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Source: Journal of Wildlife Diseases, 40(1) : 42-52

Published By: Wildlife Disease Association

URL: https://doi.org/10.7589/0090-3558-40.1.42

Journal of Wildlife Diseases, 40(1), 2004, pp. 42–52

ESTIMATING THE TRUE PREVALENCE OF *MYCOBACTERIUM BOVIS* IN HUNTER-HARVESTED WHITE-TAILED DEER IN MICHIGAN

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ABSTRACT: Apparent prevalence, although useful as a consistent index, may underestimate the true prevalence of disease. In Michigan, the ability to estimate the true prevalence of bovine tuberculosis (TB; caused by *Mycobacterium bovis*) in free-ranging white-tailed deer (*Odocoileus virginianus*) will become increasingly important to accurately assess progress towards eradication. Our objectives were threefold: to estimate the true prevalence of *M. bovis* in free-ranging deer in Michigan, to evaluate the effectiveness of existing TB surveillance methods, and to indirectly assess whether TB epidemiologic data from captive cervid herds can be meaningfully extrapolated to free-ranging populations. The study population consisted of all free-ranging deer submitted for TB testing in 2001 from six townships in northeastern Lower Michigan. Tissue samples of tonsil and cranial lymph nodes were collected bilaterally from all deer eligible for the study that did not have gross lesions suggestive of TB (n=701). Samples were subjected to histopathologic, acid-fast (AF) staining, mycobacterial culture, and polymerase chain reaction (PCR) testing. Seven deer cultured positive for *M. bovis* that would not have been detected by current surveillance, yielding apparent and true prevalence estimates (95% confidence limits) of 2.7% (1.6, 3.8) and 3.6% (2.3, 4.9), respectively. The sensitivity, specificity, and positive and negative predictive values of the current surveillance protocol were 75, 100, 100, and 99%, respectively. Histologic lesions were present only in tonsils, and ranged from simple necrosis to caseation, suppuration, and granuloma formation. Acid-fast staining and PCR detected *M. bovis* in only one of the seven culture-positive deer. Our study provides the first estimate of the true prevalence of *M. bovis* in Michigan's free-ranging deer population and suggests modest underestimation of that prevalence by current surveillance. This study also suggests that caution is warranted when extrapolating epidemiologic data on TB in captive cervids to free-ranging populations and confirms the pivotal role of the tonsil in early infections.

Key words: Bovine tuberculosis, diagnostic tests, disease frequency, *Mycobacterium bovis*, *Odocoileus virginianus*, white-tailed deer.

INTRODUCTION

Apparent prevalence (Martin et al., 1987) is the principal metric currently used by the Michigan Department of Natural Resources (MDNR) to estimate the reservoir of bovine tuberculosis (TB) in wildlife and to monitor changes in that reservoir over time. For white-tailed deer (*Odocoileus virginianus*), the primary wildlife reservoir of TB in Michigan (USA) (O'Brien et al., 2002), apparent prevalence is calculated as the number bearing gross lesions consistent with TB that test positive for *Mycobacterium bovis* on culture, divided by the total number of deer submitted for testing. Although useful as a consistent index and routinely used to measure disease frequencies in wildlife populations (Wobeser, 1994), apparent prevalence may underestimate the true prevalence of disease because it does not capture those animals infected with *M. bovis* but not as yet manifesting gross lesions. Assuming that TB control strategies (Schmitt et al., 1997) are successful in decreasing the apparent prevalence toward zero (Hickling, 2002), the ability to esti-

mate true prevalence will become increasingly important to accurately assess progress toward eradication of TB from the population. Indeed, the Governor's Executive Directive 1998-1 has mandated the state "to determine the actual prevalence of the disease and accurately evaluate trends" (Engler, 1998). Moreover, well-founded estimates of true prevalence are valuable epidemiologically (for deriving incidence rates) and politically (to address perceptions that the disease is more prevalent than is openly acknowledged).

