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# Comparison of postmortem techniques for the detection of *Mycobacterium bovis* in white-tailed deer (*Odocoileus virginianus*)

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**Abstract.** A retrospective study of various diagnostic postmortem techniques used in a 4-year surveillance program for detection of *Mycobacterium bovis* infection in wild white-tailed deer (*Odocoileus virginianus*) was conducted. The tests evaluated were routine histopathology, acid-fast staining, detection of acid-fast bacilli in culture, and an *M. tuberculosis* group-specific genetic probe applied to pure cultures. Each of these techniques were compared with a reference or ''gold standard'' of mycobacterial culture and identification. Histopathology, the most rapid form of testing for *M. bovis* infection in white-tailed deer samples, had a sensitivity of 98% and a specificity of 87%, resulting in a positive predictive value of 94%. The detection of acid-fast bacilli by staining was less sensitive than histopathology (90%), but its higher specificity (97%) resulted in a positive predictive value of 99%. The detection of acid-fast bacilli on culture was both highly specific (93%) and sensitive (100%). The group-specific genetic probe had the highest sensitivity and specificity and produced results in complete agreement with those of mycobacterial culture, suggesting that this technique could be used as the new ''gold standard'' for this particular wildlife tuberculosis surveillance program.

Ongoing control and eradication programs for an epidemic of *Mycobacterium bovis* infection in wild white-tailed deer (*Odocoileus virginianus*) in northeastern Michigan require rapid and reliable diagnostic tests for the determination of the prevalence of infection in deer.[12] Most of the recent advances in tuberculosis testing have involved antemortem serologic and hematologic assays, including enzyme-linked immunosorbent assay, interferon gamma assay, and lymphocyte blastogenic assays.[12,14–16] However, because the ongoing tuberculosis surveillance in northeastern Michigan utilizes hunter-killed wild animals, postmortem testing is of great importance. Although information comparing various postmortem testing methods for detection of tuberculosis exists for cattle and elk, no such data exist for white-tailed deer.[1,2,11] The objective of this study was to compare the relative efficiency of routine histopathology, acid-fast staining, detection of acid-fast bacilli in culture, and *M. tuberculosis* group-specific genetic probe applied to pure cultures versus the ''gold standard'' test of myobacterial culture and species identification.

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## Materials and methods

*Sample collection.* Samples were collected from the bovine tuberculosis surveillance program for deer in Michigan from 1995 to 1998. Michigan Department of Natural Resources personnel recorded the age and sex of each deer sampled, date harvested, geographic location, and hunter identification information and collected and transported samples from the endemic tuberculosis area to the Michigan State University Animal Health Diagnostic Laboratories (AHDL). The most commonly submitted samples were heads alone. Occasionally, some heads were submitted with lungs; whole carcasses were rarely submitted.

*Sample processing.* All samples collected during the first year of surveillance (1995) were tested using all available methods; in subsequent years, only samples exhibiting gross lesions suggestive of tuberculosis were tested by all methods. Gross evaluations were performed at the AHDL by veterinary pathologists or by veterinary technicians and veterinary students under direct supervision of a pathologist. All heads had the parotid, submandibular, and medial retropharyngeal lymph nodes examined and serially sectioned.

A submission was considered a gross suspect if 1 or more lymph nodes contained focal or multifocal abscesses or granulomas. When a submission was determined to be a gross suspect, each individual abnormal lymph node was collected and split into 3 samples. One sample was fixed in 10% buffered formalin for histopathology, the second sample was collected using sterile technique and delivered by courier to the Michigan Department of Community Health (MDCH) Tuberculosis Laboratory for culture and identification, and the

**Table 1.** Comparison of sensitivity and specificity of different tuberculosis laboratory techniques, using identification of culture to species as the ''gold standard'' for testing.

