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Field validation and assessment of an enzyme-linked immunosorbent assay for detecting chronic wasting disease in mule deer (*Odocoileus hemionus*), white-tailed deer (*Odocoileus virginianus*), and Rocky Mountain elk (*Cervus elaphus nelsoni*)

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Abstract. Tissue samples (n = 25,050 total) from 23,256 mule deer (*Odocoileus hemionus*), Rocky Mountain elk (*Cervus elaphus nelsoni*), and white-tailed deer (*Odocoileus virginianus*) collected statewide in Colorado were examined for chronic wasting disease (CWD) using an enzyme-linked immunosorbent assay developed by Bio-Rad Laboratories, Inc. (brELISA), in a 2-phase study. In the validation phase of this study, a total of 4,175 retropharyngeal lymph nodes (RLN) or obex (OB) tissue samples were examined independently by brELISA and immunohistochemistry (IHC). There were 137 IHC-positive samples and 4,038 IHC-negative samples. Optical density (OD) values from brELISA were classified as "not detected" or "suspect" based on recommended cutoff values during the validation phase. Using IHC-positive cases as known CWD-infected individuals and assuming IHC-negative cases as uninfected, the relative sensitivity of brELISA depending on species ranged from 98.3% to 100% for RLN samples and 92.1% to 93.3% for OB samples; the relative specificity of brELISA depending on species ranged from 99.9% to 100% for RLN samples and was 100% for OB samples. Overall agreement between brELISA and IHC was ≥97.6% in RLN samples and ≥95.7% in OB samples of all species where values could be calculated; moreover, mean brELISA OD values were ≥46× higher in IHC-positive samples than in IHC-negative samples. Discrepancies were observed only in early-stage cases of CWD. Based on the validation phase data, only RLN samples were collected for the field application phase of this study and only samples with brELISA OD values >0.1 were examined by IHC. Among 20,875 RLN samples screened with brELISA during this second testing phase, 155 of 8,877 mule deer, 33 of 11,731 elk, and 9 of 267 white-tailed deer samples (197 total) had OD values >0.1 and were further evaluated by IHC to confirm evidence of CWD infection. Of cases flagged for IHC follow-up, 143 of 155 mule deer, 29 of 33 elk, and all 9 white-tailed deer were confirmed positive. Mean (±SE) OD values for IHC-positive cases detected during the field application phase were comparable with those measured in RLN tissues during the validation phase. Based on these data, brELISA was determined to be an excellent rapid test for screening large numbers of samples in surveys designed to detect CWD infections in deer and elk populations.

Chronic wasting disease (CWD) was once considered to be enzootic in free-ranging mule deer (*Odocoileus hemionus*), Rocky Mountain elk (*Cervus elaphus nelsoni*), and white-tailed deer (*Odocoileus virginianus*) only in north central Colorado and southeastern Wyoming.[6] However, surveillance of both captive and free-ranging cervids has revealed CWD to be more widespread.[12] These recent discoveries have stimulated considerable concern among wildlife officials, hunters, and conservation agencies. One direct result of this concern is a concerted effort by wildlife agencies to determine if CWD is present in free-ranging and captive cervids in their respective states. Compounding this problem is an overwhelming demand by hunters to determine if their harvested animals are free of CWD.

The monoclonal antibody (MAb) F99/97.6.1 used to demonstrate scrapie-associated prion protein (PrP) in brain and lymphoid tissues of domestic sheep with scrapie[8] has been used in an immunohistochemistry (IHC) assay for diagnosis of CWD in mule deer and elk.[5,10,11] This is a sensitive and effective technique for detecting CWD in both species.[5,10,11] However, IHC is a costly, labor-intensive and time-consuming technique

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necessitating skilled histology technicians and experienced microscopists to diagnose CWD. Few laboratories possess the skill and experience necessary for certification to conduct IHC, and these laboratories are being overwhelmed by the increased demand for IHC service to support large-scale CWD surveys.

A rapid test designed for large-volume sample screening appears to be the most practical solution to meet this increased demand for CWD surveillance. Recently, several candidate tests have been developed in Europe to screen cattle and sheep for evidence of prion disease infections.[3] Based on previously reported similarities between performance of scrapie and CWD diagnostic tools,[7,8,10] likely one or more of these European tests could prove reliable in detecting CWD infections in deer and elk. Of the tests available for evaluation, preliminary data (Salman, unpublished data; Spraker, unpublished data) suggested that Bio-Rad Laboratories' enzyme-linked immunosorbent assay[a] (brELISA) showed the greatest promise as a reliable CWD-screening test. Thus, brELISA was chosen as a candidate screening test for CWD surveillance in deer and elk, which was evaluated in a study conducted during the 2002 deer and elk hunting seasons in Colorado. The objectives of this 2-phase study were to determine performance parameters for brELISA as compared with IHC in detecting CWD infections in mule deer, white-tailed deer, and elk and to evaluate the application and use of brELISA in a large-scale CWD surveillance program.