In Michigan, the assumption generally has been made that apparent prevalence underestimates the true prevalence of TB in free-ranging deer, but to what extent is unknown. Bacterial culture is the gold standard for establishing a diagnosis of TB (Thoen et al., 1995; de Lisle et al., 2002), but the resources (monetary, personnel, laboratory capacity, etc.) necessary to culture the thousands of test specimens needed to obtain adequate statistical power to detect the disease at low prevalence are rarely available. Consequently, screening tests, such as culturing only those specimens with evidence of gross lesions, are widely employed, and screening tests often are not as accurate as the gold standard test. Two previous Michigan studies are relevant. Palmer et al. (2000) carried out postmortem TB testing on depopulated captive deer in a facility that was diagnosed *M. bovis* positive in 1997. Based on those findings, they concluded that TB testing methods currently used for wild deer surveillance (O'Brien et al., 2002) may underestimate true prevalence by 36–57%. However, their sample size was modest ($N \cong 300$, $n = 116$, 14 TB positive), and both apparent and true prevalence (7.8% and 12.1%, respectively) in the captive population studied were higher than has thus far been recorded in free-ranging deer anywhere in the state. Moreover, how captivity might have affected the apparent- vs. true-prevalence relationship is unknown. By contrast, a study of free-ranging deer that used existing surveillance methods (Fitzgerald et al., 2000) found little evidence that the testing protocol was missing a large number of TB-positive animals. Acid-fast (AF) bacteria were detected in only 0.3% of deer without gross lesions. However, sample size once again was limited ($N/n = 354$, 18 TB-positive specimens), and pooling of samples from different deer before culture prevented direct comparison of results of AF staining and culture. To further confuse matters, Rogan and Gladen (1978) noted that apparent prevalence typically overestimates true prevalence where the true prevalence of disease in the population is small, which is likely the case in Michigan (O'Brien et al., 2002).

Consequently, a need remains to clarify the relationship between apparent prevalence of *M. bovis* in free-ranging Michigan deer, as measured by existing MDNR surveillance methods, and true prevalence. Our objectives in this study were to estimate the true prevalence of *M. bovis* among the tested population of free-ranging Michigan deer, to evaluate the effectiveness of existing TB surveillance methods, and to indirectly assess the extent to which TB epidemiological data derived from captive cervid herds can be extrapolated to free-ranging populations.

MATERIALS AND METHODS

Survey area and population

The study population inhabited a portion (44°40′–45°00′N, 83°30′–84°05′W) of the area that has come to be known as the core of the TB outbreak (O'Brien et al., 2002). Specifically, specimens were taken from six townships (township [T] 27N, range [R] 7E; T28N, R4E; T28N, R6E; T28N, R7E; T29N, R4E; and T29N, R6E) in Alcona, Alpena, Montmorency, and Oscoda counties in northeastern Lower Michigan (Fig. 1). These areas were chosen to maximize the likelihood of obtaining *M. bovis*–positive samples; between 1995 and 2000, these six townships had the highest apparent prevalence of TB in the state (4.0, 3.6, 4.0, 5.4, 7.1, and 3.7%, respectively).

The population under study consisted of all free-ranging deer from these six townships submitted for TB testing in 2001. To be eligible for inclusion in the study, a deer had to be ≥1.5 yr of age, as determined by tooth eruption/wear

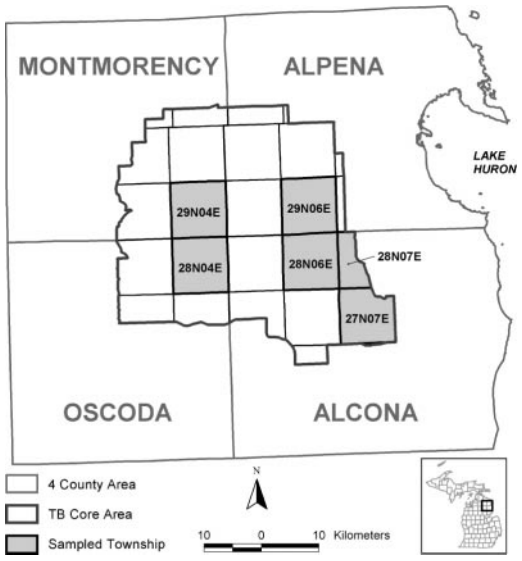

FIGURE 1. Survey area, northeastern Lower Michigan, USA. Shading indicates townships sampled; labels (e.g., 27N, 07E) signify township and range, respectively.

