in the blood of animals that are infected with a transmissible spongiform encephalopathy

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Abstract

Transmissible spongiform encephalopathies in humans and in animals are fatal neuro-degenerative diseases with long incubation times. The putative cause of these diseases is a normal host protein, the prion protein, that becomes altered. This abnormal prion protein is found mostly in the brains of infected individuals in later stages of the disease, but also can be found in lymphoid and other tissues in lower amounts. In order to eradicate this disease in animals, it is important to develop a system that can concentrate the abnormal prion protein and an assay that is very sensitive. The sensitivity that can be achieved with capillary electrophoresis makes it possible to detect the abnormal protein in blood. A peptide from the carboxyl terminal region, amino acid positions 218–232, was labeled with fluorescein during the synthesis of the peptide at the amino terminus. Antibodies that have been produced to this peptide were affinity purified and used in a capillary electrophoresis immunoassay. The amount of fluorescein labeled peptide in the capillary was 50 amol. Blood was obtained from normal sheep and elk, from sheep infected with scrapie and elk infected with chronic wasting disease. Buffy coats and plasma were prepared by a conventional method. After treatment with proteinase K, which destroys the normal protein but not the altered one, the blood fractions were extracted and tested in the capillary electrophoresis immunoassay for the abnormal prion protein. The abnormal prion protein was detected in fractions from blood from infected animals but not from normal animals. This assay makes a pre-clinical assay possible for these diseases and could be adapted to test for the abnormal prion protein in process materials that are used for manufacture of pharmaceuticals and products for human consumption.

Keywords: Prion proteins; Proteins

1. Introduction

Transmissible spongiform encephalopathies (TSEs) of humans and animals are relatively rare neuro-degenerative diseases [1,2]. Scrapie is a TSE in sheep that was first described over 200 years ago [3]. Chronic wasting disease was described in mule deer [4] and then later in Rocky Mountain elk [5]. The recent outbreak of a TSE in cattle in the United Kingdom has caused considerable concern because of the possible transmission to humans [6,7]. Many experiments have been done to investigate what

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tissues, including blood, are infectious for this disease [8–10]. The results from these studies are variable. Recently, a large systematic study has shown that fractions from blood are infectious in a rodent model but at a rather low infection rate [11]. Since the putative agent of these diseases, the abnormal prion protein [12], is rather insoluble in the typical biological buffers, analytical tests for the abnormal prion protein have been elusive. In addition, the overall concentration of the abnormal prion protein is rather low and is present in focal areas of the infected tissue. Analytical tests that are available are Western blot and plate assays [13–15]. Although the specificity in Western blot is good, the sensitivity is low. Both the sensitivity and the specificity need to be high in order to develop an early pre-clinical test for live animals. Reports using capillary electrophoresis successfully for immunoassays [16–18] led us to explore this technique. We used capillary immunoelectrophoresis to detect the abnormal prion protein in the brains as well as some of the lymphoid tissues of TSE infected animals [19]. When we used a fluorescent labeled peptide that gave a higher fluorescent signal than the one in our previous study, we were able to detect the abnormal prion protein in the blood of scrapie infected sheep and elk infected with chronic wasting disease.

2. Materials and methods

2.1. Sheep and elk blood

Sheep and elk blood was collected in commercial EDTA blood tubes (Becton Dickinson, Franklin Lakes New Jersey, USA). The blood came from 50