Materials and methods

Sample sources and collection

During August–December 2002 in Colorado, the Colorado Division of Wildlife (CDOW), the Veterinary Diagnostic Laboratory at Colorado State University (CSU) in Fort Collins, and the branch Diagnostic Laboratories at Grand Junction (western Colorado) and Rocky Ford (southeastern Colorado) assisted by the Colorado Department of Agriculture personnel and the Colorado Veterinary Medical Association (CVMA) veterinarians statewide surveyed deer and elk populations for CWD. Colorado Division of Wildlife organized the survey effort by establishing sample collection sites around the state. In most cases, CDOW personnel removed a sample of the medulla oblongata at the level of the obex (OB) or retropharyngeal lymph nodes (RLN) from submitted animals and coded the tissues with a number designating the origin of the animal; in other cases, harvested animals were taken to participating CVMA veterinarians for tissue extraction. Samples were placed in plastic bags and refrigerated. When both tissues were collected, RLN was placed in a small separate plastic bag and then into a larger bag with OB. Collected tissues were sent to one of the CSU Veterinary Diagnostic Laboratories. Samples from submitted tissues were prepared for respective assays as described below.

Samples were collected primarily from free-ranging deer and elk, although CWD-exposed mule deer from a CDOW research facility were also sampled to augment the number of positives available for assay evaluation. The vast majority of examined samples were collected from hunter-harvested deer and elk; however, clinical CWD suspects and road-killed and culled individuals were also sampled. Because both research deer and the latter free-ranging sources tend to be biased toward CWD-positive individuals,[6] overall infection rates among samples reported in this study do not reflect prevalence in sampled free-ranging populations.

Tissue preparation

In the laboratory, OB samples were cut at the level of the vagus nucleus (VN), and an approximately 2-mm-thick slice was placed into a tissue cassette for IHC. On the proximal side of the initial cut, a sample of 350 ± 40 mg was taken for brELISA. A section of RLN was taken for IHC and placed into a cassette; in cases where both OB and RLN tissues were collected from an animal, they were placed in the same cassette. A RLN sample of 200 ± 20 mg was taken for brELISA. In sampling RLN, a concerted effort was made to obtain the cortex. New blades were used for each individual tissue sample to minimize cross-contamination. Cassettes were placed in 10% neutral buffered formalin for fixation before IHC processing. Samples for enzyme-linked immunosorbent assay (ELISA) were processed immediately or were stopped after the tissue homogenization step or after the purification process and held overnight for convenience.

Immunohistochemistry assay

Validation of MAb F99/97.6.1 for IHC staining of brain and lymphoid tissues in mule deer was previously performed in the Veterinary Diagnostic Laboratory at CSU.[10] The IHC procedures for this study were as described previously[10] and will not be repeated.

Bio-Rad Laboratories' ELISA

Purification and detection of PrP^{CWD}. The Bio-Rad CWD antigen test kit, ELISA, was used for purification and detection of PrP^{CWD}. The detailed assay procedure is described in the instruction manual sent with the Bio-Rad CWD purification and detection kits. The principle of the assay is based on the selective degradation of PrP^{sen} by proteinase K (PK) treatment. The PK treatment step and centrifugation ensure selectivity of the test because PrP^{CWD} is resistant to proteolysis and copurifies with infectivity. The sample treatment procedures concentrate PrP^{CWD}, thus increasing the sensitivity of the test. Proteinase K treatment and the denaturation/renaturation process yield "cleaner" samples, minimizing nonspecific interference. Final measurements are made using the conventional 2-site sandwich ELISA assay because it provides more sensitive, more specific, and more rapid measurements than other assay formats. Monoclonal antibodies directed against PrP, by immunizing mice, are used for the detection of PrP^{CWD}. This immunometric assay can be easily adapted to automation, thus allowing high-throughput

analysis. The entire assay takes approximately 4–5 hr to complete. Toward the end of this study, Bio-Rad Laboratories' automated system[a] was used for analysis. This equipment performed some of the purification procedures automatically and increased sample throughput up to 1,000 samples/day.

