DEPARTMENT for ENVIRONMENT, FOOD and RURAL AFFAIRS **CSG 15**

Research and Development

Final Project Report

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Project title	Detection of Bovine Prions from Cattle with BSE using Transgenic Mice and Conformational Dependent Immunoassay (CDI)
DEFRA project code	**SE1756**
Contractor organisation and location	Dr. Stanley B. Prusiner University of California San Francisco - Institute for Neurdegenerative Diseases San Francisco, CA
Total DEFRA project costs	£ 1,723,692
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Project end date	28/02/03

Executive summary (maximum 2 sides A4)

We have successfully isolated three recombinant antibody fragments (Fabs) that bind tightly to denatured $BoPrP^{Sc}$ but not to the native conformation of the same protein in CDI-formatted ELISA. All three Fabs were generated against the 96-105 region of the prion protein. Clones "O" and "S" recognized only bovine PrP, whereas clone "P" bound Syrian hamster, murine, ovine, and human, as well as bovine PrPs. The "O" and "P" recombinant antibody fragments (Fabs) were isolated from mouse cDNA and cloned into a vector that expresses human-mouse (HuM) chimeric Fabs in *E. coli*. The purified Fabs were then labeled with Europium and used in the CDI to measure bovine, ovine, and cervial PrP^{Sc}. We have used transgenic mice expressing bovine PrP^{Sc} to calibrate CDI sensitivity with respect to infectious units (Safar et al. 2002). These results show that the CDI is capable of measuring PrP^{Sc} in bovine brainstems with sensitivity similar to that determined by end-point titrations in transgenic (Tg) mice expressing BoPrP.

As we originally proposed, we want to evaluate the CDI for detecting BSE prions in different tissues of BSE-infected cows and to simultaneously evaluate the specificity and sensitivity of three different methods of BSE and sheep scrapie detection by bioassay. We have concluded the first stage of validation of bovine PrP^{Sc}-specific robotic CDI for detection of BSE prions postmortem in the brainstem). We employed a high-affinity recombinant antibody fragment (recFab) reacting with residues 95-105 of bovine (Bo) PrP for detection and another recFab that recognizes residues 132-156 for capture in the CDI. We report that the CDI is capable of measuring PrP^{Sc} in bovine brainstems with a sensitivity similar to that determined by end-point titrations in transgenic (Tg) mice expressing BoPrP (Safar et al. 2002). The automated CDI achieved absolute diagnostic sensitivity and specificity in detection of BSE (Safar et al. 2002).

We continue to refine and increase the specificity and sensitivity of the CDI by employing the following strategies:

(1) We continue the purification of BSE prions from BSE brainstems for use as the antigen in immunizations of $Prnp^{0/0}$ mice;

(2) In an already initiated project we generate new sets of monoclonal anti bovine PrP antibodies using recombinant bovine PrP and rapid hybridoma techniques;
(3) We are continuously screening in CDI new antibodies acquired from commercial sources and from our collaborators.

In order to fully understand the affinity and specificity of P Fab, an antibody fragment used in the CDI for detection of BSE prions, we have solved its structure both alone as well as in complex with its cognate peptide epitope, BoPrP 95-104. This latter structure is shown in **Figure 1** with a view onto the binding region of P Fab, represented as an electrostatically colored molecular surface, allowing the bound peptide to be clearly visible. The Fab binding relies on both hydrophobic and electrostatic interactions to generate its high binding affinity. BoPrP-W99 is deeply buried in a hydrophobic cleft while BoPrP-K101 and K104 form strong ionic interactions with a large negative patch on the Fab.

