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Tonsillar Biopsy Test for Chronic Wasting Disease: Two Sampling Approaches in Mule Deer and White-tailed Deer

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ABSTRACT: Preclinical antemortem testing of deer (*Odocoileus* spp.) for chronic wasting disease (CWD) can be important for determining prevalence rates and removing infected individuals from wild populations. Because samples with high numbers of tonsillar follicles are likely to provide earlier detection of CWD than samples with fewer follicles, the method of obtaining follicular samples may be critical when investigating disease prevalence. Between January 2003 and January 2005, white-tailed deer (*O. virginianus*) in southeast and southwest Minnesota and white-tailed and mule deer (*O. hemionus*) in Wind Cave National Park, South Dakota, were sampled using dorso-lateral and ventral-medial approaches for collecting tonsillar follicles. We obtained significantly more follicles using a dorso-lateral (median number of follicles=19) rather than a ventral-medial (median number of follicles=5.5) approach. No differences were observed in collection of tonsillar follicles that were related to sex, age class, or species of deer. We recommend the dorso-lateral approach for assessing CWD prevalence in deer populations.

Key words: Chronic wasting disease, mule deer, *Odocoileus hemionus*, *O. virginianus*, prion, tonsillar biopsy, tonsillar follicles, white-tailed deer.

Chronic wasting disease (CWD) is an infectious transmissible spongiform encephalopathy of deer (*Odocoileus* spp.) and elk (*Cervus elaphus*) identified by an abnormal prion protein (PrP^{CWD}; Spraker et al., 2002a). Because of the long course of the disease, preclincial testing of animals potentially infected with the disease can be important for determining prevalence rates of CWD. Testing for CWD is usually conducted on samples collected postmortem. An antemortem test using tonsillar biopsy was developed to detect scrapie in preclinical sheep (Schreuder et al., 1996) and has been adapted for use in deer (Wild et al., 2002; Wolfe et al., 2002). Similar to scrapie, PrP^{CWD} in deer accumulates in the retropharyngeal lymph nodes and tonsillar follicles before central nervous system involvement or clinical symptoms (Sigurdson et al., 1999; Spraker et al., 2002b; O'Rourke et al., 2003). Antemortem testing of these tissues by immunohistochemistry (IHC) provides a reliable preclinical diagnosis in deer (Wild et al., 2002; Wolfe et al., 2002). Preclinical tonsillar testing for CWD in elk is less reliable due to species differences in the accumulation of PrP^{CWD} in lymphoid tissues (Williams et al., 2002).

As part of CWD monitoring in Wind Cave National Park (43°35′N, 103°30′W), South Dakota and the Farmland Region of southern Minnesota (Redwood Falls, 44°32′N, 95°07′W, and Dumfries, 44°20′N, 92°07′W; DePerno et al., 2003), we employed tonsillar biopsies to test mule deer (*O. hemionus*) and white-tailed deer (*O. virginianus*) for the disease. Our objective for these studies was to obtain tonsillar biopsy samples containing tonsillar follicles as a preclinical antemortem test for CWD to determine prevalence rates. During sample collections, we used two different approaches, ventral-medial and dorso-lateral, within the tonsillar crypt of deer to obtain samples.

This study was conducted between January 2003 and January 2005. All deer were anesthetized before processing began with an intramuscular or intravenous injection combination of ketamine or tiletamine/zolazepam mixed with xylazine or medetomidine (Kreeger, 1999). Most deer received intramuscular or intravenous injection of

yohimbine or atipamezole after processing. Deer were fitted with a very high frequency (VHF) or global positioning system (GPS) collar, an ear tag, or a passive integrated transponder electrochip. All deer were given prophylactic antibiotic. The biopsy was performed by using 30-cm Jackson biopsy forceps with a 6-mm cup size (Sontec Instruments, Englewood, Colorado, USA). The mouth of the deer was opened using a mouth gag (Design Metals, Ft. Collins, Colorado, USA), and tonsillar crypt was visually located at the back of the throat with a laryngoscope (30-cm Miller #4 blade, Jorgenson Lab, Loveland, Colorado, USA). The tonsillar crypt or sinus was located on the lateral wall of the oropharynx, near the attachment of the soft palate, which leads to the palatine tonsil (Getty, 1975). The palatine tonsil was concealed by the wall of the pharynx and was composed of numerous follicles (Getty, 1975). Biopsies were obtained from either the right or left tonsil with samples taken from only one tonsil. Biopsy tissue samples were placed in 10% neutral buffered formalin and examined by IHC (Peters et al., 2000; Spraker et al., 2002a) at Colorado State University Veterinary Diagnostic Laboratory. The Institutional Animal Care and Use Committee at South Dakota State University approved this protocol (02-A036).

