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Abundant PrP^{CWD} in tonsil from mule deer with preclinical chronic wasting disease

Katherine I. O'Rourke, Dongyue Zhuang, Amy Lyda, Gabriel Gomez, Elizabeth S. Williams, Wenbin Tuo, Michael W. Miller

Abstract. A monoclonal antibody dot-blot assay was used to evaluate detergent lysates of tonsil tissue from mule deer to detect PrP^{CWD}, the marker for the cervid transmissible spongiform encephalopathy chronic wasting disease (CWD). Samples of formalin-fixed brain and tonsil tissues from mule deer were examined for PrP^{CWD} using immunohistochemistry (IHC) with Mab F99/97.6.1, the gold standard for diagnosis of preclinical CWD. The contralateral tonsil from each of the 143 deer was prepared for confirmatory IHC and as a 10% (wt/vol) detergent lysate without purification or enrichment steps for monoclonal antibody dot-blot assay. PrP^{CWD} was detected by dot-blot assay in 49 of 50 samples considered positive by IHC. Forty-eight of the positive samples were evaluated with a quantitative dot-blot assay calibrated with recombinant PrP. Tonsillar PrP^{CWD} concentrations ranged from 34 to 1,188 ng per 0.5 mg starting wet weight of tissue. The abundant PrP^{CWD} in mule deer tonsil will facilitate development and validation of high-throughput screening tests for CWD in large populations of free-ranging deer.

Chronic wasting disease (CWD) of mule deer (*Odocoileus hemionus*) is a transmissible spongiform encephalopathy[11] characterized by accumulation of PrP^{CWD}, the disease-associated isoform of the host prion protein formed by posttranslational changes affecting secondary structure, solubility, and relative resistance to proteases. Chronic wasting disease is diagnosed by the detection of PrP^{CWD} in the central nervous system by immunohistochemistry (IHC) assay of the medulla at the level of the obex.[3] PrP^{CWD} accumulates in the lymphoid tissues of infected deer well before the central nervous system is involved.[8,10] Tonsil or lymph node testing is therefore a useful adjunct to IHC when testing deer with preclinical disease, increasing the sensitivity of surveillance testing by approximately 10%.[4] Lymphoid PrP^{CWD} is detectable by monoclonal antibody IHC,[9] but this assay is relatively expensive and time-consuming. Higher-throughput tests developed for bovine spongiform encephalopathy have been described.[1,2,7] These assays are based on the detection of the disease-associated isoform of PrP in detergent lysates of the brain, the tissue with the highest accumulation of the abnormal protein. Adaptation of high-throughput tests for early detection of CWD in clinically normal deer in large-scale CWD surveillance programs would be feasible if PrP^{CWD} were abundant in lymphoid tissues from infected animals. In this study, a semiquantitative assay was used to determine whether PrP^{CWD} could be detected in crude detergent lysates of tonsil from preclinically infected mule deer.

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Materials and methods

Tissue collection. Brain (including medulla oblongata at the level of the obex) and both palatine tonsils were collected from mule deer within and outside a well-defined endemic area in Colorado. All deer were hunter harvested and not reported as clinical suspects. However, the early signs of CWD may be subtle, and hunter reports may be unreliable. Medulla and 1 tonsil were fixed in formalin. Histologic evaluation was performed on medulla samples. Medulla and tonsil were examined by a standardized automated IHC assay.[4,9] Tonsils from mule deer at various stages of disease[4] were examined. These included deer with spongiform encephalopathy in the medulla and PrP^{CWD} in the medulla and tonsil (n = 15), PrP^{CWD} in the tonsil and the dorsal motor nucleus of the vagus nerve but with no spongiform change (n = 2), and PrP^{CWD} detected in tonsil but not in brain (n = 2). An additional 31 samples from clinically normal hunter-harvested mule deer, which were considered positive by IHC assay of the tonsil, were also tested. Negative control samples (n = 114) were collected from deer outside the endemic area; neither spongiform change nor PrP^{CWD} in medulla or tonsil was found in these samples. For these 164 (50 positive and 114 negative) samples, the second tonsil was chilled and cut into 3 pieces; the center section was fixed in formalin for confirmatory IHC, and the 2 outer sections were held at −20 C for dot-blot assay.