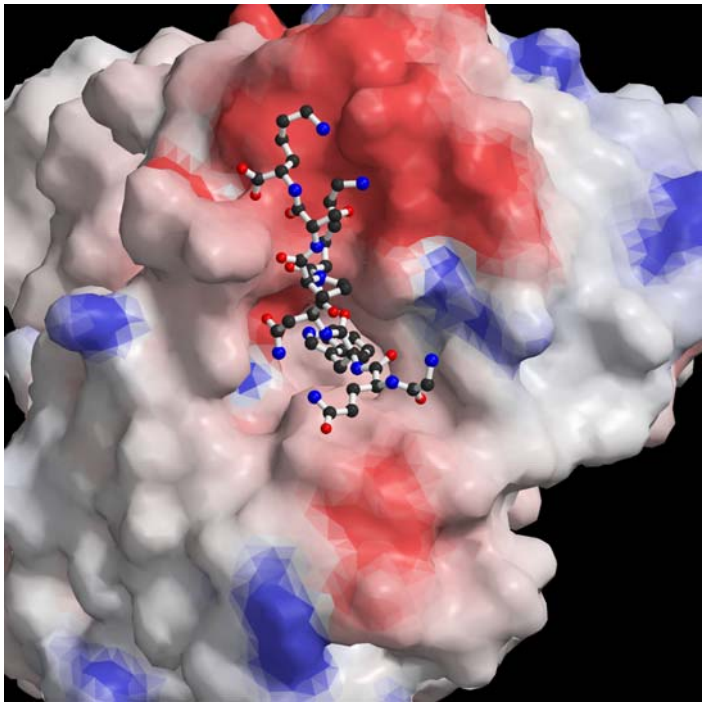

Figure 1: Crystal structure of the complex between antibody fragment P and its synthetic epitope**.**

Such structural information allows us to identify each atomic interaction and evaluate its contribution to the overall binding affinity of the Fab. This knowledge can be used most effectively in an affinity maturation scheme by limiting the combinatorial libraries to represent only those residues that are near in space to the bound peptide, yet maintaining residues deemed essential for epitope recognition, increasing the likelihood that Fab of higher affinity or increased specificity can be produced.

Using recombinant Fab P, The CDI was able to discriminate between PrP^{Sc} from BSE-infected cattle and Tg(BoPrP) mice as well as PrP^{Sc} from CWD-infected deer and elk. Preliminary data on strain typing using available scrapie sheep or bovine BSEspinal cords and brainstems demonstrate high discrimination power of CDI in distinguishing BSE from scrapie **(Figure 2)**. Our findings argue that applying the CDI to livestock and wild game animals should significantly reduce human exposure to animal prions regardless of origin.

We first obtained RIII mice at the beginning of August 2000 and spent the first several months establishing a large breeding colony. At this time, we have initiated the endpoint titration of BSE and scrapie in replicates in parallel to the endpoint study with Tg(BoPrP).

We have also recently developed several new lines of transgenic mice expressing Bo/Mo chimeric PrPs. We have previously shown that Tg(BoPrP) mice exhibit even shorter incubation periods following inoculation with sheep scrapie than following inoculation with BSE from cattle. In contrast, some of these new lines are equally susceptible to BSE as Tg(BoPrP) mice, but are relatively resistant to infection with sheep scrapie, with incubation periods several hundred days longer than those found with BSE. The best

characterized of these new lines is termed. Tg Bo3M(H155Y, V184I, E186Q, V203I, V215I) $Prnp^{0/0}$ 23953, and comprises an N-terminal region derived from BoPrP and a C-terminal region including the two disulphide-linked alpha helices from MoPrP.