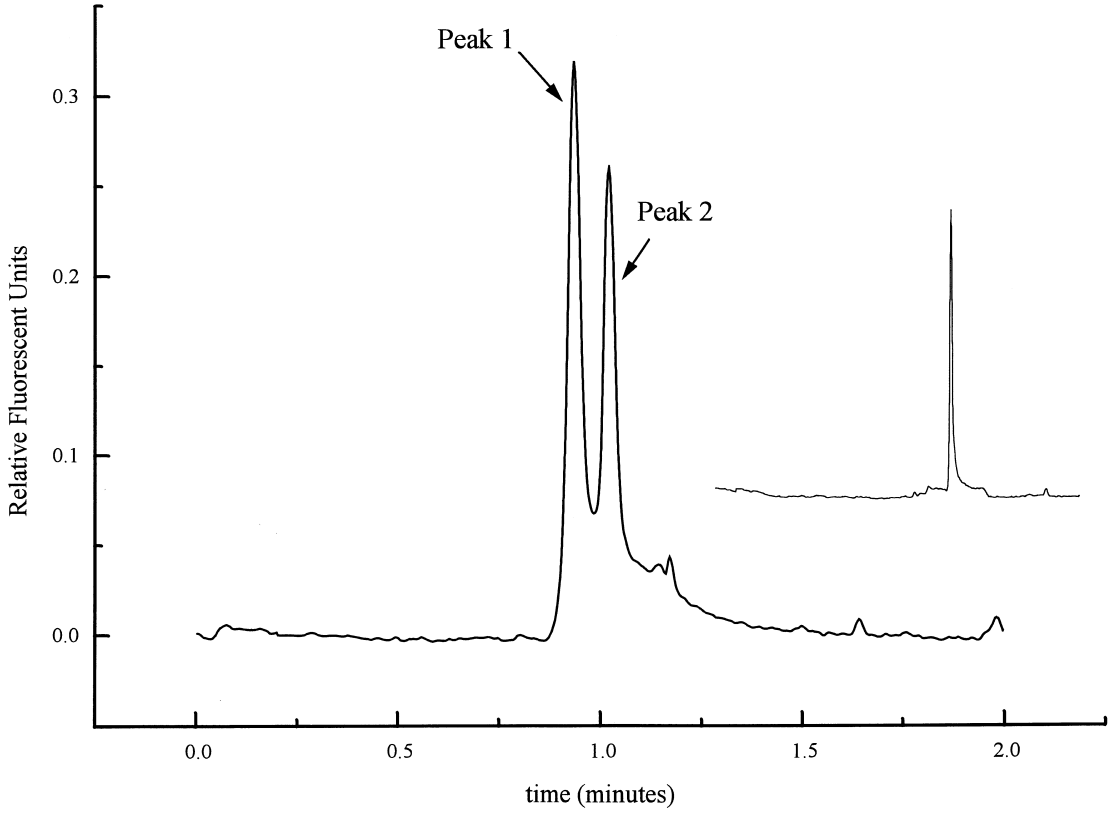

Fig. 1. Electropherogram of the labeled peptide in the presence of antibody. Inset: Electropherogram of the fluorescein labeled peptide.

sheep that were certified as scrapie free sheep. Additional blood samples were obtained from 15 other sheep that had no known history of scrapie. Blood was also obtained from another flock of sheep that were normal but had the polymorphism QQ at residue 171 of the prion protein (this genotype is more susceptible to scrapie infection [20]). The age range of these sheep was six months to nine years. Blood was collected from healthy sheep that had been exposed to scrapie and from sheep showing signs of clinical scrapie that was confirmed later to be scrapie by standard diagnostic tests [21]. Blood was obtained from elk in a similar fashion to that of the sheep. Samples from a ranch herd of elk with no known cases of chronic wasting disease were used as the normal controls. The other elk samples were obtained from animals that were in the terminal stages of chronic wasting disease or had been exposed to the disease. Sequential blood samples that were taken ~3 months apart were tested.

2.2. Buffy coat preparation

After the blood was collected, it was centrifuged at 750 *g* for 30 min to obtain the buffy coat. The plasma was removed and stored at −20°C. The buffy coat was drawn off and placed in 10× the volume of NH_4Cl and centrifuged as above. The pellet containing the buffy coat was resuspended in 1 ml of 0.05 *M* Na_2HPO_4, pH 7.0, containing 0.15 *M* NaCl (PBS) and frozen and thawed two times. It was incubated with 40 μg/ml DNase for 1 h at 37°C followed by incubation with 50 μg/ml of proteinase K for 1 h at 37°C. The treated material was extracted (by a proprietary process) and dried for the capillary electrophoresis assay. The dried samples were resuspended in 8 μl of dH_2O.

