Research Article

Antimicrobial Susceptibility Testing of *Mycobacterium bovis* Isolates from Michigan White-Tailed Deer during the 2009 Hunting Season

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Michigan has had an ongoing outbreak of endemic *Mycobacterium bovis* which has been recognized within and sustained by its free-ranging white-tailed deer population since 1994. Worldwide, organisms within the *Mycobacterium tuberculosis* complex have exhibited the ability to develop resistance to antimicrobial agents, resulting in both the multidrug-resistant (MDR) and extensively drug-resistant (XDR) strains of human tuberculosis. Michigan's Bovine Tuberculosis Working Group has conducted active antimicrobial susceptibility testing on wildlife isolates of the endemic *M. bovis* organism at five-year intervals to detect any emerging drug resistance patterns. The results of 33 white-tailed deer origin isolates collected from the 2009 hunting season are reported here. There continues to be no evidence of any drug resistance except for pyrazinamide resistance. These results are likely due to the lack of antibacterial treatment applied to either wildlife or domestic animals which would provide selection pressure for the development of drug resistance.

1. Introduction

The state of Michigan has had an ongoing free-ranging white-tailed deer tuberculosis surveillance and control program since 1994 [1, 2]. It appears that deer serve as the primary reservoir host in the state of Michigan, with elk and a variety of wild carnivores and omnivores serving as spill-over hosts, and to date there have been 50 cattle herds infected by *M. bovis* [3], While some progress has been made on controlling the spread of *M. bovis* outside of the endemic 12 county area and the overall incidence rate of infection in deer has dropped from around 4.9% to approximately 1.7%, there remain ongoing programs for surveillance, and the ultimate goal is to eradicate the disease from both domestic cattle and wild deer [4]. Since *M. bovis* is also infectious to humans, a multiagency task force consisting of personnel from state departments (Michigan Department of Natural Resources and Environment, Michigan Department of Agriculture, and Michigan Department of Community Health), from a public university (Michigan State University), and from a federal agency (United States Department of Agriculture) have formed a bovine tuberculosis task force to deal with issues concerning wildlife, domestic animals, and human health [1, 2].

Bovine tuberculosis threatens human health through direct acquisition of the disease from field dressing infected deer, from airborne transmission of the disease from infected cattle or captive deer, and from ingestion of unpasteurized milk or undercooked meat products including venison [5–7]. Over the last sixteen years, only two human cases have been identified as associated with the specific strain of *M. bovis* endemic in Michigan deer and cattle [7]. One case was an elderly individual who was raised on a farm and may have been exposed decades earlier through drinking

unpasteurized milk. A second case was a hunter who cut his hand while field-dressing a deer carcass and developed a localized cutaneous infection which has been subsequently treated successfully. Since humans do occasionally become exposed and infected by this organism, our task force has instituted regular antimicrobial susceptibility testing of deer and other wildlife isolates, so that we may recognize any developing antibacterial resistance [8].

Mycobacterial species in the *M. tuberculosis* complex undergo low-frequency spontaneous chromosomal mutations which result in genetic resistance to antituberculosis drugs. For example, *M. tuberculosis* undergoes spontaneous mutations resulting in resistance to isoniazid at a frequency of 3.5×10^{-6} and mutations resulting in rifampin resistance at a frequency of 3.1×10^{-8} [9]. However, it is the application of antituberculosis drugs which creates pressure for selection of these strains with mutations. This is generally due to improper therapeutic applications such as insufficient length of drug treatment, poor patient adherence to dosing schedules, using a single antituberculosis drug instead of the recommended multiple drug therapy, and failure to recognize pre-existing resistance in a tuberculosis case [10]. These problems then lead to the emergence of either multidrug-resistant (MDR) or extensively drug-resistant (XDR) strains of mycobacteria. MDR is defined as resistant to at least rifampicin and isoniazid, which are first-line antituberculosis drugs. XDR is defined by the World Health Organization Global Task Force on XDR-TB as resistant to rifampicin and isoniazid, as well as any member of the fluroquinolone family, and one or more of the second-line antituberculosis drugs including kanamycin, capreomycin, or amikacin [10]. Fortunately, these forms of antituberculosis drug resistance are not as prevalent in veterinary medicine as they are in human medicine because we tend to cull infected animals rather than treat them for tuberculosis [5].

The objectives for this study were to (1) take the majority of the 2009 hunter-harvested wild deer isolates of *M. bovis* from Michigan and conduct routine antimicrobial susceptibility testing by two different methodologies in order to detect any evidence of new antimicrobial resistant strains and (2) compare the 2009 data with the 1999 and the 2004 antimicrobial studies which utilized similar methods [8].