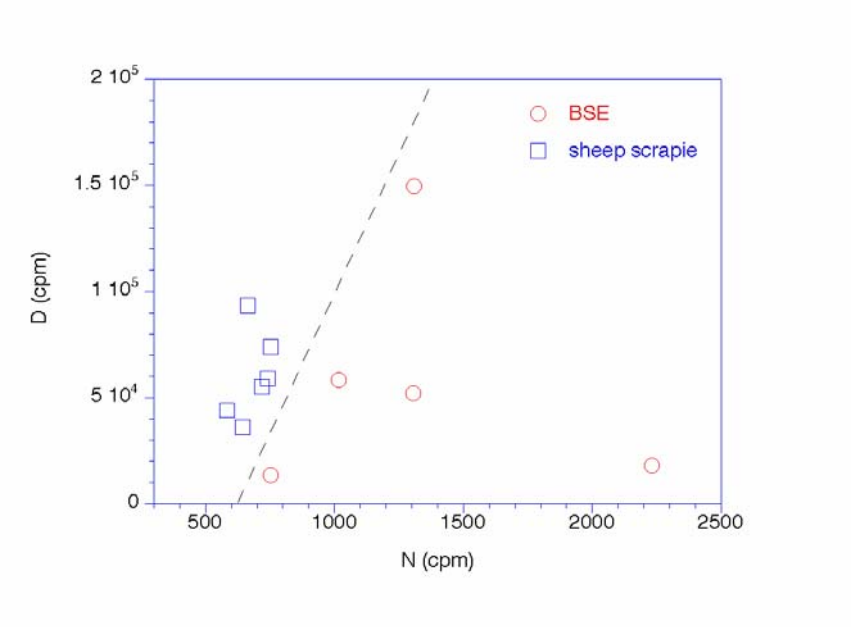

Figure 2: Strain typing of BSE and scrapie prions by CDI.

References

Safar, J. G., M. Scott, et al. (2002). "Measuring prions causing bovine spongiform encephalopathy or chronic wasting disease by immunoassays and transgenic mice." Nat. Biotechnol. **20**: 1147-1150.

Scientific report (maximum 20 sides A4)

Aim 1: Bioassay in Tg mice overexpressing bovine PrP.
Because we were unable to obtain the sample of standard BSE inoculum we requested for this series of experiments on time, we were forced to conduct an alternative study using a limited stock of characterized BSE inoculum that we had on-hand. This endpoint titration study indicates that these transgenic mice are far more sensitive to BSE in bioassay than the RIII mice that have been widely used previously **(Table 1).** However, all the originally planned replicate titrations of ovine scrapie and BSE samples were already initiated.

Table 1: End-point titer of BSE prions in PG31/90 brain homogenate bioassayd in Tg(BoPrP)4092/HOZ mice. The titer was calculated by two different statistical methods from three independent titrations.

Method	Titer per ml	Titer per g
Reed-Munsch	8.2×10^5	8.2×10^6
Spearman-Karber	6.8×10^5	6.8×10^6

Aim 2: Bioassay in wild-type RIII mice.
We first obtained these mice at the beginning of August 2000 and spent the first several months establishing a large breeding colony. At this time, we have initiated the endpoint study despite additional difficulties with obtaining the standard BSE inoculum due to the foot and mouth disease (FMD) epidemic. However, due to the shorter incubation times of BSE and scrapie in Tg(BoPrP)$Prnp^{0/0}$ 4092/HOZ mice, we expect to conclude this study in parallel with the repetition of the titration in Tg(BoPrP)$Prnp^{0/0}$4092/HOZ mice, using the previously titrated standard inoculum.

Aim 3: Detection of BSE prions by the conformation-dependent immunoassay (CDI).
We originally proposed to develop CDI for detecting BSE prions in different tissues of BSE-infected cows and to simultaneously evaluate the specificity and sensitivity of three different methods of BSE and sheep scrapie detection by bioassay. Much of the originally planned CDI experiments had to be postponed, because we have been unable to obtain the necessary samples due to the FMD epidemic with resulting embargo for imports from the U.K.. Only one set of BSE-infected brainstems that we requested was sent during the first year of funding and these have now been fully characterized by the CDI assay.

The cases of BSE-infected cow brainstems already tested by CDI were collected in the field without special precautions or selection by the CVL. The group of normal controls was composed of brainstems collected from US cows. All BSE samples tested positive and all the controls tested negative **(Table 2)**.

Table 2: Cumulated diagnostic sensitivity and specificity of the automateddirect and sandwich CDI in detecting BSE prions in bovine brainstems.

	Positive CDI		Negative CDI		Cases
	n	%	n	%	n
U.K. BSE	481	100%	0	0%	0
U.S. Controls	0	0%	1248	100%	432

We originally proposed to develop an incubation time assay for detecting BSE prions in transgenic (Tg) mice and to simultaneously evaluate the specificity and sensitivity of three different methods of BSE and sheep scrapie detection. We have been largely powerless to perform much of the originally planned experiments, because we have been unable to obtain the necessary samples on time. Only one set of tissues that we requested was sent during the first year of funding and these have now been fully characterized by the CDI assay.