(Severinghaus, 1949; Ryel et al., 1961); to be processed by the MDNR Rose Lake Wildlife Disease Laboratory (RLWDL) for TB testing between 1 October 2001 and 11 January 2002; to bear no gross lesions consistent with TB; and to have tissues sufficiently fresh to allow unequivocal anatomic identification.

Sample collection and processing

Protocols used for surveillance (O'Brien et al., 2001, 2002) and diagnosis of TB (Fitzgerald et al., 2000) have been detailed elsewhere. Briefly, free-ranging deer harvested by licensed hunters were voluntarily submitted for TB testing to the MDNR. Heads were disarticulated and identified with a jaw tag bearing a unique tracking number, sex, age, location (township, range, and section) and date of harvest, and name, address, and phone number of the hunter. Heads were shipped to RLWDL, where age and sex were confirmed, and heads harvested from the study area were sorted off. Each head's tag was marked as included in the study, and the data portion of the tag was removed, leaving only the tracking number. All tag data were archived (Microsoft Access 97, Microsoft Corporation, Redmond, Washington, USA). Gross necropsies were carried out on all heads by, or under the supervision of, wildlife veterinarians or pathologists. Heads exhibiting cranial lymph node enlargement with granuloma formation or abscessation grossly were considered suspects, and were diverted from the study back into the specimen stream for normal TB surveillance (with tissue sections processed separately for histopathology, AF staining, and bacterial culture). For heads with no gross lesions, the submandibular, parotid, and medial retropharyngeal lymph nodes and palatine tonsils were collected bilaterally and pooled for each deer, with part placed in a sterile 50-ml polypropylene screw-top centrifuge tube (Corning Incorporated, Corning, New York, USA) for culture, and the remainder placed in 10% neutral buffered formalin (NBF) in a 133-ml plastic screw-top container (VWR Scientific Products, West Chester, Pennsylvania, USA) for histopathology. Necropsy instruments were decontaminated between deer heads by immersion and agitation in 70% ethanol sand. Residual ethanol was flamed off. Instruments were cooled momentarily before dissection of the next head. Samples for culture were transferred to the Michigan Department of Community Health (MDCH). Histopathology, performed only on heads that were positive for *M. bovis,* was carried out at the Animal Health Diagnostic Laboratory, Michigan State University (East Lansing, Michigan, USA) as previously described (Fitzgerald et al., 2000). Briefly, samples were fixed in 10% NBF, where they remained for 4–12 wk until final mycobacterial isolation results were reported. Those samples with positive *M. bovis* isolation were paraffin-embedded, sectioned at 5 μm, and stained with hematoxylin and eosin, as well as Ziehl-Neelsen AF stain, for microscopic evaluation.

Bacteriologic analyses

Mycobacterial testing was performed at the MDCH Tuberculosis Laboratory (TL), designated as a Tuberculosis Genotyping and Surveillance Network Regional Laboratory and Sentinel Surveillance Site by the US Centers for Disease Control and Prevention in 1995. Tubes were identified only by each deer's tracking number; MDCH-TL was blinded to the results of previous examinations. Upon receipt, specimens were frozen at −20 C until they could be scheduled for AF bacilli (AFB) slide and culture testing. Freezing suspensions of the Michigan deer strain (strain 1315) isolate of *M. bovis* reduced the number of viable bacteria by approximately one third. Yet, culture retains its ability to detect *M. bovis* despite freezing at −20 C for at least a year (Gruft et al., 1968; Kim and Kubica, 1972). After freezing, viability of strain 1315 remains stable with no appreciable reduction for up to 4 wk (MDCH, unpubl. data).

All specimens were thawed, processed, and

tested within 28 days of receipt. One- to 2-g sections of each of the six lymph nodes and two tonsils were combined and tested as a pooled sample for each deer head. The sample was ground with individual, antiaerosol, sterile, disposable tissue grinders (The Kendall Company, Mansfield, Massachusetts, USA). Specimens were digested with *N*-acetyl-L-cysteine/sodium citrate/sodium hydroxide for 20 min (Kent and Kubica, 1985), concentrated by centrifugation at 5,000×G for 15 min, and examined according to recommended procedures (Kent and Kubica, 1985). All tissues not needed for culture were refrozen as archived samples.