2.3. Peptides and antibodies

The peptide with the sequence RE-

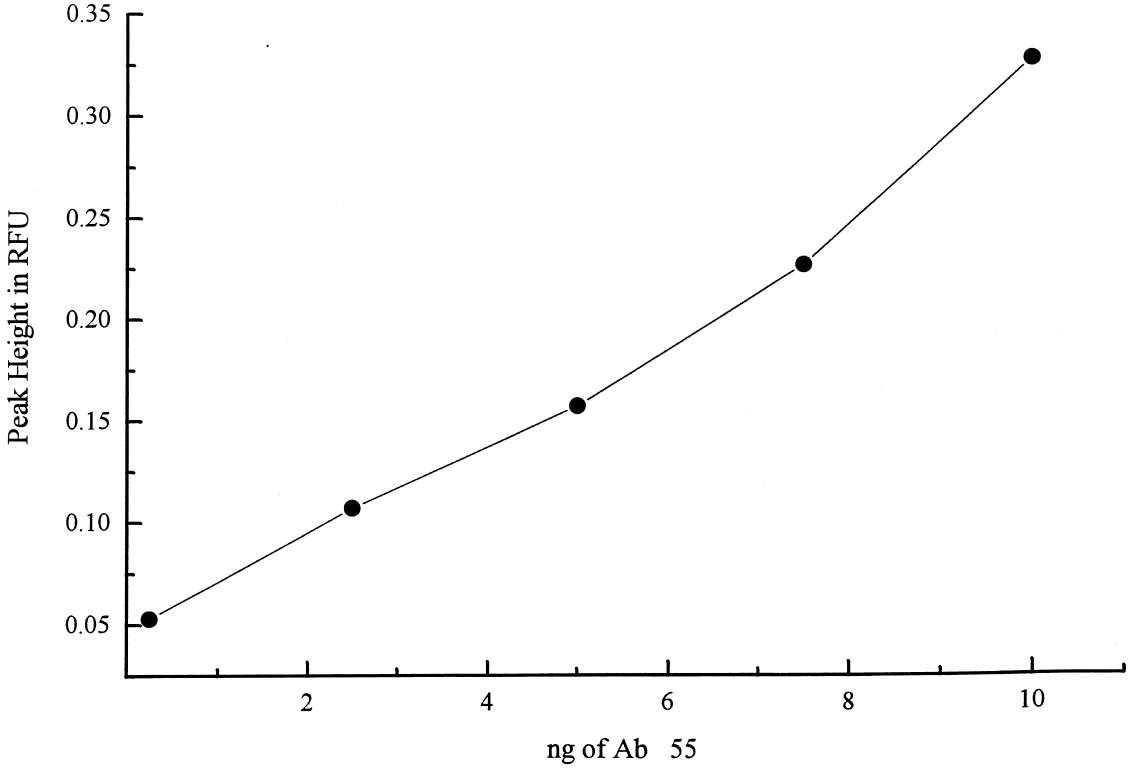

Fig. 2. Plot of antibody concentration versus the peak height of the immunocomplex.

SQAYYQRGASVIL from the prion protein was synthesized (Multiple Peptide Systems, San Diego, CA, USA). The peptide was labeled with fluorescein through a γ-butyric acid linkage on the N-terminus during synthesis. Rabbits were immunized with the peptide and specific antibodies were produced against it (Multiple Peptide Systems). After the brain samples were treated with proteinase K, this antiserum reacted with scrapie infected but not with normal brain on Western Blot analysis [13]. The rabbit antiserums were affinity purified as previously described [19].

2.4. Immunocomplex formation

To determine the amount of antibody that bound 50% of the fluorescein labeled peptide, varying amounts of purified rabbit antibody were mixed with ~50 femtomoles of the labeled peptide at 25°C and incubated for 16 h at 4°C. After the amount of antibody that bound ~50% of the labeled peptide was determined, varying amounts of unlabeled peptide or 5 μl of the re-suspended blood sample were added to the assay. The assay was incubated as above. After the electrophoresis was performed peak heights were calculated and the ratios of the bound peptide to the free peptide (peak $\text{height}_{\text{bound}}$/peak $\text{height}_{\text{free}}$) were determined.