Aim 1: Bioassay in Tg mice overexpressing bovine PrP.
Because we were unable to obtain the sample of standard BSE inoculum we requested for this series of experiments, we were forced to conduct an alternative study using a limited stock of characterized BSE

inoculum that we had on-hand. This endpoint titration study indicates that these transgenic mice are far more sensitive to BSE in bioassay than the RIII mice that have been widely used previously.

For logistical reasons, studies such as these that require large numbers of animals are best performed using transgenic mouse lines that are homozygous for the transgene. The line that we obtained originally, Tg(BoPrP)Prnp$^{0/0}$4125, could not be bred to homozygosity; consequently, we established a new line, Tg(BoPrP)Prnp$^{0/0}$4092/HOZ that was homozygous for the transgene array. Triplicate endpoint titrations of BSE prions were performed in these Tg(BoPrP)Prnp$^{0/0}$4092/HOZ mice and the endpoint titer was calculated according to the methods of Karber and Reed and Munch (Prusiner 1987). Three separate dilution series were performed in parallel, and the average endpoint titer was assessed. Using a homogenate prepared from the medulla of a Hereford bull with BSE, PG31/90, we obtained a titer of ~8 x 10^6 ID_{50} per g of brain tissue. This compares with a titer of $10^{3.1}$ mouse i.c./i.p. LD_{50} per g of tissue of BSE brain titrated in RIII mice (reported in the 8 July report of the European Commission). Thus, our preliminary data indicate that transgenic BoPrP mice are >1000 times more sensitive to BSE prions than normal non-transgenic mice. A plot of relationships between incubation time and endpoint titer has been completed to provide a calibrated incubation time assay for BSE prions in Tg(BoPrP)Prnp$^{0/0}$ mice **(Table 1 & Figure 1)**.

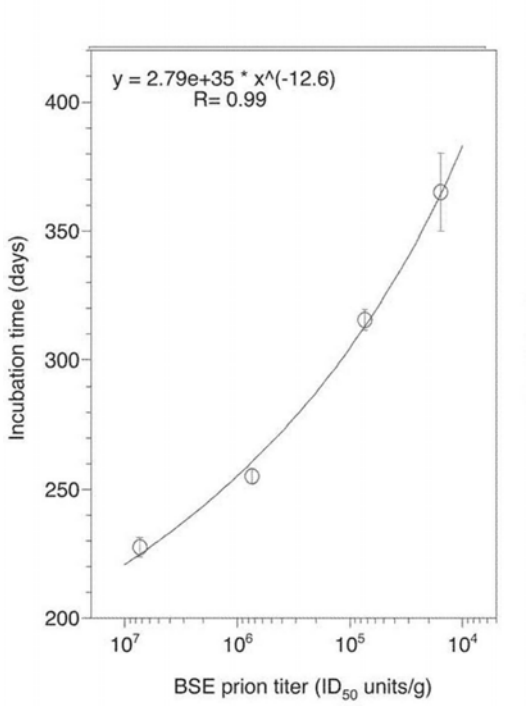

Figure 1: Inverse exponential relationship between titers of BSE prions and incubation times in Tg(BoPrP$^{+/+}$)4092/*Prnp*$^{0/0}$ mice. The data points are the average ± standard error of the mean (SEM) calculated from three independent end-point titrations.

We have used this calibration curve to estimate the titer of additional BSE brain samples we had previously obtained from CVL and consistently found that BSE brain homogenates report a titer of about 7 logs per gram brain in our system, a number equal to or greater than the reported titration in cattle and at least 3 to 4 logs higher than those found in RIII mice.

Despite these promising preliminary findings, it is impossible to make an accurate assessment of the sensitivity of the transgenic mouse bioassay compared to newborn calves. For this to be made, we are repeating the bioassay using the standardized BSE inoculum, which as explained above was not supplied to us on time. We were also unable to conclude the initiated sheep scrapie titration studies in the Tg(BoPrP)Prnp$^{0/0}$ mice, because of delayed shipment of sheep scrapie samples we requested.

Aim 2: Bioassay in wild-type RIII mice.
We first obtained these mice at the beginning of August 2000 and spent the first several months establishing a large breeding colony. At this time, we have initiated the endpoint study despite additional unforeseeable difficulties with obtaining the standard BSE inoculum due to the FMD epidemic. The shorter incubation times of BSE and scrapie in Tg(BoPrP)Prnp$^{0/0}$4092/HOZ mice will allow us to conclude this study in parallel with the repetition of the titration in RIII mice, using the previously titrated standard inoculum.