| | Histopathology | | Acid-fast bacilli | | |
|---|---|---|---|---|---|
| | Exam 1 | Exam 2 | Stain | Culture | Genetic probe |
| Test result | | | | | |
| No. positive | 267 | 229 | 199 | 225 | 216 |
| No. negative | 23 | 65 | 94 | 68 | 5 |
| Gold standard | | | | | |
| No. positive | 220 | 220 | 220 | 220 | 216 |
| No. negative | 70 | 70 | 73 | 73 | 5 |
| Sensitivity (%) | 98.18 | 98.18 | 89.54 | 100 | 100 |
| Specificity (%) | 27.14 | 87.14 | 97.26 | 93.15 | 100 |
| Predictive value, positive (%) | 80.9 | 94.32 | 98.99 | 97.78 | 100 |
| Predictive value, negative (%) | 82.61 | 93.85 | 75.53 | 100 | 100 |

third sample was collected using sterile technique and forwarded to the Diagnostic Bacteriology Laboratory, National Veterinary Services Laboratories (NVSL), Ames, Iowa, for culture and identification. In cases where only a single lesion was present and was of such small size that division into 3 pieces was difficult, optimal pieces were submitted for culture and less representative pieces were submitted for histopathology.

*Histopathology* Formalin-fixed tissues were embedded in paraffin and cut into 6-μm sections, and histopathologic slides were prepared using standard hematoxylin and eosin (HE) staining methods.[6] Slides were evaluated microscopically by a veterinary pathologist, and histologic lesions were characterized according to 2 classification schemes. Initially, any microscopic abnormalities consistent with inflammatory disease (e.g., lymphoid hyperplasia, leukocyte infiltration, necrosis, mineralization) were considered suggestive of mycobacteriosis and thus a positive sample by histopathology (designated exam 1; Tables 1–3). A more critical microscopic review followed (designated exam 2; Tables 1–3). A sample was recorded as positive only if it consisted of granulomatous inflammation associated with central necrosis and with no evidence of other nonmycobacterial etiologies (i.e., no bacterial colonies, fungal hyphae, or Splendore-Heoppli reaction).

*Acid-fast staining.* Acid-fast staining was applied to all suspect samples using 2 different methods. Six-micrometer tissue sections from paraffin blocks of suspect samples were stained using the Ziehl-Neelson method.[6] In addition, fresh tissues forwarded to the MDCH Tuberculosis Laboratory were homogenized, digested, and concentrated.[3] Smears of the resultant material were stained using a fluorescent acid-fast staining procedure.[3] If acid-fast bacilli were detected by either of these techniques, the sample was considered positive by acid-fast staining.

*Mycobacterial culture and identification.* Fresh samples were collected in duplicate and forwarded to both the MDCH Tuberculosis Laboratory and the Diagnostic Bacteriology Laboratory, NVSL. The culture procedures used at both laboratories have been described previously.[12] If acid-fast bacilli were detected on culture medium by weekly staining, irrespective of their eventual species determination, the samples were considered positive for acid-fast bacilli by culture.

Identification of mycobacterial isolates to species at both laboratories involved a battery of biochemical tests and

**Table 2.** Comparison of sensitivity and specificity of different tuberculosis laboratory techniques based on gender, using identification of culture to species as the ''gold standard'' for testing.

| | Females | | | | | Males | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | Histopathology | | Acid-fast bacilli | | Genetic probe | Histopathology | | Acid-fast bacilli | | Genetic probe |
| | Exam 1 | Exam 2 | Stain | Culture | | Exam 1 | Exam 2 | Stain | Culture | |
| Test result | | | | | | | | | | |
| No. positive | 107 | 84 | 75 | 85 | 82 | 159 | 144 | 123 | 138 | 133 |
| No. negative | 7 | 32 | 40 | 30 | 3 | 12 | 27 | 50 | 35 | 2 |
| Gold standard | | | | | | | | | | |
| No. positive | 82 | 82 | 82 | 82 | 82 | 136 | 136 | 136 | 136 | 133 |
| No. negative | 33 | 33 | 33 | 33 | 3 | 35 | 35 | 37 | 37 | 2 |
| Sensitivity (%) | 98.78 | 98.78 | 90.24 | 100 | 100 | 98.53 | 98.53 | 89.71 | 100 | 100 |
| Specificity (%) | 18.75 | 93.93 | 96.97 | 98.67 | 80 | 28.57 | 71.42 | 97.3 | 94.59 | 100 |
| Predictive value, positive (%) | 100 | 96.43 | 90.91 | 96.47 | 100 | 84.28 | 93.06 | 99.19 | 98.55 | 100 |
| Predictive value, negative (%) | 100 | 96.88 | 100 | 100 | 100 | 83.33 | 92.59 | 72 | 100 | 100 |

