Preclinical diagnosis of chronic wasting disease in captive mule deer (*Odocoileus hemionus*) and white-tailed deer (*Odocoileus virginianus*) using tonsillar biopsy

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The usefulness of tonsillar biopsy on live deer for preclinical diagnosis of the transmissible spongiform encephalopathy chronic wasting disease (CWD) was evaluated. Disease was tracked in a CWD-endemic herd using serial tonsillar biopsies collected at 6 to 9 month intervals from 34 captive mule deer (*Odocoileus hemionus*) and five white-tailed deer (*O. virginianus*). Tonsillar biopsies were examined for accumulation of PrP^{CWD}, the protein marker for infection, using immunohistochemical (IHC) staining. 26/34 (76%) mule deer and 4/5 (80%) white-tailed deer had PrP^{CWD} accumulation in tonsillar biopsies; CWD was subsequently confirmed by post-mortem examination in all 30 of these tonsillar-positive deer. Six mule deer with IHC-negative tonsillar biopsies had positive brain and tonsillar IHC staining upon death 12 to 40 months following the last biopsy. PrP^{CWD} accumulation in tonsillar biopsy was observed 2 to 20 months before CWD-related death and up to 14 months before onset of clinical signs of CWD. Tonsillar biopsies from 3-month-old mule deer ($n = 6$) were IHC negative, but PrP^{CWD} accumulation was detected in tonsillar biopsies from 7/10 mule deer by 19 months of age. Tonsillar biopsy evaluated with IHC staining is a useful technique for the preclinical diagnosis of CWD in live mule deer and white-tailed deer when intensive management approaches are possible.

Introduction

Chronic wasting disease (CWD) is a naturally occurring transmissible spongiform encephalopathy (TSE) of North American deer (*Odocoileus* spp.) and elk (*Cervus elaphus*) (Williams & Young, 1980, 1982). Like other TSEs, CWD is identified by an accumulation of a characteristic partly protease-resistant isoform of the prion protein, referred to as PrP^{res} or PrP^{CWD}, in the brain of affected animals (Spraker *et al.*, 1997). Lymphoid accumulation of PrP^{CWD} has been reported in mule deer (*O. hemionus*) with naturally occurring CWD (Spraker *et al.*, 2002a, b) and early lymphoid tropism of PrP^{CWD} has been documented following experimental oral inoculation in mule deer (Sigurdson *et al.*, 1999). Detection of tonsillar PrP^{CWD} by immunohistochemical (IHC) staining is therefore a sensitive indicator of CWD infection.

In addition to application to post-mortem samples, IHC staining of lymphoid tissue has been used as an ante-mortem test to detect preclinical scrapie in sheep (Schreuder *et al.*, 1998; O'Rourke *et al.*, 2000) and to diagnose variant CJD in humans (Hill *et al.*, 1999). Such a preclinical test for CWD could greatly enhance epidemiological studies and potentially aid in management activities where maintenance of live animals is indicated. The objective of this study was to determine the feasibility of using tonsillar biopsies obtained from captive

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mule deer and white-tailed deer (*O. virginianus*) as a preclinical test for CWD. Serial tonsillar biopsies collected from mule deer and white-tailed deer confirmed that PrP^{CWD} accumulations occur prior to onset of clinical CWD and can precede CWD-related death by at least 20 months.

Methods

■ **Animals.** Thirty-four captive mule deer and five white-tailed deer were sampled at a CWD-endemic site, the Colorado Division of Wildlife's Foothills Wildlife Research Facility (FWRF; Fort Collins, CO, USA). CWD has occurred in mule deer housed at the facility since at least 1967 (Williams & Young, 1992). Deer used in the study were females, males and castrated males born at the facility or acquired from CWD-endemic and non-endemic areas of Colorado (Miller *et al.*, 2000). Deer were maintained in 1 to 4 ha pastures and had ad libitum access to alfalfa hay, a high-energy pelleted supplement (Baker *et al.*, 1998), pelleted browser supplement (Mazuri browser maintenance, PMI Feed), a trace mineral block and water. Except for commercially obtained evaporated bovine milk fed to some fawns, no ruminant-derived feed material was included in any of the food sources. Sampled deer ranged in age from 3 months to 11 years. Most deer were more than 2 years of age when the biopsy collection protocol was developed; however, one cohort of ten mule deer born in 1997 was sampled at 3, 10 and 19 months of age.