In order to determine the amount of perturbation that a sample would introduce into the assay, TSE-free animals were tested. All of the TSE-free animals, both the sheep and the elk, had peak ratios that were greater than 75% of the control. As a result, samples with a peak ratio<70% were set as positive.

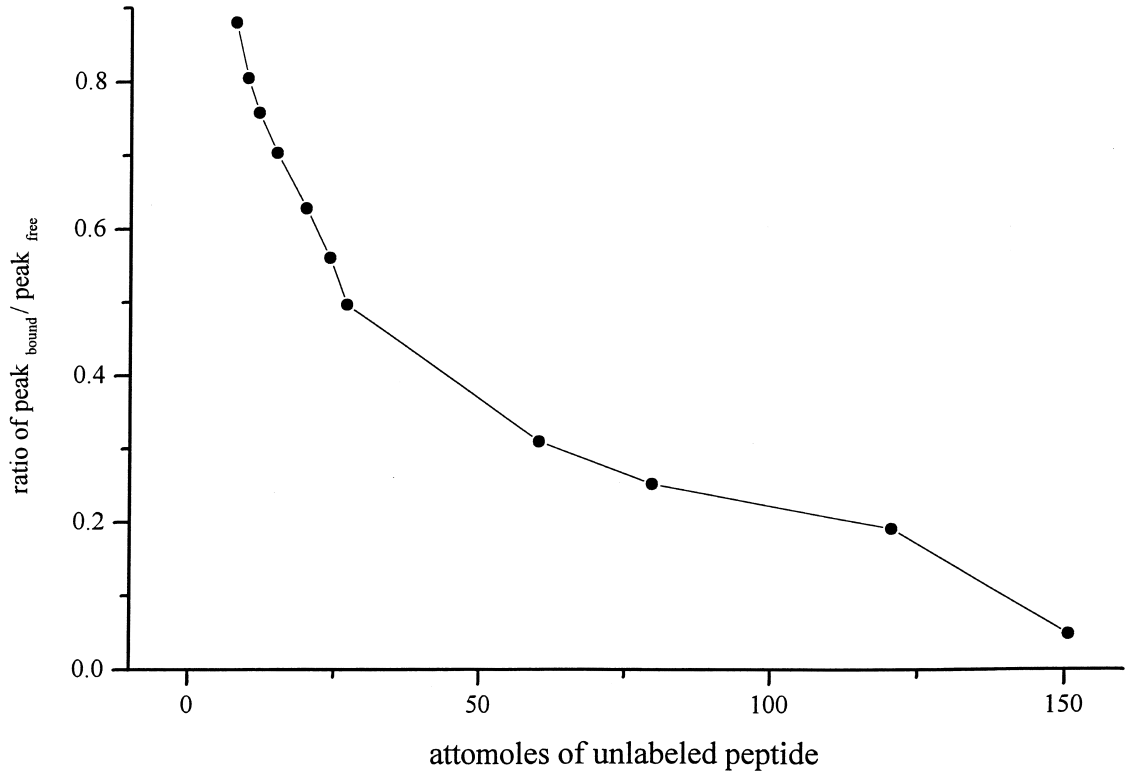

Fig. 3. Competition curve of unlabeled peptide for labeled peptide binding to antibody versus the ratio of the peak $\text{height}_{\text{bound}}$/peak $\text{height}_{\text{free}}$.