Aim 3: Detection of BSE prions by the conformation-dependent immunoassay (CDI).
We have successfully isolated three recombinant antibody fragments (Fabs) that bind tightly to denatured BoPrP^{Sc} but not to the native conformation of the same protein in CDI-formatted ELISA. All three Fabs were generated against the 96-105 region of the prion protein. Clones "O" and "S" recognized only bovine PrP, whereas clone "P" bound Syrian hamster, murine, ovine, and human, as well as bovine PrPs. The "O" and "P" recombinant antibody fragments (Fabs) were isolated from mouse cDNA and cloned into a vector that expresses human-mouse (HuM) chimeric Fabs in *E. coli*. The purified Fabs were then labeled with Europium and used in the CDI to measure bovine, ovine, and cervial PrP^{Sc}. Transgenic mice expressing bovine PrP^{Sc} are currently being used for the calibration of CDI sensitivity with respect to the infectious units.

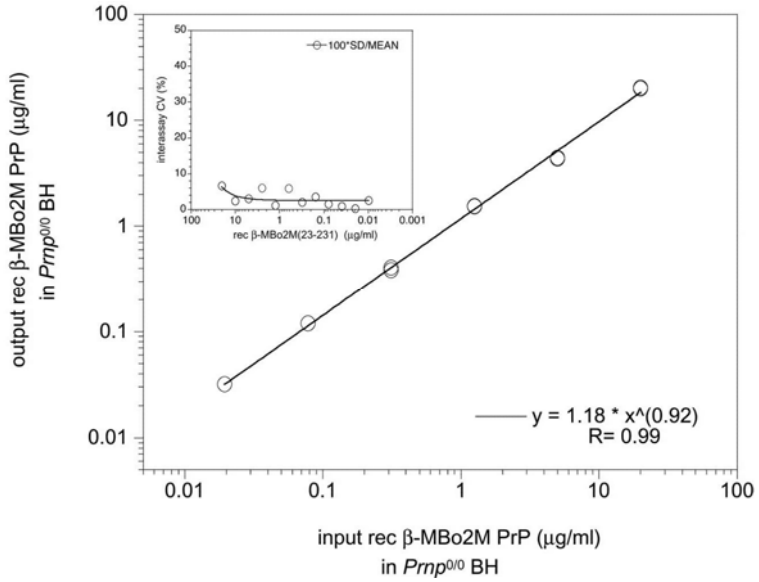

Figure 2: Calibration of the CDI and high interassay reproducibility. The CDI was calibrated with recombinant MBo2M PrP(23-231) purified from *E. coli* and refolded into ß-sheet conformation. Rec ß-MBo2M PrP was quantified by absorbance at 280 nm (Mehlhorn et al. 1996) and at the indicated concentrations, spiked into brain homogenate (BH) from a *Prnp*$^{0/0}$ mouse. The input values were then correlated with the concentration calculated from CDI data measured in duplicate as described (Safar et al. 1998). Interassay reproducibility is shown in the inset and was determined as an interassay coefficient of variation (CV = 100*(standard deviation)/mean) from six different plates**.**

Using newly generated recombinant chimeric (HuM) Fabs against epitope 96-105 of bovine PrP, the CDI for bovine PrP^{Sc} achieved sensitivity equivalent to Europium-labeled 3F4 IgG used in detection of hamster and human PrP. This sensitivity was further increased by using recombinant Fab D 18 as a capture antibody in sandwich-formatted CDI (Safar et al. 2002). The linear correlation between the absolute amount of PrP spiked into Prnp$^{0/0}$ brain homogenate and the concentration of PrP calculated from the CDI (R = 0.99) within a 100-fold range is evidence of CDI sensitivity and robustness. The assay is also reliable with an inter-assay variation of less than 7% **(Figure 2).**

End-point titration of BSE-infected brain homogenates in transgenics harboring multiple copies of the bovine PrP gene indicates a titer of ~10^7 infectious units per g (ID_{50}/g) of brain tissue. Slightly less sensitive bioassays in cattle demonstrated 10^6 ID_{50}/g and the least sensitive is apparently the bioassay in RIII mice with only$10^{3.1}$ ID_{50}/g. The current sensitivity of the manual and robotic protocol for CDI in BSE-infected transgenics is close or equivalent to that of the most sensitive bioassay for BSE prions now available, and significantly higher than the sensitivity of titrations for BSE prions in RIII mice **(Figure 3**).