Two smears were prepared from concentrated specimen sediments for microscopic examination. One was heat-fixed and stained with the fluorescent auramine-O (AO) AF staining method (Kent and Kubica, 1985) and examined microscopically for the presence of AFB. When AO smears were positive or suspicious for the presence of AFB, the second smear was stained by using the Ziehl-Neelsen method and examined to confirm the presence of AFB. The remaining specimen sediment was resuspended with 1.5 ml of phosphate-buffered saline. Using the entire resuspended specimen, equal aliquots were inoculated to one each of a Lowenstein Jensen medium slant (Becton-Dickinson, Cockeysville, Maryland, USA), a Middlebrook 7H11S medium slant (Becton-Dickinson) and a Bactec 12B broth vial (Becton-Dickinson, Sparks, Maryland). All media were examined for growth at least weekly for 8 wk. Bacterial growth was examined microscopically by using a Ziehl-Neelsen–stained smear to determine if growth was due to AFB. Acid-fast bacterial growth was subsequently tested by genetic probe (Accuprobe, Gen-Probe, San Diego, California, USA) to determine whether the bacteria were members of the *M. tuberculosis* complex (Risner et al., 1994). Complete species identification was performed by biochemical testing and high-performance liquid chromatography to differentiate *M. bovis* from other members of the *M. tuberculosis* complex and other mycobacteria (Butler et al., 1991; Metchock et al., 1995).

Polymerase chain reaction analyses

After isolation of *M. bovis,* archived frozen tissues were thawed, fixed in 10% NBF, and paraffin-embedded, and the blocks were forwarded to the National Veterinary Services Laboratory (Ames, Iowa, USA) for polymerase chain reaction (PCR) testing to determine whether lymph nodes, tonsils, or both contained genetic evidence of *M. bovis.* Polymerase chain reaction testing was performed to detect *IS6110,* an insertion sequence specific for *M. tuberculosis* complex, by using the following primers: 5′-CTCGTCCAGCGCCGCTTCGG and 5′-CCTGCGAGCGTAGGCGTCGG (Eisenach et al., 1990). The procedure followed Miller et al. (2002), except the amplification conditions were 94 C for 10 min, 50 cycles of 94 C for 45 sec followed by 72 C at 135 sec, and a single cycle of 72 C for 10 min.

Quantitative analysis

All free-ranging deer tested for TB from the six survey townships were divided into three groups for analysis: 1) deer enrolled in the study (sample deer), 2) deer ineligible for the study (exclusions), and 3) deer eligible for the study that were not enrolled (eligible but unenrolled population [EUP]). To assess the extent to which the study sample adequately characterized the study population, the proportion of does, proportion originating from each township, and mean age were compared between the sample and the EUP via chi-square test, two-tailed Fisher's exact test (Stata, Version 5, Stata Corporation, College Station, Texas, USA), and two-tailed Student's *t*-test (Proc TTest, Statistical Analysis System, Version 8, SAS Corporation, Cary, North Carolina, USA), respectively (Thrusfield, 1995). Statistical significance was considered attained at $P \leq 0.05$.

To determine the true prevalence of disease in a population, it is necessary to know apparent prevalence (as detected by some screening test), as well as sensitivity and specificity of that test (Rogan and Gladen, 1978). In this study, the screening test was the existing surveillance protocol (i.e., only specimens with gross lesions are submitted for AF staining, histopathology, and culture). Sensitivity is the proportion of infected (here, TB-positive) animals that test positive on the screening test (Martin et al., 1987), which in this study was calculated as the number of grossly lesioned *M. bovis* culture–positive specimens, divided by the total number of *M. bovis* culture–positive specimens. Specificity is the proportion of noninfected (here, TB-negative) animals that test negative. In the existing surveillance protocol, culture-negative specimens from deer with and without gross lesions are pooled for calculation of specificity. With apparent prevalence (the number of grossly lesioned, *M. bovis* culture–positive specimens/total number of specimens tested), sensitivity and specificity were used to calculate the true prevalence of TB in the tested population by using the formula of Rogan and Gladen (1978):

TABLE 1. Comparison of selected characteristics of the enrolled sample with the eligible, but unenrolled, population of white-tailed deer in Michigan, USA, 2001.