2.5. Capillary electrophoresis conditions

Free zone capillary electrophoresis was performed on a Beckman P/ACE 5500 (Beckman Instruments, Fullerton, CA, USA) controlled by P/ACE Station software (Beckman Instruments). LIF detection was done using an air-cooled argon laser (Beckman Instruments) with excitation at 488 nm and emission at 520 nm. Unmodified capillaries were obtained from Beckman Instruments. A 20 cm (length to the detector)×20 μm I.D. capillary was used with a 250 m*M* Tricine buffer that was adjusted to pH 8.0 by 6 *M* NaOH. This buffer contained 0.1% *n*-octylglucoside (Boehringer Mannheim, Indianapolis, IN, USA) and 0.1% bovine serum albumin (Sigma, St. Louis, MO, USA). In preparation for the separation, the capillary was rinsed for 1 min with 0.25 *M* NaOH, rinsed for 1 min with water, and then rinsed 2 min with buffer. The separating conditions were 30 kV for 3 min at 20°C. The current was ~25 μA. The sample was injected for 15 s followed by a 5 s injection of running buffer. The sample volume was ~0.95 nl. Rinses were carried out under high pressure and sample injection carried out under low pressure.

3. Results

The sensitivity in this study was greater by 25-fold than our previous study [19]. The labeled peptide in this study has a greater fluorescent signal than the

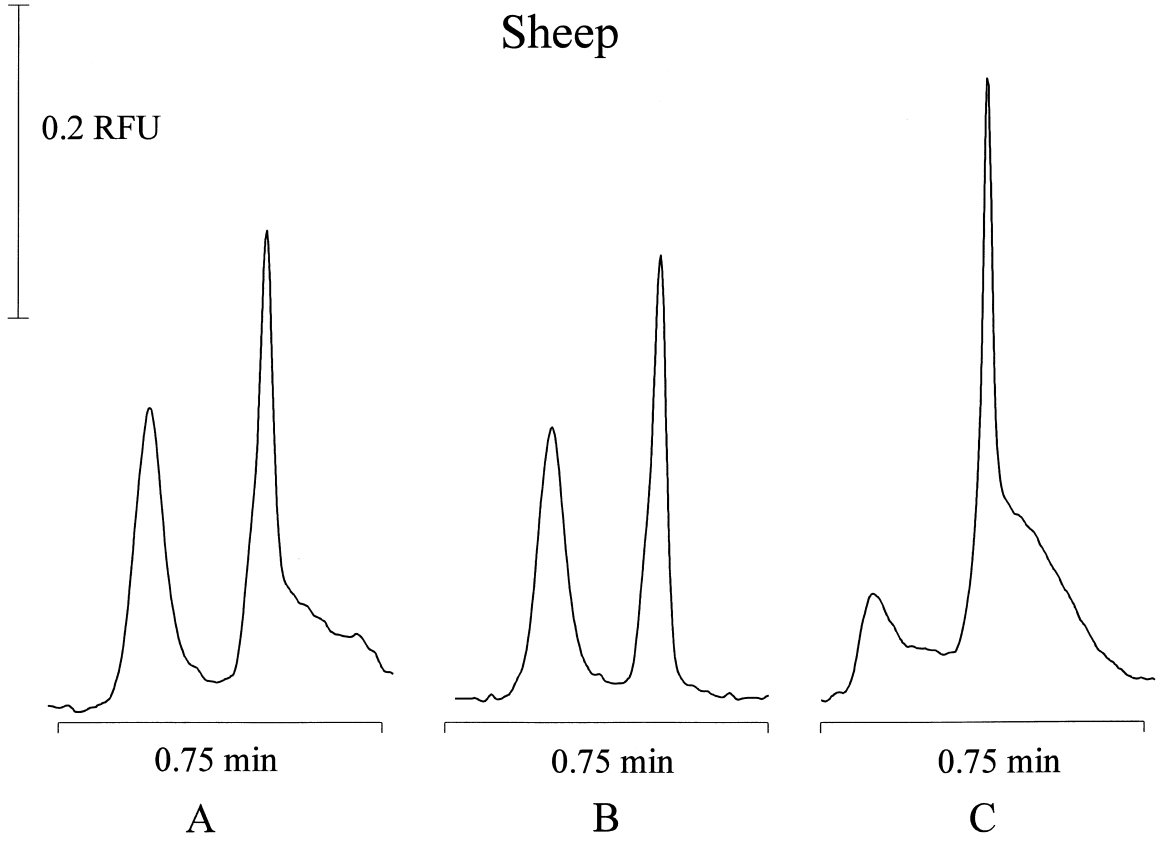

Fig. 4. Electropherograms of normal and scrapie infected sheep. (A) Antibody and the labeled peptide; peak height$_{bound}$/peak height$_{free}$= 0.67. (B) Representative sample from a normal sheep; peak height$_{bound}$/peak height$_{free}$=0.62. (C) Representative sample of a scrapie infected sheep; peak height$_{bound}$/peak height$_{free}$=0.17.