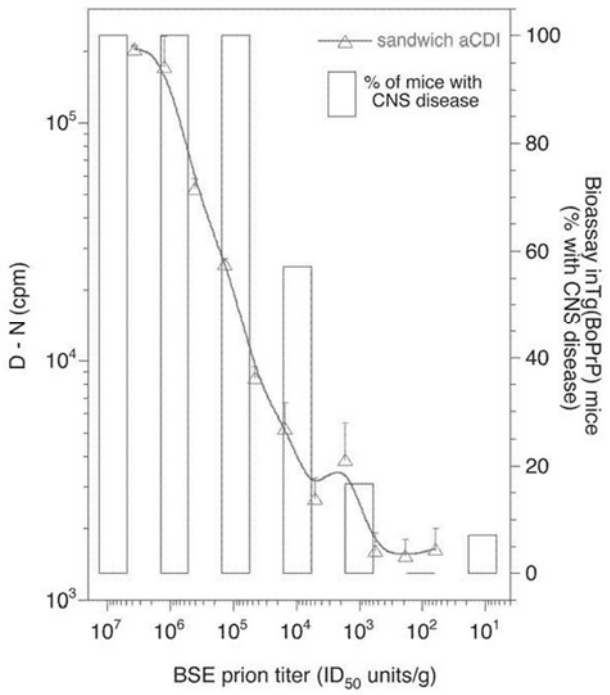

Figure 3: Direct relationship between $BoPrP^{Sc}$ detected by CDI and BSE prions measured in Tg(BoPrP)*Prnp*$^{0/0}$ mice. The (D – N) value is directly proportional to the concentration of PrP^{Sc} (Safar et al. 1998). The percentage of ill mice at each BSE sample dilution used in the bioassay was calculated from three independent end-point titrations.

The variability of CDI between 10^{-4} and 10^{-6} dilution is expected and corresponds to the variability of the end-point titration in the same dilution zone. Apart from differences in protocols and tissue origin, it is due to the statistical nature of 1 infectious unit, which is by definition, the dilution point with an average of a 50% survival rate (for BSE ~10^{-4}) in bioassay. Therefore, less than 100% of the positive rate of detection between 10^{-4} and 10^{-5} correlates with bioassay results and **Table 4**.

Table 4: Comparison of sensitivity of different CDI protocols and available bioassays.

		Sensitivity cut-off brain dilution with 50% pos. CDI	Sensitivity cut-off in ID_{50}/g		
			RIII mouse bioassay	Cow bioassay	Tg(BoPrP) bioassay
Manual CDI	BSE	5.21E-05	0.07	52	354
Manual CDI	Tg(BoPrP)	6.05E-06	0.01*	6*	41*
Robotic CDI	BSE	2.08E-04	0.26	208	1417

*Extrapolated values from BSE titrations; back-titrations of Tg(BoBSE) in TgBoPrP, RIII mice and cow are not available.

The performance characteristics of CDI in detection of PrP^{Sc} in BSE-infected and CWD-infected brain homogenates using Eu-(HuM)Fab P:

1. Assay is conformation-sensitive, quantitative, and robust;
2. Signal ratio of positive/control sample is up to 1,000 in BSE-infected and 4,000 in CWD-infected samples;
3. Intra-assay coefficient of variation (CV) is less than 7%;
4. Sensitivity is less than ≤1 ng/ml of PrP^{Sc} in 5% brain homogenate;
5. Detection limit is close to the sensitivity of bioassays for BSE prions in cattle;
6. Prospect of detecting PrP^{Sc} through the presymptomatic latent period of the experimental prion infection;
7. Diagnostic sensitivity and specificity of CDI in detection of BSE is 100% in symptomatic stage of the disease;
8. Simultaneous prion strain typing;
9. No interference, background, or false positive signal from natural samples;
10. Working scaled-up and robotic version of CDI for high flow-through.