■ **Sample collection.** Serial tonsillar biopsies were collected from mule deer at 6 to 9 month intervals between July 1996 and January 1999 and from white-tailed deer in April 1998. Deer were anaesthetized by intramuscular administration of 5 to 12·5 mg/kg ketamine.HCl (Ketaset, Fort Dodge Animal Health) combined with 1 to 2·5 mg/kg xylazine (Cervizine, Wildlife Laboratories or Rompun, Bayer Corporation). Alternatively, deer received 4 mg/kg Telazol (Fort Dodge Animal Health) combined with 2 mg/kg xylazine. When anaesthetized, deer were blindfolded and placed in sternal recumbency for sampling. The mouth was held open with a metal mouth gag and the palatine tonsil visualized using a laryngoscope with a 30 cm blade (Jorgensen Laboratories). The biopsy was collected with a 30 cm Jackson endoscopic forceps with 4 mm cup (Sontec Instruments, Inc.). The cup of the forceps was placed in the tonsillar crypt and pressed against the ventral-medial wall to obtain one to several tissue samples. Biopsy specimens were placed in 10% buffered neutral formalin. Deer were given prophylactic antibiotics and analgesic. The mouth gag and blade of the laryngoscope were wiped with 20% bleach solution and rinsed in water. The Jackson forceps was cleaned, soaked in 20% bleach solution for 30 min, and then rinsed in water.

Deer were euthanized after showing clinical signs of CWD or, in some cases, died naturally with or without clinical signs of CWD. Brain, and in most cases tonsil, was collected and formalin fixed to determine CWD status by histology and IHC.

■ **Immunohistochemical staining.** Biopsy specimens were processed and embedded in paraffin blocks within 10 days of collection. Tissue sections were mounted onto positively charged glass slides, deparaffinized and hydrated in preparation for IHC. Tissue treatment performed prior to IHC consisted of slide immersion in 88% formic acid solution for 5 min followed by a rinse in water. Tissue sections were then autoclaved for 12 min at 121 °C in a buffer solution (DAKO target antigen retrieval) and cooled for 30 min.

The IHC protocol employed an automated immunostainer (Ventana Medical Systems) and PrP monoclonal antibody (mAb) F99/97.6.1, a biotinylated secondary antibody, an alkaline phosphatase–streptavidin conjugate, a substrate chromagen (fast red A), and a haematoxylin and bluing counterstain (Ventana Medical Systems). mAb F99/97.6.1 binds residues 220–225 of the cervid PrP protein (QYQRES) (O'Rourke *et al.*, 2000) and has been validated for IHC staining of brain and tonsil in mule deer (Spraker *et al.*, 2002a). Positive and negative control deer brain and tonsil sections were included in each run. CWD-negative deer were harvested from the CWD non-endemic area (non-endemic area established by Miller *et al.*, 2000) and were confirmed as CWD negative by the absence of histological brain lesions and negative IHC staining for PrP^{CWD} in brain and tonsil.

Number of follicles staining positive (Spraker *et al.*, 2002a) and total number of intact tonsillar follicles were determined for each section. Results were categorized into one of three interpretations based on Schreuder *et al.* (1998): positive (PrP^{CWD} in any number of follicles), negative (no detectable immunostaining in at least three follicles) or inconclusive (less than three follicles in the sample with no detectable immunostaining). We refer to biopsies where positive or negative interpretation was possible as adequate samples.