Biopsy instruments were cleaned in the field between animals. Tissue and blood were removed with disposable brushes and cotton swabs, then sterilized by soaking for ≥1 hr in LpH solution (≥9% concentration; Ernst and Race, 1993) in individual pans. We used a rotational system of six marked instruments to ensure at least 1 hr of sterilization time between sampling. Instruments were autoclaved between captures; instruments used in South Dakota were not used on deer in Minnesota. Mouth gags and laryngoscopes were wiped down with LpH solution (Wild et al., 2002). All instruments were washed with water before use. Latex gloves were changed between sampling each deer.

Two different biopsy sampling approaches were used during this study. For deer captured in Minnesota and Wind Cave National Park in 2003, we collected biopsies from the ventral-medial portion of the tonsillar crypt as described by Wild et al. (2002). This approach involved placing one of the two biopsy cups inside of the tonsillar crypt and taking a sample or "bite" from the ventral-medial wall of the crypt. The laryngoscope was held in the free hand and was often used to depress the tongue. In some cases, an assistant would have to physically restrain the tongue. For the ventral-medial approach, the biopsy cups were angled downward and in toward the base of the tongue. The top cup was visualized on the lateral wall of the pharynx, while the bottom cup was not visible inside the tonsillar crypt. Once the top cup was completely covering tissue, the forceps were tightly closed. Tissue samples were removed by pulling straight back out of the deer's mouth while holding the forceps closed. A second bite was taken from the same location using the same procedure to improve the likelihood of penetrating the mucosal tissue and accessing follicles. The sampling protocol was for two bites, but additional bites were taken at the same location if the sample tissue was deemed insufficient.

Because of inconsistency in obtaining tonsillar follicles from biopsies taken from the ventral-medial location, we used a modified approach that involved taking a sample from the dorso-lateral wall of the crypt. With this approach, the biopsy instrument cups were pointed up and out toward the base of the ear. Similarly, two bites were taken at this location, both with one of the biopsy forceps' cups inside the crypt and one outside the crypt. The second bite was taken at the same location on the crypt wall as the first bite. However, we also took a third bite by closing the forceps and placing both cups inside the tonsillar crypt. Once inside the crypt, the forceps were opened, pressed up and in toward the base of the ear, closed, and the

sample was removed. Again, additional bites were taken if the sample was deemed insufficient.

For the tonsillar biopsy procedure, a testable sample was defined as a tissue sample containing at least one tonsillar follicle. A failure was a sample without any tonsillar follicles. As a post hoc analysis of the two approaches, a chi-square analysis of difference in probabilities was conducted to determine whether the acquisition of testable samples was similar (Conover, 1999). To determine which approach had higher quality samples (more tonsillar follicles per tissue sample), we performed a median test for differences in testable samples (Conover, 1999); only testable samples were included in this analysis. Finally, separate chi-square analyses for each sampling approach were used to determine bias in sampling by deer sex, species, and age.

Overall, failure rates for approaches were 36.2% for ventral-medial and 5.1% for dorso-lateral; these rates differed significantly ($\chi^2_1=17.33$, $P<0.0001$). The dorso-lateral approach (median number of follicles=19; Table 1) consistently yielded samples with higher numbers of tonsillar follicles than the ventral-medial (median number of follicles=5.5, $\chi^2_1=27.75$, $P<0.0001$) approach. Number of collected follicles for tonsillar biopsies did not differ by sex for ventral-medial ($\chi^2_1=0.18$, $P=0.67$) or dorso-lateral ($\chi^2_1=0.03$, $P=0.86$) approaches. Similarly, rates did not differ by species for ventral-medial ($\chi^2_1=1.38$, $P=0.24$) or dorso-lateral ($\chi^2_1 = 0.35$, $P=0.55$) approaches. We did not sample juvenile (<1-yr-old) deer using the ventral-medial approach; however, follicular samples from testable biopsies did not differ ($\chi^2_1=0.35$, $P=0.55$) between juvenile and adult deer using the dorso-lateral approach.