Diagnostic sensitivity and specificity of robotic CDI and test behavior for other TSEs.

We originally proposed to develop CDI for detecting BSE prions in different tissues of BSE-infected cows and to simultaneously evaluate the specificity and sensitivity of three different methods of BSE and sheep scrapie detection by bioassay. We have been powerless to perform much of the originally planned CDI experiments, because we have been unable to obtain the necessary samples. Only one set of BSE-infected brainstems that we requested was sent during the first year of funding and these have now been fully characterized by the CDI assay.

The 100 cases of BSE-infected cow brainstems already tested by CDI were collected in the field without special precautions or selection by the CVL. The group of normal controls was composed of 432 brainstems collected from US cows. All BSE samples tested positive and all the controls tested negative **(Table 2)**.

Based on epitope mapping, preliminary experiments, and GenBank data, the epitope of PrP recognized by Eu-(HuM)Fab P is shared by cattle, elk, deer, sheep, goats, and most of the other ungulates. Therefore, we are confident that the CDI developed using Eu-(HuM)Fab P for detection of BSE in cows will perform equally well in detecting scrapie in sheep or goat **(Figure 4).**

The availability of positive and negative controls for CDI, prepared from inoculated and uninoculated transgenic mice expressing bovine PrP genes and inoculated with relevant prion strains, facilitates data evaluation. Such controls are critical for comparison of the data from day to day. In contrast with BSE-infected cow brain, samples of transgenic mice available in the laboratory are renewable and are routinely amplified into large pools.

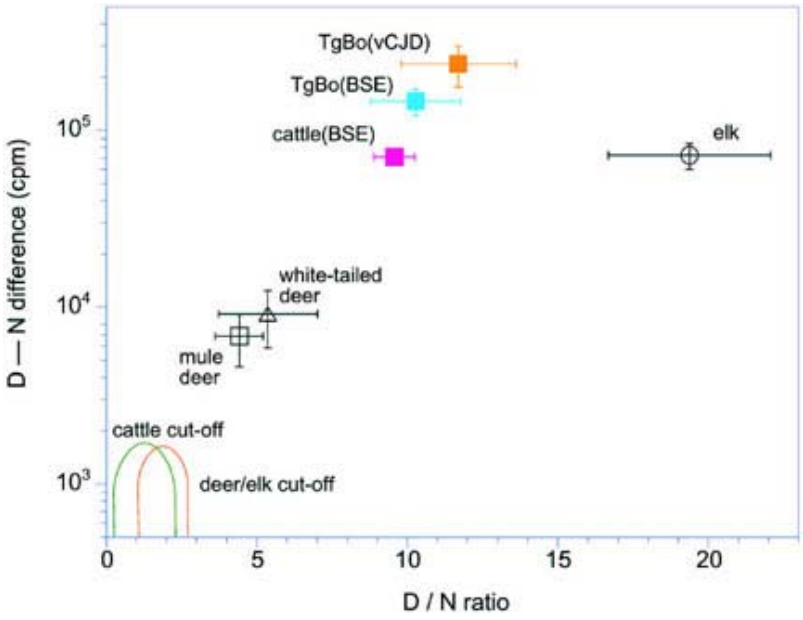

Figure 4: Different conformational characteristics of ungulate prion strains revealed by direct CDI. CDI data were plotted as D/N ratios against (D – N) values recorded for BSE-infected British cattle (*n* = 100; magenta square), first passage of pooled brain homogenates from BSE- and vCJD-infected Tg(BoPrP)*Prnp*$^{0/0}$ mice (cyan square and orange square, respectively), CWDinfected mule deer (*n* = 10; open square), white-tailed deer (*n* = 6; open triangle) and elk (*n* = 19; open circle). The arcs link cut-off values for differences and ratios in normal U.S. cattle (green; *n* = 432) and in normal deer and elk (orange; *n* = 60).

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Safar, J. G., M. Scott, et al. (2002). "Measuring prions causing bovine spongiform encephalopathy or chronic wasting disease by immunoassays and transgenic mice." Nat. Biotechnol. **20**: 1147-1150.

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