Briefly, the ELISA procedure was as follows: samples were placed into grinding tubes provided by the manufacturer for processing. Each tube was given a number; in addition, grinding tubes were designated with an "O" or "L" to indicate the source tissue. Obex tissue of 350 ± 40 mg or RLN tissue of 200 ± 20 mg was used. To ensure proper homogenization of the lymphoid tissue, 1 large grinding bead provided by the manufacturer was added to each homogenization tube. Because lymphoid follicles are the focus of PrP^{CWD} deposition in RLN, the cortex of RLNs was preferentially sampled. Tubes were closed firmly and placed in the FASTPREP 120.[b] Samples were homogenized for 45 sec at a speed setting of 6.5 units. For RLN samples, two 45-sec agitation cycles were performed to ensure complete homogenization. When 2 agitations were performed, the homogenization tubes were cooled to room temperature between each cycle. Tubes were removed from the homogenizer, and the homogenate was resuspended by inversion. Using a calibration syringe provided by the manufacturer, 500 µl of the sample was collected by carefully immersing the syringe at the bottom of the homogenization tube to avoid sampling poorly homogenized tissue fragments. Unused homogenate was frozen at −20 C. The sample aspirated from the homogenization tube was transferred to a 2-ml Eppendorf tube.

Selective purification of PrP^{CWD} was achieved by adding 500 µl PK solution to each sample and incubating at 37 C in a heating block for 10 min from the time at which the first sample was placed in the heating block. Five hundred microliters of clarifying solution was added to each sample and mixed gently by inversion. Selective concentration of PrP^{CWD} was achieved by centrifuging for 7 min at 15,000 × *g* at room temperature. The supernatant was decanted into a waste bottle, and the tubes were "tapped" onto an absorbent pad or paper towel to remove all excess liquid from the sample. Fifty microliters of the resuspension buffer was added to each sample, and tubes were incubated in a 100 C heating block for 5 min. After incubation, each sample was vortexed for 5 sec. At this point, the samples could be used in the detection step listed below or stored at −20 C indefinitely. However, if the samples were frozen, then they were reheated to 100 C before use in the detection plate.

The detection kit components were brought to room temperature at least 30–60 min before use. The lyophilized positive control, a noninfectious recombinant PrP, was resuspended in 2 ml buffer provided in the Bio-Rad CWD kit. The negative control was phosphate-buffered saline buffer (pH 7.4) supplemented with bovine serum albumin, which is also provided as part of the detection kit components.

Samples were thoroughly mixed by pipetting and transferred into the appropriate well of the plate provided in the kit. One hundred microliters of the prepared negative and positive controls as well as the samples were added to plates according to the loading order indicated in the detection kit instructions. The plate was covered with a sealing film and incubated on a 37 C microplate heating block for 75 min. During sample incubation, the 1× wash buffer (WB) was prepared by adding 100 ml of 10× WB to 900 ml of deionized water. Once incubation was complete, unbound proteins were removed by washing the plate 3 times with 800 µl WB using Model 1575 Immunowash Microplate Washer.[a] The 800-µl wash uses the overflow function of the strip washer for a better wash. Excess WB was removed from the plate by "clapping" it on an absorbent pad or paper towel.

The conjugate was prepared in a 15-ml conical tube by adding 1 ml of 10× conjugate to 9 ml of 1× WB. The contents of the tube were mixed by inversion and 100 µl added to each well. The plate was covered with a sealing film and incubated at 4 C for 1 hr. After conjugate incubation, the plate was washed using the Model 1575 Immunowash Microplate Washer 5 times with 800 µl of WB.

Before the end of the conjugate incubation, the chromagen solution was prepared in another 15-ml conical tube by the addition of 1 ml of 10× chromagen to 10 ml peroxidase substrate buffer. The tube was mixed well by inversion, and 100 µl of chromagen mixture was added to each plate well and incubated in the dark at room temperature for 30 min. After incubation, 100 µl of stop solution was added to each well.

Light absorbency of samples was measured in each well of the plate using a Model 550 Microplate Reader[a] with 450- and 620-nm filters. The 450-nm filter was optimum for the substrate used by the assay; the 620-nm filter was used to help reduce background absorbance due to scratches in the plate or other abnormalities of the plastic.

Calculation and interpretation of results. Microplate Manager software was used to analyze assay results. A subroutine within this software calculated the mean optical density (OD) (absorbance reading) of 4 negative controls and determined the cutoff value as the mean OD of the negatives plus 0.21. Samples with OD values lower than that of the cutoff were classified as negative; samples with OD values greater than or equal to the cutoff value were classified as positive. Test runs were considered valid only if the following conditions were met: 1) both positive controls produced ODs greater than 1.0, and 2) negative controls used for determining the cutoff value produced ODs less than 0.150.