Parameter	Sample (n=701)	Eligible unenrolled population (n_u=53)	P
Female[a]	309 (44.1)	29 (54.7)	0.13[b]
Mean age[c] (years) ± SE	2.76 ± 0.06	2.99 ± 0.21	0.31[d]
Geographic origin			0.13[e]
T27N, R07E	143 (20.4)	5 (9.4)	
T28N, R04E	142 (20.3)	9 (17.0)	
T28N, R06E	115 (16.4)	13 (24.5)	
T28N, R07E	81 (11.6)	5 (9.4)	
T29N, R04E	117 (16.7)	8 (15.1)	
T29N, R04E	103 (14.7)	13 (24.5)	

[a] One deer in the sample population was a hermaphrodite, and no gender was recorded for one deer in the eligible unenrolled population.
[b] Pearson chi-square, uncorrected, one degree of freedom.
[c] No age was recorded for one deer in the sample population.
[d] Student's two-tailed t-test, equal variances, 751 degrees of freedom.
[e] Fisher's two-tailed exact test.

$$\hat{p} = \frac{\hat{t} + \beta - 1}{\alpha + \beta - 1}$$

where $\hat{p}$ is true prevalence, $\hat{t}$ is apparent prevalence, β is specificity, and α is sensitivity.

The positive and negative predictive values of the existing surveillance protocol also were calculated (Thrusfield, 1995). The former is the probability that a deer diagnosed as TB positive on screening actually is positive, whereas the latter is the probability that a deer testing TB negative actually is negative. Ninety-five percent confidence limits on the apparent and true prevalence, sensitivity and specificity, and predictive values were calculated by methods described by Thrusfield (1995). All measures were calculated by using freely available software (de Blas et al., 2000).

TABLE 2. Comparison of the results of this study vs. the existing surveillance protocol for *Mycobacterium bovis* in free-ranging white-tailed deer in Michigan, USA.

	Existing surveillance protocol			
	Gross lesions present			
This study	Culture positive	Culture negative	Gross lesions absent	Total
Positive	21	0	7	28
Negative	0	6	747[a]	753
Total	21	6	754	781

[a] Includes 694 sample deer and 53 deer from the eligible unenrolled population.

RESULTS

Of 24,271 deer tested for TB in 2001, 906 (3.7%) originated in the six survey townships and composed the study population. Of these, 125 (13.8%) were exclusions: 88 (70.4%) were fawns, 20 (16.0%) had incomplete or ambiguous locations, 14 (11.2%) were processed outside the effective study dates, and three (2.4%) yielded inadequate specimens. Twenty-seven animals (3.0% of the study population) were classified as suspects because they had gross lesions consistent with TB. This left 754 deer (83.2% of the study population) eligible for enrollment in the study; 701 (92.9%) were enrolled in the sample, leaving 53 deer (7.0%) in the EUP.

No significant difference was found in the composition of the sample and the EUP with respect to sex, mean age, or geographic origin across the study townships (Table 1), suggesting that the sample adequately characterized the study population.

Comparing the results of this study with the existing TB surveillance protocol (Table 2), seven sample deer (1.0%) with no

evidence of gross tuberculous lesions were *M. bovis* positive. The apparent prevalence of TB among the 781 deer (sample + EUP + suspects) processed in the study was 2.7% (95% confidence limits: 1.6, 3.8). The sensitivity of the existing TB surveillance protocol was 75.0% (59.0, 91.0), whereas its specificity was 100.0%. Because all TB-negative deer test negative (a positive culture is necessary to be considered TB positive), specificity is predetermined at 100% (or, as close as the accuracy of culture, the gold standard, allows). Given these values, the true prevalence of *M. bovis* in the study population was estimated to be 3.6% (2.3, 4.9). The positive and negative predictive values of the existing surveillance protocol were 100.0% and 99.1% (98.4, 99.8), respectively. As with specificity, positive predictive value is predetermined at 100% (because a positive culture is necessary to be considered TB positive).