Tissue preparation. Dot-blot analysis was performed using 150 mg of frozen deer tonsil tissue. The tissue was finely minced with scissors and mixed with 900 μl of lysis buffer (0.5% nonylphenol ethoxylate (NP-40), 0.5% sodium deoxycholate, 10 mM Tris–HCl, pH 7.5). The sample was incu-

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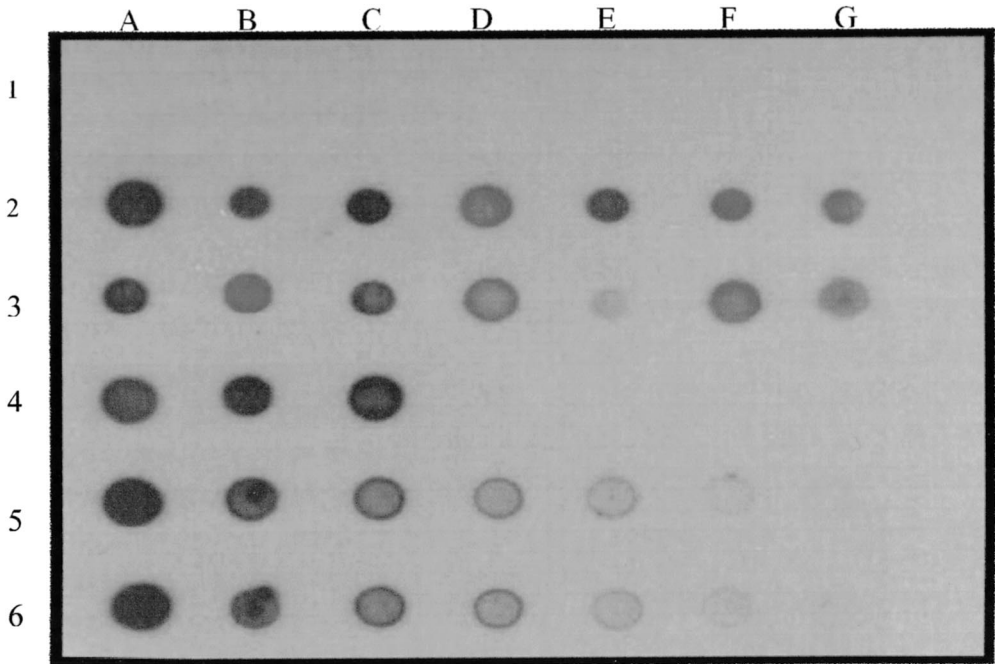

Figure 1. Dot-blot analysis of crude tonsil homogenates from deer with CWD—row 2, columns A–G and row 3, columns A–C, negative control deer (row 1, columns A and B). Calibration standards (rows 5 and 6) are serial 2-fold dilutions of recombinant ovine PrP ranging from 240 ng (column A) to 3.25 ng (column G).

bated at room temperature (RT) for 2 hr and then centrifuged at 1,000 × *g* for 5 min. Supernatants were held at −20 or −80 C. Immediately before use, 20-μl aliquots of supernatant from each prepared sample were incubated with proteinase K (PK+) or without PK (PK−) (final concentration 100 μg/ml) at 50 C for 40 min. The samples were mixed with an equal volume of 2× dot-blot sample buffer (125 mM Tris–HCl, pH 6.8, 6 mM ethylenediamine tetraacetic acid, 8 M urea, and 8% 2-mercaptoethanol) and boiled for 5 min.