Results

Tonsillar biopsy collection

Effectiveness of collecting tonsillar follicles in the biopsy specimens varied greatly. Some samples yielded no follicles while others produced > 25. At least one adequate tonsillar biopsy was collected from 34 mule deer and five white-tailed deer. In early samplings, we failed to direct the biopsy instrument to the ventral–medial aspect of the tonsillar crypt and therefore collected many samples void of tonsillar follicles; these samples are excluded from the study. Otherwise, success did not appear to vary over time, i.e. repeated sampling of the tonsil did not appear to reduce successful collection of follicles.

Immunohistochemical staining

Positive staining was characterized by coarse granular, bright red material in lymphoid follicles (Fig. 1). With one exception (Table 1, animal M92), all follicles within an individual biopsy stained uniformly either positive or negative. No staining was seen in negative control samples from deer harvested outside the CWD-endemic area.

Clinical and post-mortem findings

All deer used in this study had died by February 2001. Overall, 32/34 (94%) mule deer and 4/5 (80%) white-tailed deer were infected with CWD. Thirty mule deer and three white-tailed deer died, or were euthanized at varying stages during the clinical course of CWD. Clinical signs observed in these deer included weight loss, behavioural change, depression, excessive salivation, excessive oesophageal reflux and polyuria/polydipsia (Williams & Young, 1992). Date of onset of CWD clinical signs was recorded for 12 mule deer and two white-tailed deer. Clinical signs of CWD were usually observed 1 to 4 months prior to euthanasia or death; however, two mule deer exhibited prolonged clinical presentations of 9
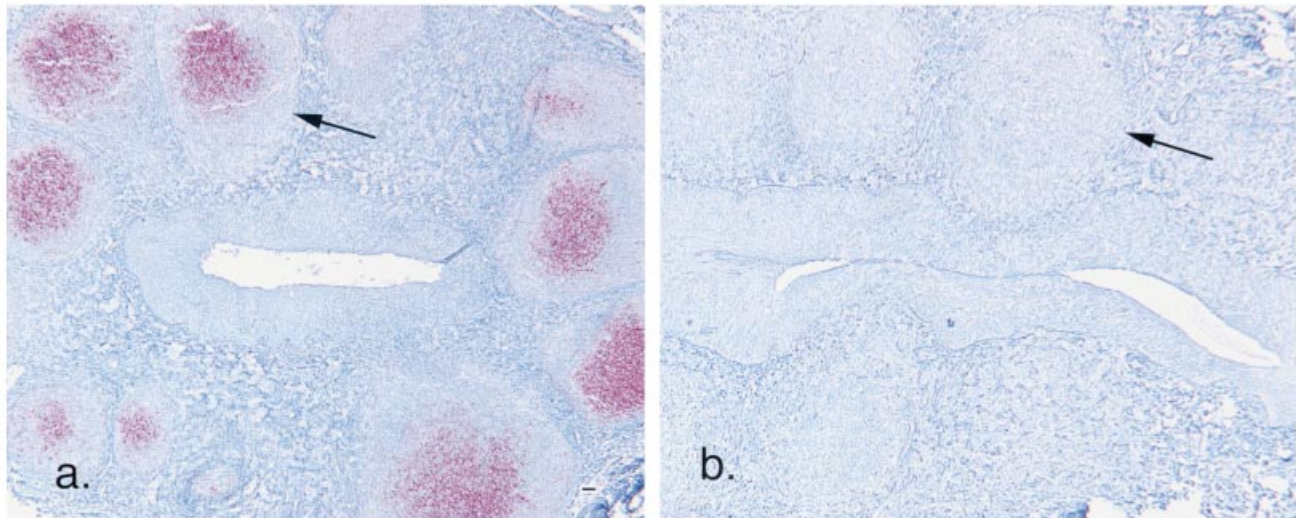
Fig. 1. Tonsillar biopsy collected from CWD-positive (a) and negative (b) captive mule deer as determined by IHC using anti-PrP monoclonal antibody 99/97.6.1. Dark granular red immunostain is apparent in the lymphoid follicles (arrows) of the CWD-positive deer but not the negative deer.

and 13 months (Table 1). Appearance of clinical signs of CWD followed positive IHC staining of tonsillar biopsy by up to 14 months (Table 1).