The characteristics and histopathologic results for seven deer without gross lesions that cultured *M. bovis* positive are summarized in Table 3. Although six of the seven were bucks, no significant difference was found in the percent of males among these false-negative deer and either the 21 positives with gross lesions (Fisher's, two-tailed $P=0.63$) or the 701 sample deer as a group (Fisher's, two-tailed $P=0.14$). No significant difference was found via Student's t-test in the mean ($\pm$SE) ages of the seven false-negative deer (2.64 ± 0.26) compared to the 21 deer with gross lesions (2.79 ± 0.27; $P=0.77$), or the sample deer as a group (2.76 ± 0.06; $P=0.68$). No significant histologic lesion or AF bacteria were found in any tissue sections of lymph node examined. In tonsil, histologic lesions were noted in all seven deer, and ranged in severity from simple necrosis to caseation, suppuration, and granuloma formation. Multinucleated giant cells were present in the tonsil of only one deer, as were AF bacteria.

Polymerase chain reaction testing detected evidence of *M. bovis* in only one of

TABLE 3. Summary of characteristics, histopathology, presence of acid-fast (AF) bacteria, and polymerase chain reaction (PCR) results for seven non–grossly lesioned, *Mycobacterium bovis* culture–positive free-ranging white-tailed deer, Michigan, USA.

Case	Sex	Age (yr)	Township and range	Date processed (2001)	Histopathology/PCR[a]	
					Cranial lymph nodes	Tonsils
90648	Male	2.5	T29N, R06E	26 November	NSL, AF−, PCR−	Necrosis and granuloma formation, AF−, PCR−
94956	Male	2.5	T29N, R04E	25 November	NSL, AF−, PCR−	Necrosis, AF−, PCR−
114094	Male	3.5	T29N, R04E	21 November	NSL, AF−, PCR−	Necrosis and granuloma formation, AF−, PCR−
117315	Female	2.5	T27N, R07E	6 December	NSL, AF−, PCR−	Multifocal granulomas, AF+, PCR+
139621	Male	3.5	T27N, R07E	19 November	NSL, AF−, PCR−	Suppuration and granuloma formation, multinucleated giant cells, AF−, PCR−
160114	Male	1.5	T29N, R04E	29 November	NSL, AF−, PCR−	Necrosis, AF−, PCR−
176928	Male	2.5	T27N, R07E	20 November	NSL, AF−, PCR−	Necrosis, AF−, PCR−

[a] NSL = no significant lesions; − = negative; + = positive.

14 tissue samples taken from the seven culture-positive deer (Table 3).

DISCUSSION

Although many aspects of pathogenesis and epidemiology of TB in white-tailed deer have been described in recent years, uncertainties about the true prevalence of TB in the free-ranging population have remained. The inability to plausibly estimate true prevalence has obscured the actual extent of the TB problem in Michigan. Because change in prevalence is used to assess progress toward disease eradication, this has led to confusion on the part of policymakers and the public. Moreover, misconceptions about the extent of the TB outbreak also have encumbered wildlife agencies competing for limited disease control funding. Our study provides the first scientifically credible estimate of the true prevalence of *M. bovis* in Michigan's wild deer population. Notably, because the apparent vs. true prevalence relationship remains linear for a given screening test as prevalence varies (Rogan and Gladen, 1978), our results can be extrapolated to areas of Michigan outside those where the study was conducted even though TB prevalence across those areas varies.

Questions have been raised (Palmer et al., 2000) about the number of infected deer the current surveillance program might be missing by screening only for grossly lesioned individuals. Our results suggest that apparent prevalence underestimates true prevalence by about 25%. Although by no means negligible, this underestimation is modest compared to that anticipated (36–57%) by Palmer et al. (2000). Considering the increased costs that would be incurred by culture of every animal tested, the benefit in diagnostic accuracy seems likely to be minimal, especially given that true prevalence can now be estimated from current surveillance. However, comparing our results with those of Fitzgerald et al. (2000) also suggests that counting on demonstration of AF bacteria to indicate early TB infection is unwise.