peptide used in the previous study and thereby increased the sensitivity of the assay. As can be seen in Fig. 1, two peaks are observed, one for the labeled peptide complexed to the specific antibody and one for the free labeled peptide. As can be seen in the inset of this figure, the labeled peptide alone has one peak. In Fig. 2, it is shown that the peak height of the immunocomplex peak is dependent upon the concentration of the antibody in the assay. The competition curve of the unlabeled peptide with the labeled peptide for binding to the antibody is shown in Fig. 3. A competition curve was not generated for abnormal prion protein from blood because of the very limited amount of starting samples. Previously, this was determined for abnormal prion protein from a brain sample [19]. In Fig. 4, electropherograms are shown that represent the antibody and the labeled peptide which is the control (A), a representative sample from a normal sheep (B), and a representative sample from a scrapie infected sheep (C). The change in the peak height of the immunocomplex can be easily seen when the abnormal prion protein is present in a sample from a scrapie-infected sheep. In Fig. 5, the elk blood samples showed similar results to those of the sheep. Blood from the elk with chronic wasting disease, showed a considerable reduction in the immunocomplex peak with a concurrent increase in the free peptide peak indicating competition for the binding sites on the antibody. Table 1 shows the results of blood samples from five sheep from a high risk scrapie flock that were confirmed to be positive by Western blot analysis of