Dot-blot assay. Polyvinylidene difluoride membranes were pretreated with methanol for 15 sec and soaked in distilled water for 5 min. After the membrane was completely dried, 6-μl aliquots of prepared tonsil sample (duplicate aliquots of PK+ and PK− samples) were applied to the membrane in a 0.8-cm grid and allowed to dry for 2–18 hr at RT. The membrane was blocked with 1% casein adjusted to 0.05% Tween-20 (blocking buffer) (20 ml per 7- × 8-cm membrane) at RT for 1 hr, incubated with 10 ml of primary antibody (F99/97.6.1[a] 3.5 μg/ml in blocking buffer) at RT for 1 hr with constant agitation, and then washed 3 times, 5 min each, with 0.01 M Tris–HCl, pH 8.0, 0.15 M NaCl, and 0.05% Tween 20 (wash buffer). The membrane was incubated with 10 ml of secondary antibody (1:5,000 dilution in blocking buffer goat anti-mouse IgG_1 conjugated with horseradish peroxidase[b]) at RT for 30 min with constant agitation and washed 5 times, 5 min each, with wash buffer. The membrane was developed for enhanced chemiluminescence[c] and exposed to film. The density of the signal for each dot was determined by a digital imaging system.[d]

PrP^{CWD} quantitation. Adequate tissue was available for quantitation from 48 PrP^{CWD}-positive samples. Samples were assayed as described above on filters loaded with calibration standards prepared from recombinant ovine PrP[6] that shares the conserved epitope (QYQRES) for Mab F99/97.6.1[5] with cervid PrP. Serial 2-fold dilutions of the recombinant PrP were prepared in lysis, denaturation, and dot-blot sample buffers as described above without PK digestion. Calibrators were assayed in duplicate, and the mean density for each calibrator was plotted against concentration for linear regression analysis. Tonsil samples were assayed twice on independent runs as described above. The PrP^{CWD} concentration for each tonsil sample was extrapolated from the standard curve for each filter, the mean values for replicate runs were determined, and the results were expressed as nanograms of PrP^{CWD} per 0.5 mg starting wet weight of tissue. Samples with densitometry readings greater than those of the 240-ng calibration standard were diluted 1:2, 1:5, or 1:10 before addition of denaturation buffer or dot-blot sample buffer.

Results

A monoclonal antibody dot-blot assay for detection of PrP^{CWD} in deer tonsil was used to evaluate the feasibility of assays using crude detergent lysates without further enrichment or purification steps. Using this assay, 164 tonsil samples were studied for detectable PrP^{CWD}. The mean densitometry reading for the samples considered true negatives (collected from outside the reported CWD endemic area) was 45 (range = 16–78; SD = 15). Forty-nine of 50 samples that were considered CWD positive by IHC had densitometry readings that were significantly higher ($P < 0.01$) than