Assay evaluation

Sample testing for this study was divided into 2 phases. Initially, an attempt was made to collect both RLN and OB tissues from each submission to gather data for assessing brELISA performance as compared with IHC. During this validation phase, RLN and OB tissues were independently evaluated for the presence of PrP^{CWD} and categorized as positive or negative based on IHC staining (present or absent) or brELISA OD values (positive OD > ~0.21; negative OD < 0.21, calculated as described above). Laboratory personnel had no knowledge regarding geographic origin or other

Table 1. Data describing performance of the Bio-Rad Laboratories' enzyme-linked immunosorbent assay (brELISA) as compared with immunohistochemistry (IHC) (chronic wasting disease diagnosis).*

Tissue	Species	brELISA+/IHC+	Relative sensitivity (%) (95% CI)	brELISA−/IHC−	Relative specificity (%) (95% CI)	Test agreement (95% CI)
Retropharyngeal lymph node	mule deer	59/60	98.3 (91.1 to >99.9%)	1,097/1,097	100 (99.7 to 100%)	0.991 (97.3 to 100%)
	elk	21/21	100 (84 to 100%)	814/815	99.9 (99.3 to >99.9%)	0.976 (92.9 to 100%)
	white-tailed deer	3/3	100 (28.9 to 100%)	130/130	100 (97.2 to 100%)	1.0 (98.9 to 100%)
Obex	mule deer	35/38	92.1 (78.5 to 98.4%)	958/958	100 (99.6 to 100%)	0.957 (90.9 to 100%)
	elk	14/15	93.3 (67.9 to >99.8%)	1,028/1,028	100 (99.6 to 100%)	0.965 (89.7 to 100%)
	white-tailed deer	0/0	NC	10/10	100 (69.1 to 100%)	NC

* CI = confidence interval; NC = not calculated because of small sample size; number of false-negatives = (IHC+) − (brELISA+); number of false-positives = (IHC−) − (brELISA−).

case details of individual samples that might influence test interpretations. Paired RLN and OB tissues were collected from 984 mule deer and 800 elk; because white-tailed deer were less abundant and rarely harvested in early sampling seasons, only 10 paired samples were collected from this species. Retropharyngeal lymph node tissues alone were collected from 173 more mule deer, 123 more white-tailed deer, and 36 more elk; OB tissues alone were collected from 12 more mule deer and 243 more elk. Total numbers of samples with IHC results that were used to assess brELISA performance are shown in Table 1. Relative sensitivity (number brELISA positive/number IHC positive), relative specificity (number brELISA negative/number IHC negative), and agreement of results[4] from brELISA for RLN and OB samples were estimated using IHC results from respective tissues as the gold standards.[5]

Once sufficient data were gathered to assure that RLN brELISA was performing well as a screening test, the study entered a second phase. In this field assessment phase, only RLN (or OB, when RLN was unavailable) tissue was collected from submissions, and only samples with OD > 0.1 were examined with IHC to confirm the presence of PrP^{CWD}. The decision to streamline sampling and testing was based on previous observations on reliability of RLN tissue for detecting CWD in deer and elk using IHC as the assay[5,9,10,11] as well as on observations made during the initial phase of this study. For the field assessment, samples with OD < 0.1 were classified as "CWD not detected," and samples with OD ≥ 0.1 were classified as "CWD suspect" and referred for IHC. The 0.1 OD value was selected based on observation of frequency distribution data (Fig. 1). Throughout both study phases, sampled animals were not regarded as CWD-infected unless at least 1 tissue was IHC positive.

Descriptive statistics for brELISA data were calculated using a computerized spreadsheet.[c] Ninety-five percent confidence intervals were constructed using either exact or approximation methods depending on sample size.[1] Relative sensitivity (number brELISA positive/number IHC positive), relative specificity (number brELISA negative/number IHC negative), and agreement of results[2] from brELISA for RLN and OB samples were estimated using IHC results from respective tissues as the gold standards.[5] A receiver operating characteristic curve using OD values and IHC data was generated using a computerized spreadsheet.[c]

Results

A total of 25,050 samples from 23,256 deer and elk were collected and examined for CWD during August–December 2002 using brELISA. For the initial validation phase, 4,175 samples were examined independently by brELISA and IHC. There were 137 IHC-positive tissues and 4,038 IHC-negative tissues. Among the 1,794 cases for which paired tissue samples were available, 45 mule deer and 15 elk showed positive IHC staining in RLN tissue; 35 of 45 (78%) RLN-positive mule deer and 14 of 15 (93%) RLN-

positive elk also showed positive IHC staining in OB tissues. Therefore, 22% of the positive mule deer and 7% of the positive elk had PrP^{CWD} detected only in the RLN tissue. None of the 10 white-tailed deer cases for which paired samples were available tested positive. Of the additional 587 cases sampled during the validation phase for which only RLN or OB tissue was available, 28 more positive samples were obtained: 15 RLN and 3 OB from mule deer, 6 RLN and 1 OB from elk, and 3 RLN from white-tailed deer. The remaining samples were negative. Data from paired and single tissues are combined in the calculations shown in Table 1.