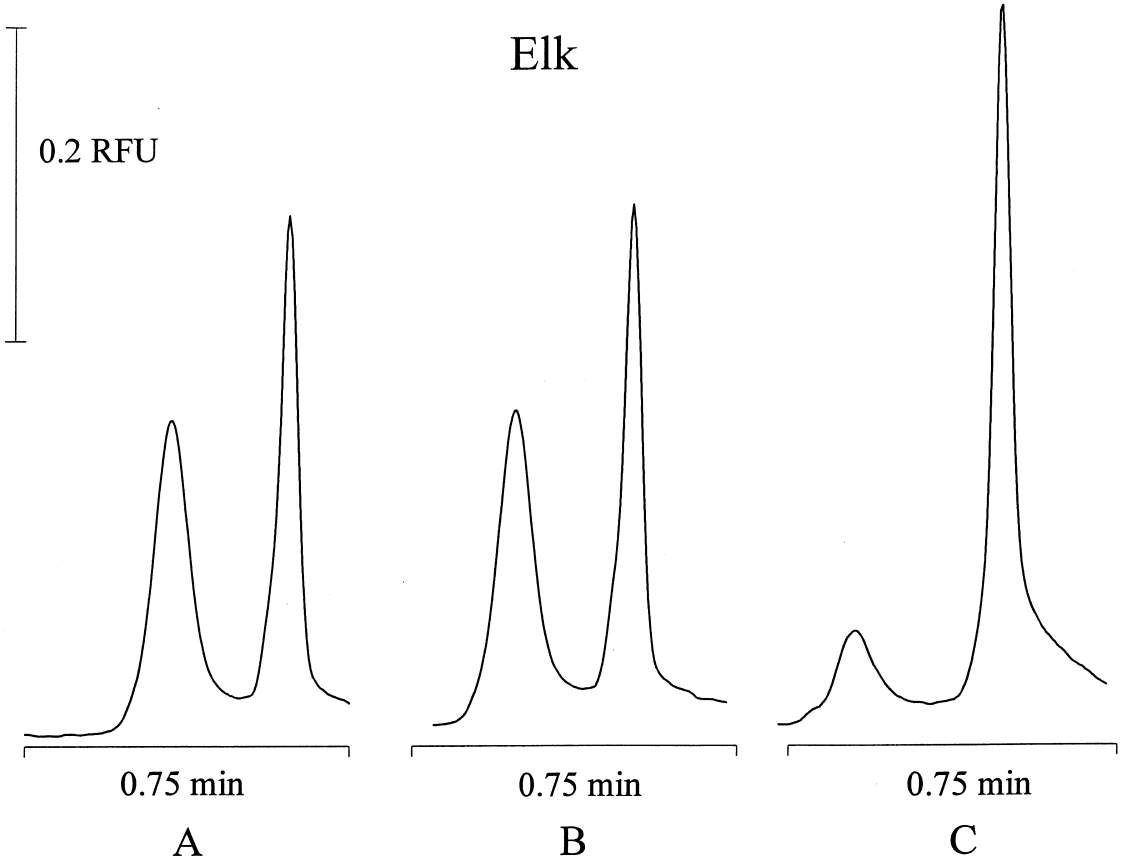

Fig. 5. Electropherograms of a normal elk and of an elk infected with chronic wasting disease. (A) Antibody and the labeled peptide; peak height$_{bound}$/peak height$_{free}$=0.67. (B) Representative sample from a normal elk; peak height$_{bound}$/peak height$_{free}$=0.61. (C) Representative sample of an elk infected with chronic wasting disease; peak height$_{bound}$/peak height$_{free}$=0.13.

Table 1
Correlation of the Western blot with the capillary electrophoresis assay on blood

Sheep number	Western blot result	% of Control ratio[a]
1	+	64.8
2	+	64.8
3	+	53.7
4[b]	+	47.2
5	+	54.2

[a] Positive<70% of control.
[b] This sheep was showing clinical signs of scrapie.

a brain sample. Table 2 shows the results of sequential bleedings of five elk. There was one elk that became positive on the second assay. In an attempt to calculate inter-assay variation, two tubes of blood were obtained from the same animal and processed separately in different assays on different days. The variation was ~15%. This error may have been introduced by the processing of the samples. Even though the variation was large, the sample still remained in the positive category. When two samples were processed together and tested in the same assay, the error for the ratio was 4%. Because the amount of sample was limiting, we were unable to do extensive calculations regarding the error in the assay.

4. Conclusions

This is the first report of an analytical method that detects the abnormal prion protein in the blood of animals infected with a transmissible spongiform encephalopathy. The capillary immunoelectrophoresis assay that we used had the sensitivity and specificity that were necessary to detect the abnormal prion protein in the blood of TSE infected animals. Although we report here the results of blood samples from sheep and elk, this assay also could detect the abnormal prion protein in other species of animals as well. The animals used in this study were naturally infected. Most of the previous studies investigating the role that blood may play in transmission of these diseases were done using infectivity studies in rodents. Because of the manner in which these studies need to be performed, there was considerable variation in the early studies [8–10] and sometimes conflicting results from different laboratories. The sensitivity of the capillary electrophoresis immunoassay makes it possible to detect the abnormal prion protein at levels that are present in the blood. Since blood is a readily accessible tissue and can be sampled repeatedly, the introduction of a practical pre-clinical diagnostic test may be possible for this group of diseases. In addition, examination of animal derived process material that is used in cosmetics and human medicine could be done with this test. The ability to measure the abnormal prion protein at these levels may contribute also to understanding the mechanism of transmission and the pathogenesis for naturally infected animals.

Disclaimer: No endorsements are herein implied. Brand names are necessary to report factually on available data; however, the USDA neither guarantees nor warrants the standards of the products, and the use of the names by the USDA implies no approval of the products to the exclusion of others that may also be suitable.

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Table 2
Results of two bleedings (three month interval) of elk exposed to chronic wasting disease

Elk number	% of control 1st sample[a]	% of control 2nd sample[a]
1	91.8	59.7[b]
2	100	86.8
3	90.7	107.4
4	59.6	58.7
5	98.9	91.4

[a] Positive<70% of control.
[b] This may indicate that this animal has become infected with chronic wasting disease between bleeding intervals.

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