CWD was confirmed by post-mortem examination of brain in all mule deer ($n = 30$) and white-tailed deer ($n = 3$) with clinical signs indicative of CWD. All deer with confirmed CWD for which tonsil samples were available ($n = 28$) showed positive immunostaining in tonsil tissue collected post-mortem. Two mule deer and one white-tailed deer that died or were euthanized without clinical signs of CWD were IHC positive on post-mortem examination of brain. The remaining two mule deer and one white-tailed deer were IHC negative on tonsillar biopsy and on post-mortem examination of brain.

Live animal testing

Ten mule deer had positive tonsillar IHC when first sampled (Table 1, group a) at ages ranging from 10 to 64 months. Those ten deer died or were euthanized with clinical signs of CWD 2 to 20 months after sampling (Fig. 2, group a). CWD was confirmed post-mortem by examination of brain and tonsil tissue in all ten of these deer.

Sixteen mule deer converted from negative to positive IHC staining on biopsy samples over the 3 year sampling period (Table 1, group b). Once an IHC-positive biopsy was obtained from an individual, all subsequent biopsies from that individual were positive. Age at initial biopsy ranged from 3 to 128 months and the interval from the last negative biopsy sample to the first positive biopsy sample ranged from 6 to 23 months. All 16 deer died or were euthanized with clinical CWD at ages 28 to 145 months, 6 to 19 months after the first IHC-positive tonsillar biopsy (Fig. 2, group b).

Six mule deer with negative biopsy samples (Table 1, group c) died or were euthanized 12 to 40 months after the last biopsy collection at ages ranging from 31 to 80 months. Death occurred 27 to 55 months after the first biopsy sampling. Two of these deer (Table 1, C92 and J93) died due to causes other than CWD; however, CWD was confirmed in all six deer by post-mortem IHC analysis of the brain. Tonsil samples collected post-mortem from each deer were also IHC positive.

Two mule deer remained negative throughout (Table 1, group d). These deer died or were euthanized due to causes other than CWD 7 and 13 months after the first biopsy was obtained. Deer were age 11 and 98 months at death.

Of the 34 mule deer sampled, ten were members of a cohort born in 1997. These deer were evaluated by sequential sampling at 3, 10 and 19 months of age, although not all samplings yielded adequate biopsy samples. None of the six deer from which adequate tonsillar biopsies were collected at 3 months of age were IHC positive. Seven of the ten deer had at least one positive tonsillar biopsy by 19 months of age: 2/7 deer successfully sampled at 10 months and an additional 5/5 deer successfully sampled at 19 months of age were tonsillar IHC positive. The remaining three deer were test negative at 3 and 10 months and no adequate sample was collected at 19 months. One of these deer died at 11 months of age from non-CWD related causes while the remaining nine all died from CWD at ages 26 to 42 months.

White-tailed deer were 58 months of age at sampling. Four of five white-tailed deer had IHC-positive tonsillar biopsy. CWD was confirmed in all four deer at death 2 to 18 months after sampling. One of these deer had not exhibited clinical signs of CWD when it was euthanized for non-CWD related causes 18 months following a positive tonsillar biopsy. The

Table 1. Age of mule deer at onset of clinical signs of CWD, first and last IHC-negative tonsillar biopsy, first positive tonsillar biopsy and death.