**Table 3.** Comparison of sensitivity and specificity of different tuberculosis laboratory techniques by age, using identification of culture to species as the "gold standard" for testing.

| | Deer <2 yr | | | | | Deer 2–5 yr | | | | | Deer >5 yr | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Histopathology | | Acid-fast bacilli | | Genetic | Histopathology | | Acid-fast bacilli | | Genetic | Histopathology | | Acid-fast bacilli | | Genetic |
| | Exam 1 | Exam 2 | Stain | Culture | probe | Exam 1 | Exam 2 | Stain | Culture | probe | Exam 1 | Exam 2 | Stain | Culture | probe |
| Test result | | | | | | | | | | | | | | | |
| No. positive | 78 | 56 | 45 | 51 | 47 | 166 | 143 | 142 | 158 | 154 | 17 | 26 | 9 | 11 | 11 |
| No. negative | 10 | 37 | 43 | 37 | 3 | 7 | 13 | 32 | 16 | 2 | 1 | 9 | 10 | 8 | 0 |
| Gold standard | | | | | | | | | | | | | | | |
| No. positive | 48 | 49 | 48 | 48 | 47 | 156 | 141 | 156 | 156 | 154 | 11 | 26 | 11 | 11 | 11 |
| No. negative | 40 | 44 | 40 | 40 | 3 | 17 | 15 | 18 | 18 | 2 | 7 | 9 | 8 | 8 | 0 |
| Sensitivity (%) | 100 | 98.0 | 89.6 | 100 | 100 | 98.7 | 98.6 | 91.0 | 100 | 100 | 90.9 | 96.2 | 81.8 | 100 | 100 |
| Specificity (%) | 25 | 81.8 | 95 | 92.5 | 100 | 29.4 | 73.3 | 100 | 88.9 | 100 | 0 | 88.9 | 100 | 100 | 100 |
| Predictive value, positive (%) | 61.5 | 85.7 | 95.6 | 94.1 | 100 | 92.8 | 97.2 | 100 | 98.7 | 100 | 100 | 96.1 | 100 | 100 | 100 |
| Predictive value, negative (%) | 100 | 97.3 | 88.4 | 100 | 100 | 71.4 | 84.6 | 56.3 | 100 | 100 | 0 | 88.9 | 80 | 100 | 0 |

high-performance liquid chromatography techniques that have been previously described.[12] Those isolates identified as *M. bovis* were considered gold standard positives and served as the reference against which all other testing techniques were measured.

*Genetic probe.* The MDCH Tuberculosis Laboratory applied a commercial genetic probe[a] to cultures of *Mycobacterium* spp. as soon as growth was evident on weekly examination of medium. This DNA probe is a nonisotopic probe that uses an acridinium-ester label for chemiluminescence. The probe is applied to supernatent from the cultures, and the DNA hybridizes with ribosomal RNA found in the mycobacteria. The genetic probe is *M. tuberculosis* group specific, meaning it will react positively to *M. tuberculosis, M. bovis, M. africanum,* or *M. microti* but will not react to *M. paratuberculosis, M. avium,* or various environmental mycobacterial species.[7]

*Statistical analysis.* Descriptive statistics were generated using the SAS 6.12 software package.[b] Sensitivity, specificity, positive predictive values, and negative predictive values were computed[13] for all samples and for samples categorized by age group (<2 yr, 2–5 yr, and >5 yr of age) and gender. Test sensitivity is the percentage of true positive samples that were positive by the test, and specificity is the percentage of true negative samples that were negative by the test. Positive predictive value is the probability that a test-positive sample is truly positive, and negative predictive value is the probability that a test-negative sample is truly negative.

## Results

*Sample collection.* Over 17,200 deer from the tuberculosis-endemic area were submitted for testing, with 354 submissions in 1995, approximately 4,000 in 1996, approximately 4,000 in 1997, and approximately 9,000 in 1998. Of these 17,200 samples, 294 exhibited gross lesions within their lymph nodes, which were submitted for complete diagnostic evaluation for mycobacteriosis. Comparison of testing techniques are reported in Table 1. Based on mycobacterial culture and identification, 220 samples were confirmed *M. bovis* positive by culture, and 73 samples with suspicious lesions based on gross examination were confirmed negative by culture.