Using IHC-positive cases as known infected individuals and assuming IHC-negative cases as uninfected, brELISA showed high relative sensitivity and specificity for detecting CWD infection in all 3 species. Relative sensitivity of brELISA using the manufacturer's classification algorithm for positives ranged from 98.3% to 100% for RLN samples and 92.1% to 93.3% for OB samples in the 3 species (Table 1). Relative specificity of brELISA using the manufacturer's classification algorithm for negatives ranged from 99.9% to 100% for RLN samples and was 100% for OB samples in the 3 species (Table 1). Overall agreement between brELISA and IHC was ≥97.6% for RLN and ≥95.7% for OB in all species for which values could be calculated (Table 1). Moreover, brELISA OD values provided clear separation between IHC-positive and -negative samples in most cases (Fig. 1A–1C). Only 7 IHC-negative RLN and 4 IHC-negative OB samples had OD values >0.1; similarly, only 1 IHC-positive RLN and 4 IHC-positive OB samples had OD values <0.2. Two of the last 5 cases had OD values >0.1, and all were judged to be early cases of CWD with minimal IHC staining. All 4 OB samples were positive in the RLN by brELISA and IHC. Mean OD values for positive samples of both RLN and OB averaged >46× the mean values for negative samples (Table 2).

Among 20,875 RLN samples screened with brELISA during the field application phase, 155 of 8,877 mule deer, 33 of 11,731 elk, and 9 of 267 white-tailed deer had RLN OD values >0.1 and were further evaluated by IHC to confirm evidence of CWD infection. Of these cases flagged for follow-up, 143 of 155 (92.3%) mule deer, 29 of 33 (87.9%) elk, and all 9 (100%) white-tailed deer were IHC positive. Mean (±SE) OD values for positives detected during the field application phase (Table 3) were comparable with those measured in RLN tissues during the validation phase (Table 2), and frequency distribution of OD values resembled the distribution of validation phase data. Fifteen of 16 IHC-negative samples that were flagged because of an OD value >0.1 had OD values >0.1 but <0.21. Three mule deer and 1 elk had OD values >0.1 but <0.21 and were IHC positive. An elk sample that initially had an OD value of >2.0 was IHC negative and had OD < 0.1 when the homogenate was rerun (Table 3).

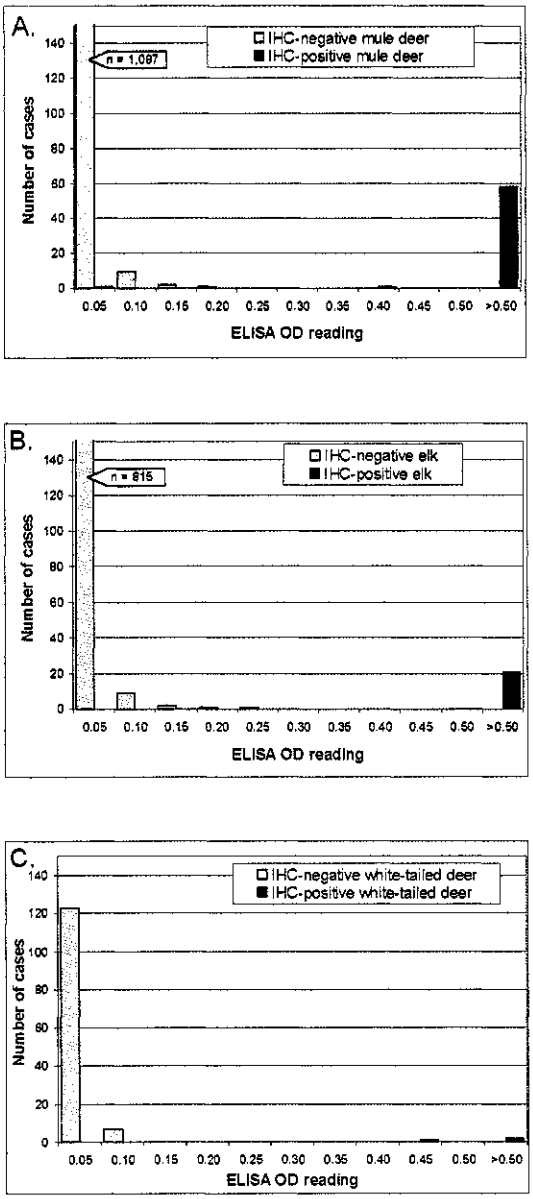

Figure 1. Frequency distributions of Bio-Rad Laboratories' enzyme-linked immunosorbent assay (brELISA) optical density readings from immunohistochemistry (IHC)-positive and -negative retropharyngeal lymph node tissues screened for evidence of chronic wasting disease infection. *A–C*, data from validation phase of study wherein all tissues were examined independently using brELISA and IHC.