Biopsy groups: a, IHC positive on first biopsy; b, IHC-negative biopsy followed by IHC-positive biopsy, c, IHC-negative biopsy followed by CWD-positive brain post-mortem; d, IHC-negative biopsy and CWD-negative brain post-mortem.
NA, Not applicable; Unk., unknown.

| Animal ID | Group | Age (months) on: | | | | |
		Clinical CWD	First negative biopsy	Last negative biopsy	First positive biopsy	Death
Ea93	a	40	NA	NA	37	42
K92	a	63	NA	NA	56	66
L94	a	40	NA	NA	32	44
M92	a	Unk	NA	NA	64	84
N92	a	70	NA	NA	56	72
Ra397	a	Unk.	NA	NA	10	26
Rb91	a	69	NA	NA	61	70
Rc91	a	69	NA	NA	61	70
Sa97	a	Unk.	NA	NA	19	36
Y92	a	58	NA	NA	56	58
B93	b	Unk.	37	52	58	72
Db97	b	Unk.	3	3	19	37
F92	b	58	49	49	64	71
G92	b	Unk.	64	64	70	85
H92	b	Unk.	64	70	79	88
Ma97	b	Unk.	10	10	19	32
Mb97	b	Unk.	10	10	19	28
O92	b	Unk.	49	49	64	82
R193	b	Unk.	37	44	67	74
Rb697	b	Unk.	3	10	19	38
V92	b	Unk.	56	56	70	85
W92	b	74	49	49	70	76
W97	b	Unk.	3	3	10	29
X92	b	69	49	56	64	73
Y86	b	144	128	128	136	145
Za93	b	Unk.	37	44	58	69
C92*	c	NA	56	56	NA	76
Da97	c	Unk.	3	10	NA	31
J93*	c	NA	37	52	NA	66
Sb97	c	Unk.	3	10	NA	42
T94	c	Unk.	25	40	NA	80
Z93	c	55	52	52	NA	64
Ra697	d	NA	3	3	NA	11
Z89	d	NA	85	92	NA	98

* Deer died of causes unrelated to CWD, but was IHC positive on brain post-mortem.

deer that was negative on tonsillar biopsy was CWD-negative on post-mortem examination of brain when it was euthanized for non-CWD related causes 18 months after sampling.

Discussion

Accumulation of PrP^{CWD} in lymphoid tissue has been documented post-mortem in mule deer with experimental CWD infections (Sigurdson *et al.*, 1999) and in mule deer (Spraker *et al.*, 2002a, b) and white-tailed deer (T. R. Spraker,

unpublished data) with natural CWD infections. In mule deer experimentally inoculated orally with CWD-positive brain material, PrP^{CWD} accumulation was observed in tonsils prior to brain and as early as 78 days post-inoculation (Sigurdson *et al.*, 1999). In our study, tonsillar biopsy provided a means for the preclinical diagnosis of CWD in live mule deer and white-tailed deer. PrP^{CWD} accumulation in ante-mortem tonsillar biopsy samples was documented in 26/32 (81 %) mule deer and 4/4 (100 %) white-tailed deer subsequently diagnosed with CWD. Although rare, lack of lymphoid accumulation of
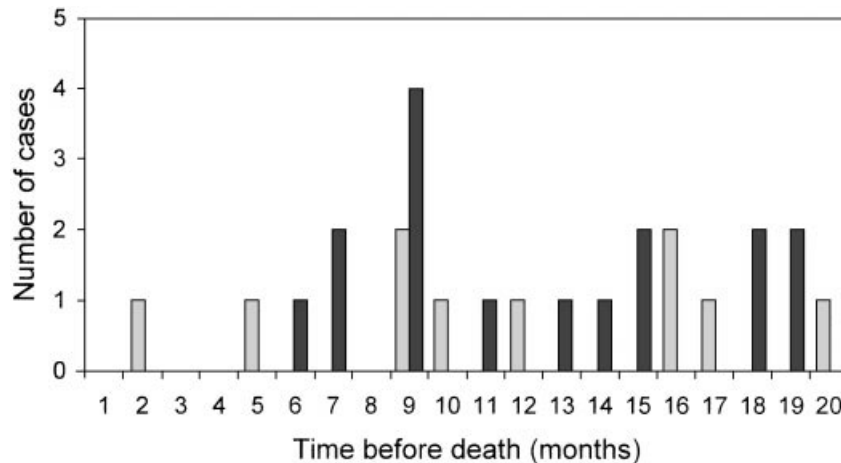
Fig. 2. Time between first IHC-positive tonsillar biopsy and CWD-related death in captive mule deer in cases where initial tonsillar biopsy was IHC positive (group a; grey bar) and cases that converted from IHC negative to positive on tonsillar biopsy (group b; black bar).