Because of difficulties inherent in studying diseases in free-ranging cervids, epidemiologic studies of captive cervids have sometimes been suggested as surrogates. Given the large areas encompassed by some captive cervid enclosures, which could seem to mimic a free-ranging situation, this is not entirely implausible. However, management conditions under which cervids are maintained in captivity vary widely, and factors such as stress, high stocking densities, alterations in movement and social behavior, and aggregation of animals due to feeding can create an artificial environment that can reasonably be expected to affect transmission of infectious diseases. Irrespective of the underlying reasons, the apparent vs. true TB prevalence relationship measured in our study, and that predicted by the study of naturally infected, captive Michigan deer (Palmer et al., 2000), differ substantially. Although the limitations inherent in drawing conclusions from a single study are obvious, the different results of these two studies nonetheless suggest that epidemiologic data on TB in captive cervids may be of limited relevance to free-ranging cervid populations.

A minor finding of this study, albeit one with practical implications for Michigan TB surveillance programs, was the quantification of the predictive value of a negative test. An essential part of our current surveillance program is notification of the hunter who harvested a tested deer of its test status. Most hunters consume their venison, often after cooking to temperatures insufficient to destroy *M. bovis.* Consequently, the probability that a deer testing TB negative is truly negative is of practical importance. Examination of our data suggests that more than 99% of the time, when hunters are informed that their deer was TB negative, it actually was negative. Beyond any public health implications, this level of confidence is critical in main-

taining the credibility of testing, and in ensuring continued hunter participation.

The palatine tonsils are anatomically situated so that they may serve as initial points of infection in the body whether the exposure occurs by nasal (aerosol) or oral (ingestion) routes (Palmer et al., 2002a). This makes the tonsils an efficient tissue to survey for infection with *M. bovis* when attempting to determine true prevalence. Experimental studies of *M. bovis* in deer routinely use intratonsilar instillation as the preferred route of infection (Palmer et al., 1999). Pathogenesis studies of *M. bovis* in deer indicate that the tonsil can be the earliest site of active infection, with organisms cultured from the tonsils as early as 15 days postinfection (PI). No microscopic inflammatory lesions were detectable before 28 days PI (Palmer et al., 2002b).

Although the tonsil is an excellent sampling site for early detection of *M. bovis* in white-tailed deer, it is unlikely to be suitable for large-scale surveillance. First, the tonsils are not readily visible grossly in the oral cavity because they are concealed by the oral mucosa of the palate. Second, no gross lesions were detected among the seven culture-positive animals in this study, suggesting that gross examination alone is unlikely to reveal typical tuberculosis lesions in infected individuals. Indeed, gross examination of nearly 90,000 Michigan deer for tuberculosis surveillance since 1995 has not noted a single positive animal exhibiting gross tonsilar lesions. This is in contrast to the presentation of *M. bovis* in the two positive free-ranging elk (*Cervus elaphus nelsoni*) from Michigan identified to date, both of which had gross lesions in the tonsil. If the tonsil cannot be screened through rapid gross examination, more labor-intensive and expensive techniques, such as tonsil collection for culture and histopathology, would be required. Thus, tonsilar examination is unlikely to become a routine part of TB surveillance in Michigan.

The microscopic tonsil lesions found in the seven culture-positive cases were generally nonspecific (Table 3). Only one animal in this study exhibited tonsil lesions typical of mycobacteriosis (granulomatous inflammation with intralesional AFB). Yet, non–*M. tuberculosis*-group mycobacteria could produce similar histologic lesions, necessitating culture to establish a definitive diagnosis. The other six TB-positive deer had histologic lesions less specific for TB. Both necrosis and suppuration of the tonsil may frequently result from a wide range of insults and bacterial infections. Granulomatous inflammation, particularly in the presence of multinucleated giant cells, is more highly associated with mycobacteriosis, but foreign bodies, several fungi, and higher bacteria incite a similar response.

Although PCR generally is regarded as highly sensitive, only one of seven *M. bovis* culture–positive cases was positive by PCR on formalin-fixed tissues. Detection of *M. bovis* by PCR on formalin-fixed tissues has a reported sensitivity of 81–93% (Miller et al., 1997). Notably, the technique was specifically developed for use in cases where typical inflammatory lesions and AFB were present histologically, but fresh tissues for mycobacterial isolation were not available, or where a more rapid test was desired (Miller et al., 1997). In our study, PCR detected *M. bovis* only in the tissue sample that displayed typical microscopic lesions and AFB. The failure to detect *M. bovis* in the other culture-positive samples may be attributable to repeated freezing and thawing, the length of time held in storage, or the possibility that trimming of tissue samples for initial isolation may have removed a focal tuberculous lesion, or may simply reflect the lower sensitivity of PCR on fixed, compared to fresh, tissue samples.