Discussion

Based on data reported in this study, brELISA was an excellent and effective diagnostic tool for screen-

Table 2. Descriptive statistics for optical density (OD) data from Bio-Rad Laboratories' enzyme-linked immunosorbent assay (brELISA) of retropharyngeal lymph node and obex tissues from 3 cervid species evaluated independently by immunohistochemistry (IHC).

Tissue	Species	IHC-positive samples			IHC-negative samples		
		Number	Mean OD (±SE)	OD Range	Number	Mean OD (±SE)	OD Range
Retropharyngeal lymph node	mule deer	60	2.931 (±0.114)	0.042 to >3.5	1,097	0.025 (±0.0003)	0.004–0.161
	elk	21	2.711 (±0.188)	0.82 to >3.5	815	0.026 (±0.0004)	0.01–0.204
	white-tailed deer	3	1.664 (±0.854)	0.412 to 3.297	130	0.036 (±0.0008)	0.016–0.053
Obex	mule deer	38	2.062 (±0.225)	0.042 to >3.5	958	0.026 (±0.0003)	0.002–0.148
	elk	15	2.633 (±0.313)	0.034 to >3.5	1,028	0.023 (±0.0002)	0.01–0.145
	white-tailed deer	0	NC*	NC	10	0.029 (±0.002)	0.019–0.042

* NC = not calculated because of small sample size.

ing RLN or OB tissues from deer and elk for evidence of CWD infection. There were several operational advantages evident in incorporating brELISA as a screening test in a large-scale surveillance program. The protocol for IHC assay required a minimum of 3 to 5 days of preparation time (if samples were already adequately preserved in formalin) before specimens were ready for microscopic evaluation, whereas brELISA required only 5 hours before results were available. The brELISA protocol could be stopped after the homogenization or purification steps and held overnight for convenience. Specimens were held overnight during the validation phase because the requirement to simultaneously process double samples (one for IHC and the other for brELISA) delayed delivery of prepared samples for ELISA testing. During the field application phase of this study, results from brELISA typically were available within 24 hours after receipt of the samples, but "suspects" were not reported until confirmed by IHC; "not detected" (=negative) brELISA results were reported within 10–48 hours.

Field validation studies provide an opportunity to obtain information regarding the accuracy of new tests and to examine the pitfalls facing analysts. Immunohistochemistry and brELISA appear to be equally sensitive for detection of CWD in established infections. Moreover, likely neither test is completely accurate in early cases with minimal PrP^{CWD} deposition. When performing validation studies, care must be exercised to ensure that tissues taken for each test are equitably sampled. This was especially evident while reviewing data from OB tissue samples. In very early cases of CWD, PrP^{CWD} deposition is limited.[5,9,10,11] In evaluations where the OB, of necessity, must be divided between 2 techniques, sample selection could inadvertently favor one technique over the other. This may account for the 4 discrepancies observed in OB results from mule deer and elk (Table 1) in the validation phase of the study. Immunohistochemistry detected PrP^{CWD} deposition around 1–2 neurons in the VN of all 4 of these animals; although brELISA did not formally identify these animals as "suspect," the test did register relatively high OD values in 2 of the 4 cases. In contrast, RLN tissues were positive in all 4 of these animals, and both tests indicated CWD infection.

Some discrepancies also were noted in early cases of CWD for RLN in both mule deer and elk. A RLN sample from 1 mule deer that registered a low OD value on brELISA was identified as positive by IHC. In this case, PrP^{CWD} deposition was localized at 1 pole of the RLN, and repeating brELISA on a second sample of this lymph node produced a high OD value. One elk RLN sample registered a high OD by

Table 3. Descriptive statistics for optical density (OD) data from Bio-Rad Laboratories' enzyme-linked immunosorbent assay (brELISA) of retropharyngeal lymph node tissues from 3 cervid species during the field application phase. Cases were flagged for immunohistochemistry (IHC) confirmation because initial OD values were >0.1.