PrP^{CWD} has been reported in mule deer with CWD (Spraker et al., 2002a); however, all CWD-infected deer in this study had accumulation of PrP^{CWD} in tonsil at the time of post-mortem examination. Negative findings in six mule deer that subsequently died with CWD 12 to 40 months after the last negative sampling may have been due to inadequate biopsy sample size or sampling too early in the disease course. Follicles within a biopsy stained uniformly either all positive or all negative in 74/75 (99 %) of the adequate samples; therefore it is unlikely that positive cases were misdiagnosed in serial sampling of these captive deer. However, deer will test IHC negative on tonsillar biopsy samples early in disease progression, before lymphoid accumulation begins (Sigurdson et al., 1999). Therefore, negative tonsillar biopsy does not assure that deer are free of infection.

Although biopsy collection was primarily from adult deer, one cohort of young mule deer born at this CWD-endemic facility was followed and provided insight into early exposure to CWD. All biopsies ($n = 6$) collected at 3 months of age were negative. The youngest deer to test IHC positive were two 10-month-old mule deer. Five other mule deer tested IHC positive at 19 months of age.

PrP^{CWD} accumulation in tonsillar biopsies was documented up to 20 months prior to death and 14 months prior to onset of clinical signs of CWD (Table 1). However, the period of preclinical lymphoid accumulation of PrP^{CWD} in our study was likely underestimated in some cases due to the time lapse between sample collections and because we sampled primarily adult deer. Many adult deer may have had disease exposure and progression prior to initiation of biopsy sampling. In fact, 5/8 mule deer with IHC-positive tonsillar biopsies $\geqslant 16$ months prior to death (Table 1) were from the 1997 cohort, the only deer that we began sampling as juveniles. Therefore, reported preclinical periods should be considered minimum estimates in these captive deer.

Although use of the 4 mm Jackson endoscopic forceps was adequate for collection of biopsies in many cases, a modified technique that more reliably obtains numerous tonsillar follicles in the biopsy should be developed for highest efficiency. Availability of an affordable disposable biopsy instrument also would be useful to minimize the risk of unintentional PrP^{CWD} transmission. Although we believed that our inactivation technique for surgical instruments was adequate, recent research underscores the difficulty in fully inactivating PrP^{res} (Taylor, 2000), particularly on stainless steel surgical instruments (Zobeley et al., 1999). Although we cannot exclude the possibility that iatrogenic transmission occurred, high rates of CWD in deer used in our study (94 % in mule deer and 80 % in white-tailed deer) should not be regarded as evidence that cross-contamination from the biopsy technique occurred. From 1970 to 1981, 90 % of mule deer that were resident at this same facility for 2 years or longer developed CWD (Williams & Young, 1992). In our study, the mean interval from first biopsy collection to death from CWD in deer with negative initial samples was 36 months (SD = 7 months) for deer ranging in age from 3 to 128 months at sampling. In a group of non-biopsied mule deer ($n = 8$) held at this facility concurrently, mean time from arrival at the facility to death from CWD was also 36 months (M. W. Miller, unpublished data).

Tonsillar biopsy was successful in identifying CWD-affected live deer, in most cases well before the onset of clinical signs of the disease. Although few white-tailed deer were available for biopsy, findings were consistent with those in mule deer and support similarity in lymphoid accumulation of PrP^{CWD} between the species that has been observed post-mortem. However, because PrP^{CWD} does not appear to accumulate in lymphoid tissue to the same degree in elk as deer (T. R. Spraker, unpublished data), the technique is not currently applicable to elk. However, where intensive management actions are possible, use of this technique will aid in the clinical

2633

diagnosis of CWD in individual captive or free-ranging deer and in epidemiological investigations of CWD in populations of mule deer and white-tailed deer.

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