Limitations of this study must be kept in mind. First, despite its name, true prevalence as derived here remains an estimate. True prevalence could only be determined with absolute accuracy by using a perfectly sensitive and specific diagnostic test applied to an entire population. Such

circumstances are unlikely to arise in studies of free-ranging wildlife. Some TB-infected deer possibly could have cultured negative in our study, because of the insufficient time elapsed since initial infection, or because of decreased sensitivity due to freezing and processing of tissues. However, a study of lesion development (Palmer et al., 2002b) found culture to be quite sensitive for detecting early infection, recovering *M. bovis* from the medial retropharyngeal lymph nodes of three of three white-tailed deer 15 days after being inoculated intratonsillarly. Notably, the dose used in that study was low (300 colony-forming units per animal). Freezing of *M. bovis* at −20 C for more than 1 yr does not affect the ability to recover viable mycobacteria on culture (Gruft et al., 1968; Kim and Kubica, 1972). Heavy microbial contamination of deer specimens with saprophytes is a reality of our field surveillance, and the fastidious, slow growth of *M. bovis* can be easily overwhelmed by these more vigorous contaminants. Those contaminants multiply the longer a sample is held at ambient temperatures. The small risk of decreased sensitivity of culture due to freezing is tolerated to avoid the higher risk that *M. bovis* might not be recovered from a sample at all because of contaminant overgrowth. Thus, although not perfect, culture continues to be the gold standard test for diagnosis of infection with *M. bovis,* and, at present, no more sensitive technique (including PCR) is available. Any infected deer not detected by culture simply cannot be captured by current testing.

Second, our study sampled only cranial tissues, so TB infections present only in extracranial sites would have been missed. However, other studies suggest that was unlikely. Only 15 (0.06%) of 25,000 free-ranging Michigan deer tested for TB in 1999 had extracranial, but not cranial lesions (O'Brien et al., 2001). All of those animals had gross lesions sufficiently advanced to be noticed by the hunter and reported. Griffin and MacKintosh (2000) noted a relationship between lesion location and management circumstances, with thoracic and abdominal lesions most common in captive cervids, and cranial lesions most common in free-ranging cervids. Notably, Palmer et al. (2002b) found the medial retropharyngeal lymph node to be more sensitive in detecting *M. bovis* than any extracranial lymph nodes or lung, irrespective of whether microscopic lesions or positive culture was being assessed. Third, our combination of the sample and EUP deer to form the denominator for calculation of apparent and true prevalence could be questioned as being a source of bias. However, given that no significant difference was found between the sample and the EUP for measured parameters, or between the apparent and true prevalences calculated with and without the inclusion of the EUP, little evidence exists of any such bias. Further, if the rate of false negatives derived from the sample is applied to the EUP, the resulting likely number of non–grossly lesioned, culture-positive animals in the EUP is negligible ((7/701)×53=0.53 deer). Because both the sample and EUP are part of the study population, it is appropriate to combine them to obtain prevalence estimates for the study population as a whole. Finally, because the possibility cannot be excluded that the deer tested were not a random subpopulation of all deer harvested, or of the free-ranging deer population as a whole, our estimates should strictly be considered to apply to the tested population of free-ranging deer only.

ACKNOWLEDGMENTS

The authors gratefully acknowledge the assistance of S. Hogle in mapping; J. Gabbard, C. Stickney, G. Hickling, and J. Sikarskie in sample collection; as well as staff from MDNR Wildlife Division and US Department of Agriculture–Animal and Plant Health Inspection Service–Wildlife Services for their roles in surveillance efforts. In addition, gratitude is extended to M. Palmer and an anonymous reviewer for useful and insightful comments on the manuscript. This work was supported by the Federal Aid in Wildlife Restoration Act un-

der Michigan Pittman-Robertson Project W-127-R.

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Received for publication 15 November 2002.