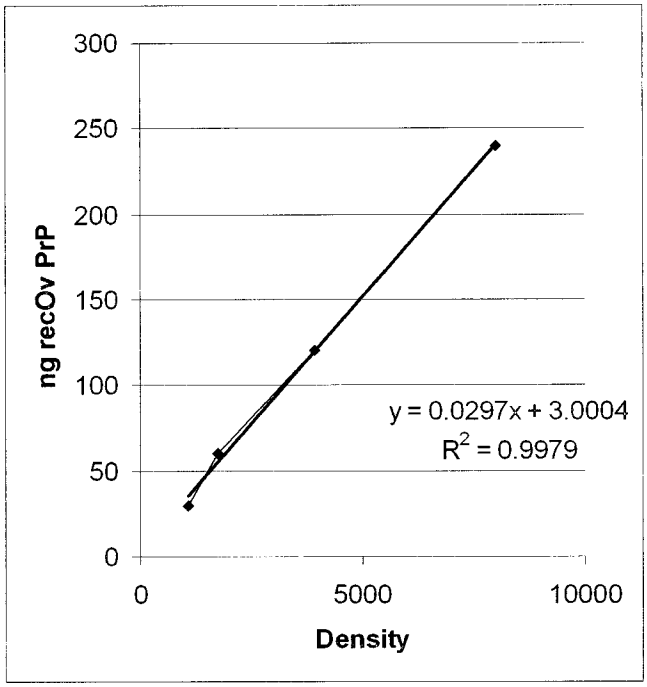

Figure 2. Mean densitometric readings by immunodot-blot assay for calibrators prepared from recombinant ovine PrP. Calibration standards were prepared in duplicate as serial 2-fold dilutions of recombinant ovine PrP (recOvPrP) ranging from 3.25 to 240 ng.

the mean of the negative samples (mean reading = 2,650; range 159–5,468; SD = 1,267). One sample considered positive by IHC was negative by dot-blot densitometry (densitometry reading = 75) and by Western blot (data not shown).

Sufficient tonsil tissue from 48 CWD-positive deer was available for quantitation using the same dot-blot assay calibrated with serial 2-fold dilutions of a recombinant ovine PrP. Representative results are shown in Fig. 1. Calibrators between 15 and 240 ng were assayed in duplicate. The R^2 value for linear regression of the calibration curve between 30 and 240 ng was 0.9979 (Fig. 2). Tonsil lysates with densitometry readings higher than those of the 240-ng calibration standard were diluted 1:2, 1:5, or 1:10 before addition of the lysis, denaturation, and sample buffers. PrP^{CWD} concentration in each sample was determined by extrapolation from the standard curve and adjusted for dilution if necessary. PrP^{CWD} concentrations ranged from 34 to 1,188 ng in 0.5-mg equivalent wet weight of starting tissue (mean concentration 336 ng; SD = 229).

Discussion

This study examined the feasibility of using crude detergent lysates of tonsil tissue for inexpensive dot-blot diagnostic testing of mule deer for CWD. Using a quantitative dot-blot assay, PrP^{CWD} levels in mule deer tonsil exceeded 34 ng/0.5 mg of starting tissue, readily detectable without enrichment steps. One sample, considered positive for PrP^{CWD} by IHC, showed no signal on dot-blot analysis. Discordant results may be attributed to differences in assay sensitivity or to differences in tissue processing. Immunohistochemistry analysis can detect single PrP^{CWD}-positive cells and may be a more sensitive test for specimens with occasional single-cell staining. However, PrP^{CWD} immunostaining, when detected, typically involves more than half the lymphoid follicles in a section.[9] In the case of the false-negative sample in this study, the IHC assay of the center tissue fixed in formalin showed clear and abundant staining, although the dot-blot and Western blot analyses (data not shown) on the flanking tissue sections were negative. Relative sensitivity of the dot-blot system was less likely a cause for the discordant event than improper trimming of the relatively small tonsil. Likewise, the wide variation in PrP^{CWD} levels is largely attributed to the regional distribution of the immunoreactive material in scattered germinal centers. This effect is even more pronounced in retropharyngeal nodes, a tissue preferred over the tonsil for necropsy specimens because of its size and ease of collection in the field. Standardization of tissue collection and trimming techniques will be critical to the accuracy of high-throughput tests. Efficient methods for tissue dispersion, selection of appropriate indicator tissues, and conversion to a 96-well plate suitable for laboratory robotics and a digital readout will be necessary for increasing throughput to meet the anticipated demands for CWD surveillance testing. However, the abundant levels of PrP^{CWD} in lymphoid tissues of mule deer with CWD suggest that high-throughput, rapid, inexpensive tests for deer-associated CWD are feasible.

Acknowledgements

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Sources and manufacturers

a. VMRD, Inc., Pullman, WA.
b. Southern Biotechnology Associates, Birmingham, AL.
c. Enhanced Chemiluminescence, Amersham Pharmacia, Piscataway, NJ.
d. ChemiImager 4000, Alpha Innotech Corp., San Leandro, CA.

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