It is critical that tonsillar biopsy samples contain tonsillar follicles if collected to determine CWD status. During the course of our study, we modified our sampling approach in hopes of obtaining higher quality

TABLE 1. Number of deer sampled, median and mode for number of follicles per sample for Minnesota (MN) and Wind Cave National Park (WC) by capture operation (helicopter net-gun [HNG], clover trap [CT], or chemically immobilized by a dart) and year detailed by white-tailed deer (WT) or mule deer (MD), male (M) or female (F), adult or juveniles (<1 y old). Number of deer sampled are shown in parentheses.

Location/capture	Year	Sampling technique	Species	Sex	Age	n	Follicles/sample		Failures
							Median	Mode	
MN/HNG	2003	Ventral-medial	WT	F	Adult	30	1.75	0	13
WC/HNG	2003	Ventral-medial	MD	M (7) F (21)	Adult	28	3	0	8 (2 M, 6 F)
WC/CT/dart	2003-2005	Dorso-lateral	WT (6)	M (9)	Adult (33)	35	20	20	1 (MD adult F)
			MD (29)	F (26)	Juvenile (2)				
WC/HNG	2004	Dorso-lateral	MD	M (8)	Adult (20)	24	15	15	2 (1 adult M, 1 adult F)
				F (16)	Juvenile (4)				

samples. With the ventral-medial approach, there was substantial variation in obtaining testable samples. In contrast, the dorso-lateral approach resulted in an increase in the proportion of testable samples and number of follicles in samples. Although a learning curve might have been associated with sampling, the immediate increase in sample success and quality indicates that the dorso-lateral approach likely samples an area that contains more follicles and less connective tissue in the tonsil (Velinova et al., 2001). Also, Wild et al. (2002) had variation in effectiveness of the ventral-medial approach in obtaining successful samples but did not provide success rates. They proposed that failures may have been due to the smaller 4-mm-cup Jackson biopsy instrument used to obtain samples (Wild et al., 2002). Wolfe et al. (2002) had a high success rate for obtaining samples with a 6-mm biopsy cup but did not detail directional rotation of biopsy instruments from the starting rostral position.

Because tonsillar biopsy samples only a portion of the tonsil, infected follicles are more likely to be included if the sample contains a greater number of follicles, particularly early in infection. Wolfe et al. (2002) suggested that ≥9 follicles were necessary to accurately test deer for CWD infection assuming minimal infection. The dorso-lateral biopsy approach had a median value of 19 follicles, whereas the ventral-medial approach had a median value of 5.5 follicles per sample. Thus, the dorso-lateral approach was better at preventing false-negative results from samples containing low numbers of follicles (Wolfe et al., 2002).

Assessing the value of tonsillar biopsy as a preclinical diagnostic tool for CWD testing is critical. Our study at Wind Cave National Park resulted in two adult female mule deer and one yearling female white-tailed deer testing positive by tonsillar IHC for CWD. These deer were positive in 20 of 20, 23 of 25, and 30 of 30 follicles in the sample. All deer had PrP^{CWD} accumulation in the retropharyngeal lymph nodes and brain in postmortem examination. During sampling, minor bleeding occurred in only three mule deer (one using the ventral-medial approach, two using the dorso-lateral approach); applying mild pressure to the site of biopsy with a gauze pad controlled bleeding. All deer survived for at least 2 months postcapture, indicating that biopsies had negligible effects on survival.

Employing an antemortem preclinical test for free-ranging deer is difficult. It requires trained personnel and ample funding to cover the costs associated with deer capture. Our goal in capturing deer was to effectively test them for CWD; therefore, it was critical the sampling method was consistently successful in providing biopsies that contained tonsillar follicles. These results indicate the dorso-lateral approach consistently yielded higher tonsillar biopsy sample success and quality for CWD testing. However, because all samples included in this study were taken by a single person (K.L.S.), and sampling success may vary between individuals collecting biopsies, individual variation in success rates achieved with these two approaches may occur and should be assessed in future studies. Because PrP^{CWD} accumulation occurs in the lymphoid tissues of the alimentary tract at the onset of infection (Sigurdson et al., 1999), early detection and removal of deer testing positive for CWD via tonsillar biopsy may aid in reducing transmission rates of CWD in wild deer populations (Gross and Miller, 2001).

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