*Gross pathology.* Gross lesions were most frequently seen in the medial retropharyngeal lymph nodes and were present unilaterally or bilaterally in similar numbers; lesions in either the parotid or submandibular lymph nodes were uncommon. The majority of lymph node lesions from which *M. bovis* was isolated appeared grossly as abscesses filled with pale purulent material, and <25% of the lesions appeared as solid granulomas. With the exception of the first year of the study, gross lesions were used as an initial screening step, and full diagnostic work-ups were not performed on grossly normal samples. Consequently, evaluation of the gross lesions as compared with the gold standard for determination of specificity, sensitivity, and

predictive values could not be conducted. Data collected from a total of 354 deer examined in 1995 revealed that 15 deer with gross lesions had histologic lesions consistent with tuberculosis, and acid-fast bacilli were identified in tissues from all 15 animals.[12] Conversely, of the remaining 339 deer with no gross lesions, only 4 animals exhibited histologic lesions consistent with tuberculosis, and only 1 of these lesions contained acid-fast bacilli.

*Histopathology.* Histopathology was performed at the same laboratory where samples were collected and was typically completed within 48 hours of sample processing. Histopathology was evaluated using 2 different sets of criteria as explained in the methods; exam 1 was a screening process for evidence of inflammation, and exam 2 included specific histomorphologic criteria and cases that had obvious etiologies other than mycobacteria were excluded. Both histopathology techniques resulted in high sensitivities (98% for both). Although exam 1 had a low specificity (27%), exam 2 exhibited significantly higher levels of specificity (87%) and improved positive and negative predictive values (94% for both).

*Acid-fast staining.* Acid-fast staining was available at the same time as histopathology, generally within 48 hours of sample processing. Acid-fast staining was performed on both fixed tissue sections and fresh homogenized tissue, and similar results were obtained by both methods, although a few more acid-fast-positive results were obtained with homogenized tissue preparations. This technique resulted in moderately high sensitivity (90%), high specificity (97%), high positive predictive value (99%), but somewhat lower negative predictive values (76%). The number of acid-fast bacilli seen was extremely variable from case to case; some had very rare bacilli (1 or 2 per slide) and others had moderately high numbers of bacilli (>10 bacilli/high-power field). The location of these acid-fast bacilli was also variable. The organisms were most frequently seen within the cytoplasm of multinucleate giant cells and less frequently within macrophage cytoplasm or free in caseous debris.

The presence of acid-fast bacilli on culture was a technique applied by the MDCH Tuberculosis Laboratory and often provided results within 1–2 weeks, but in some evaluations took up to 8 weeks (maximum length of time cultures were held). This technique provided high levels of sensitivity (100%), specificity (93%), positive predictive value (98%), and negative predictive values (100%).

*Genetic probe.* The group-specific genetic probe applied to mycobacterial cultures was dependent on the initial positive culture growth of acid-fast bacilli, taking anywhere from 1 to 8 weeks from initial sample processing to conclusion depending on how rapidly cultures of acid-fast bacilli were isolated. In many cases, the genetic probe results were available in only 1–2 weeks. This technique had 100% correlation with the final culture and identification results, giving the highest possible sensitivity, specificity, and predictive value.

*Testing based on age and gender.* Analysis of each testing method was conducted with samples divided by gender (Table 2) and age of deer (<2 years, 2–5 years, >5 years) (Table 3). The resulting test sensitivities, specificities, and predictive values were similar to those obtained by analyzing the entire sample set.

## Discussion

The decision to use the presence of gross lesions as the primary screening test for further diagnostic workup was based on earlier tuberculosis screening results,[1,2,9,14] on an initial first-year deer survey in which all tests were applied regardless of the presence of gross lesions, and on available resources to process the additional tests as the number of annual samples surveyed climbed into the thousands in subsequent years of the study. The specificity and predictive values of gross lesions were <100%; 73 samples exhibiting gross lesions were culture negative for mycobacteria. The purulent abscesses and caseogranulomas commonly seen in deer in this study were similar to tuberculous lesions previously reported in other cervids (elk, fallow deer, red deer, sika deer).[4,5,8,10]