Tissue	Species	IHC-positive samples			IHC-negative samples		
		Number	Mean OD (±SE)	OD Range	Number	Mean OD (±SE)	OD Range
Retropharyngeal lymph node	mule deer	143	2.991 (±0.067)	0.113 to >3.5	12	0.121 (±0.013)	0.075–0.205
	elk	29	2.194 (±0.172)	0.17 to 3.415	4	0.659 (±0.517)	0.113–2.208*
	white-tailed deer	9	2.612 (±0.347)	0.304 to >3.5	0	NC†	NC

* Rerun of this sample had OD < 0.1.

† NC = not calculated because of small sample size.

brELISA but was negative by IHC; the repeat brELISA was also a strong "suspect," but repeat IHC remained negative. The reason for this brELISA discrepancy could not be explained. Selection and subsampling of RLN tissue may influence the outcome of both brELISA and IHC. During the field application phase, 2 or 3 sections of RLN (depending on the size of the lymph node) from brELISA "suspects" were prepared for IHC examination. In some cases, only 1 or 2 follicles on 1 section stained positive. In 4 cases where samples were "suspect" by brELISA and initially negative by IHC, 4–5 sections were examined before finding 1 or 2 follicles with PrP^{CWD} accumulation; all these cases were prepared twice before they could be verified as positive by IHC. These observations suggest that the practice of using 1 section of lymph node per animal for IHC may result in false-negative results in some early cases.

In presumably early cases of CWD,[5,9] the need to prepare additional lymph node samples for IHC to corroborate the results obtained by brELISA can be interpreted in 1 of 2 ways. For brELISA, 180- to 220-mg samples of RLN tissue are collected, and the cortex is preferentially sampled to improve accuracy. For IHC, fixed tissue sections inherently include both cortex and medulla; preparing them otherwise would not be feasible. Moreover, a 5- to 7-μm section of RLN contains only a fraction of the tissue sampled by brELISA. Therefore, it should be expected that several sections sometimes must be prepared for IHC before positive follicles are observed in early CWD cases. In addition, it is also possible that the anti-PrP antibody used in brELISA has greater affinity for PrP^{CWD} than MAb F99/97.6.1. In light of these results, it seems prudent to examine multiple RLN sections using IHC to confirm or refute CWD infection in cases where brELISA data identify suspect cases.

In a field study involving more than 25,000 samples from animals, some mechanical problems are inevitable. For both the IHC and brELISA, human error was occasionally encountered, irrespective of the skill and experience of the analysts. In the IHC assay, technical errors in processing can lead to a need to repeat the test in some cases, resulting in additional expense. Furthermore, occasionally, spurious staining occurs, which could lead an inexperienced microscopist to interpret the tissue as positive. Microscope fatigue can be a major factor with IHC; fatigue becomes a definite factor after examination of about 200 slides. Mistakes, such as "carry over" of materials from tube to tube or from one plate well to adjacent wells, can lead to false-positive results with brELISA. Reagent failure or failure to add sample or reagents can lead to false-negative or false-positive brELISA results. Fortunately, failure to detect CWD cases or incorrect classification of cases as positive using the combination of brELISA screening and IHC confirmation were unlikely, as indicated by the high relative sensitivity and relative specificity estimates. Although problems generally do not appear to occur except in early cases of CWD, hunters typically will not select an animal that does not appear healthy, so more early cases may be encountered in harvest surveys than in other applications to CWD surveillance in free-ranging or captive deer and elk.

As the validation phase progressed and submission volumes increased, problems securing a brain stem sample adequate for a meaningful evaluation soon became apparent. Removing a brain stem is a difficult and labor-intensive process. More often than not, by the time a hunter brought the head to a collection station considerable autolysis had occurred, or the head was removed leaving behind the area of the brain stem needed for the sample. This was especially true of mule deer or white-tailed deer samples. Obviously, brain stem from deer are much smaller than brain stem from elk, compounding the problem. By the time an autolyzed sample was removed, placed in a bag, labeled, identified, and subsequently received for analysis in the laboratory, the critical area needed for sampling was often unidentifiable. This problem occurred in approximately 10% of the samples obtained. The proportion of unusable samples negates any advantage that might be gained by sampling both tissues, especially when the data from this study and others[5,9,10,11] have shown that RLN is the tissue of choice for diagnosing CWD in both deer and elk. The proportions of RLN-positive but OB-negative cases encountered in this study (22% of mule deer and 7% of elk) were similar to rates in other studies.[5,6]