Histopathology was highly sensitive and had good predictive value for a rapid test, even when using the less selective criteria of general inflammation (exam 1). The commonly observed histopathologic features in the positive deer consisting of caseous necrosis with or without mineralized foci and surrounded by mixed mononuclear leukocytes and multinucleate giant cells were similar to those previously reported in other cervids.[6–9] When these ''typical'' histopathologic findings were used as the defining criteria as with exam 2, the specificity and positive predictive values were significantly higher than in exam 1. In no cases were microscopic features indicative of another causative etiology (i.e., significant eosinophilic infiltrates, presence of Splendore-Hoeppli reaction, presence of bacterial colonies or fungal hyphae visible by HE staining) associated with positive mycobacterial culture results. Sensitivity for histopathologic evaluation may have been reduced because several grossly suspect animals had such small focal lesions that the they could not be easily divided into 3 separate samples for further testing, and those portions exhibiting gross lesions were preferentially selected for mycobacterial culture rather than histopathology.

The presence of acid-fast bacilli on staining was not only rapid but also was highly specific and had a high

positive predictive value. The lower sensitivity was explained by isolation of a number of acid-fast non-*M. bovis* organisms, including 2 samples identified as *M. terrae*, 1 as *M. avium*, 1 as *M. fortuitum*, 1 as *M. gordonae*, and 1 as an unidentified *Mycobacterium*. The great variability in numbers of acid-fast bacilli present in samples was a likely source of the lower negative predictive value, which explains the failure of the rapid acid-fast staining technique to identify mycobacteria in samples that were later positive on culture.

The presence of acid-fast bacilli on culture took several weeks compared with the more rapid histopathology and acid-fast staining techniques; however, the culture technique provided very high sensitivity and predictive values. There was also a high, but slightly lower (93%), level of specificity. The presence of samples infected with non-*M. bovis Mycobacterium* species contributed to the decrease in specificity for this test.

When the genetic probe was applied to the acid-fast cultures, the results achieved were in 100% agreement with the reference standard. Even though several weeks were sometimes required to obtain mycobacterial cultures for genetic probe testing, these results suggest that the tedious, time-consuming, and labor-intensive methods used for definitive mycobacterial culture and identification may not be required in future years for the continuing surveillance of *M. bovis* in wild deer in Michigan.

The choice of mycobacterial isolation and identification as the gold standard is well established in mycobacteriologic diagnostics, and this standard forms the basis for definitive diagnosis in current USDA Uniform Methods and Rules for bovine tuberculosis eradication. However, this technique is not completely accurate. Failure to isolate *M. bovis*, resulting in false-negative results, does occur and is dependent on total number of infectious organisms, destruction of viable organisms by overgrowth of other bacteria, state of preservation of tissues, and use of decontaminates during tissue processing.[12] Considering these hunter submissions were voluntary and may have spent days to weeks at ambient temperatures or in freezers, it is reasonable to assume that some false-negative results would have been obtained. Our goal was not to evaluate the sensitivity of the gold standard itself but to compare these other diagnostic techniques with the gold standard.

The close agreement in results for all testing methods irrespective of deer gender and age illustrates that testing methods were not influenced by these factors. Neither gender nor age-related factors have previously been associated with variability in tuberculosis testing sensitivity or specificity in other animal species.

In addition to providing a better understanding of the capabilities and limitations of these current postmortem testing techniques for detection of *M. bovis* in deer, this study also provides us with the background data necessary to evaluate new testing methods as they become available. Because the genetic probe has proven to be highly sensitive and specific, it is reasonable to concentrate on developing more rapid, efficient, and affordable genetic probe methods. One technique now undergoing evaluation is the application of a genetic probe directly to suspect tissues; this approach would be significantly more rapid than the several weeks needed for isolation of acid-fast bacilli. A second technique that appears both highly accurate and rapid is the use of polymerase chain reaction techniques on formalin-fixed, paraffin-embedded tissues to identify the species of *Mycobacterium* recognized by acid-fast staining.[4]

The expansion of surveillance testing could be facilitated by the development of new tests, with high levels of sensitivity and specificity, that can be performed rapidly, inexpensively, and with minimal labor costs. The development of rapid field tests for *M. bovis* screening would improve the efficiency of surveillance programs by reducing the large numbers of negative specimens currently sent to high-biosecurity testing facilities and the huge amount of potentially infectious material that must be disposed of in large medical-waste incinerators. Ultimately, all advances made in testing for infectious zoonotic diseases such as tuberculosis will result in healthier animal populations, which in turn will reduce the public health risk of infection to humans.

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b. SAS Institute, Cary, NC.

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