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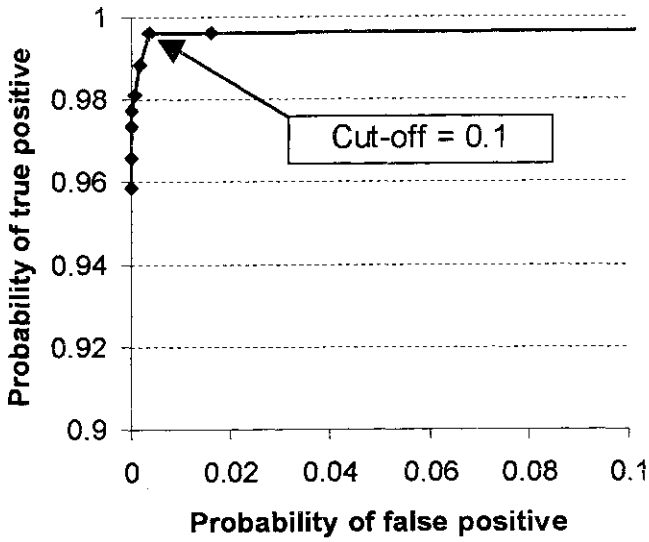

Figure 2. Receiver operating characteristics (ROC) curve for performance of Bio-Rad Laboratories' enzyme-linked immunosorbent assay (brELISA) on retropharyngeal lymph node tissue using optical density (OD) cutoff values ranging from 0.05 to 0.5; all test data from mule deer, elk, and white-tailed deer were combined to generate this curve. The strong discriminating capability of brELISA in distinguishing chronic wasting disease (CWD)–positive and –negative samples is evident from the shape of the ROC curve (note scales of x and y axes). Interpreting brELISA using a cutoff value of OD $\geq$ 0.1 should provide a CWD-screening test with high sensitivity (about 99.6%) that still minimizes the number of unnecessary immunohistochemistry confirmations (about 0.3%) run on truly negative samples.

to become a problem, necessitating more careful evaluation. Based on observations made during the validation phase, the cutoff OD value used to flag samples for IHC evaluation was lowered to $\geq$0.1 for the field application phase. Even with this adjustment, only 197 (<1%) of the 20,875 screened RLN samples were flagged for IHC. Nearly 92% of the samples flagged as "suspects" under this classification scheme, including 4 with OD values below the original BSE cutoff, were IHC positive. In light of the relatively minor increase in "unnecessary" IHC evaluations (about 0.3%) arising from using this lower cutoff value in screening (Fig. 2), the use of an OD value of $\geq$0.1 is recommended in screening RLN tissues from deer and elk for IHC evaluations in large-scale CWD surveys. This ensures that false-negative cases are minimized and should provide a CWD-screening test with high sensitivity (about 99.6%). It is important, however, to assure that a reliable and experienced laboratory performs IHC follow-up and to rely on IHC for final determination of CWD infections in deer and elk because in CWD-negative populations it appears that about 0.3% of the samples would be identified as "suspect" using this lower cutoff.

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a. Bio-Rad Laboratories, Inc., Hercules, CA.
b. FASTPREP 120, Q-Biogene, San Diego, CA.
c. Excel, Microsoft Corp, PO Box 100140, Pasadena, CA.

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brELISA but was negative by IHC; the repeat brELISA was also a strong "suspect," but repeat IHC remained negative. The reason for this brELISA discrepancy could not be explained. Selection and subsampling of RLN tissue may influence the outcome of both brELISA and IHC. During the field application phase, 2 or 3 sections of RLN (depending on the size of the lymph node) from brELISA "suspects" were prepared for IHC examination. In some cases, only 1 or 2 follicles on 1 section stained positive. In 4 cases where samples were "suspect" by brELISA and initially negative by IHC, 4–5 sections were examined before finding 1 or 2 follicles with PrP^{CWD} accumulation; all these cases were prepared twice before they could be verified as positive by IHC. These observations suggest that the practice of using 1 section of lymph node per animal for IHC may result in false-negative results in some early cases.

In presumably early cases of CWD,[5,9] the need to prepare additional lymph node samples for IHC to corroborate the results obtained by brELISA can be interpreted in 1 of 2 ways. For brELISA, 180- to 220-mg samples of RLN tissue are collected, and the cortex is preferentially sampled to improve accuracy. For IHC, fixed tissue sections inherently include both cortex and medulla; preparing them otherwise would not be feasible. Moreover, a 5- to 7-μm section of RLN contains only a fraction of the tissue sampled by brELISA. Therefore, it should be expected that several sections sometimes must be prepared for IHC before positive follicles are observed in early CWD cases. In addition, it is also possible that the anti-PrP antibody used in brELISA has greater affinity for PrP^{CWD} than MAb F99/97.6.1. In light of these results, it seems prudent to examine multiple RLN sections using IHC to confirm or refute CWD infection in cases where brELISA data identify suspect cases.

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