2. Materials and Methods

All culture positive deer *M. bovis* samples submitted to the Michigan Department of Community Health Tuberculosis Laboratory during the 2009 wild white-tailed deer hunting season were tested for antimicrobial susceptibility. The antimicrobial agents tested included isoniazid, streptomycin, rifampin, ethambutol, ethionamide, kanamycin, ciprofloxacin, cycloserine, capreomycin, and pyrazinamide. Isolates from 33 deer were utilized in this study.

2.1. Proportion Plate Method. Isolates were subcultured into Middlebrook 7H9 broth which was then incubated at 35°C for 2 to 6 days. Subcultures were then used to conduct 1% proportion plate susceptibility assays by previously described

methods [11, 12]. Middlebrook 7H10 agar plates were prepared with 5 mL of medium into each of four quadrants in the Petri dishes, which then had drug-impregnated discs placed into each quadrant, with one drug-free quadrant serving as a control. Powdered suspensions were utilized for the cycloserine and capreomycin preparations. Final drug concentrations in these plates were 0.2 and 1.0 μg/mL isoniazid, 2.0 and 10.0 μg/mL streptomycin, 1.0 μg/mL rifampin, 5.0 μg/mL ethambutol, 5.0 μg/mL ethionamide, 6.0 μg/mL kanamycin, 2.0 μg/mL ciprofloxacin, 30.0 μg/mL cycloserine, 10.0 μg/mL capreomycin, and 100 μg/mL pyrazinamide. Plates were inoculated to reach a colony count of between 100 and 200 colony forming units on the control quadrant containing no drug. Plates were incubated as 35°C with CO_2 for 3 weeks. Percentage resistance for each drug was calculated by dividing the total number of colonies in a quadrant by the total number of colonies in the control quadrant and multiplying the result by one hundred. A 1% standard cutoff value was used for the interpretation of resistance. Therefore, a culture with a percent resistance of less than 1% was considered susceptible to that particular drug at that concentration while a culture with a percent resistance greater than or equal to 1% was considered resistant to that particular drug.

2.2. Bactec Method. Bactec radiometric susceptibility testing was the second method used to evaluate *M. bovis* isolates for antimicrobial resistance and followed previously described methods [13]. The following antimicrobial drugs were added to Bactec 12B vials: isoniazid at 0.1 g/mL, streptomycin at 2.0 g/mL, rifampin at 2.0 g/mL, and ethambutol at 2.5 g/mL. A suspension of each isolate was diluted to a 0.5 MacFarland suspension and then inoculated into vials containing each of the drugs. A drug-free control vial was also prepared by diluting the 0.5 MacFarland suspension by 1 : 100. Vials were read each day using the Bactec 460 TB instrument until the growth index (GI) of the control vials reached 30 or greater. Susceptibility results were determined by comparing the ΔGI (difference between the current day's GI value with the GI value from the previous day's reading) of the drug vial to the ΔGI of the control vial. A resistant isolate had a ΔGI value greater than the ΔGI value of the control vial.

The Bactec method for determination of pyrazinamide was somewhat different from the other drugs tested. A 0.5 MacFarland suspension of each isolate was inoculated into a Bactec 12B vial and then grown out to a GI value of greater than 300. Next, these vials contents were inoculated into 2 pyrazinamide test medium vials. Pyrazinamide was added to one vial at a concentration of 100 μg/mL while the second vial served as a drug-free control. Vials were read each day using the Bactec 460 TB instrument until the control GI value reached greater than or equal to 200. Bactec pyrazinamide results were interpreted by dividing the GI of the drug vial by the GI of the control vial and multiplying the result by 100 to calculate the percentage resistance. Percentage of resistance less than 9% was interpreted as susceptible to pyrazinamide while percentage of resistance greater than 11% was interpreted as resistant. Percentages between 9 and

Table 1: Antimicrobial susceptibility results for Michigan deer from 2009.

Host Species	Bactec method	Bactec PZA[1] method	Proportion method	Proportion method PZA[1]
Deer	0/33[2]	33/33[2]	0/33[2]	33/33[2]

[1]PZA: pyrazinamide
[2]Number of resistant isolates over the total number of isolates tested.

11% were considered equivocal, and repeat of the assay was performed; this repetition of the assay was rarely needed.

3. Results

For the 2009 deer isolates of *M. bovis*, all 33 were susceptible to isoniazid, streptomycin, rifampin, ethambutol, ethionamide, kanamycin, ciprofloxacin, cycloserine, and capreomycin by the proportion plate method (Table 1). All 33 isolates were also susceptible to isoniazid, streptomycin, rifampin, and ethambutol by the Bactec method. All 33 isolates were resistant to pyrazinamide (100%) by both the proportion plate and the Bactec methods. These results indicating no drug resistance except for pyrazinamide were identical to our findings in the 1999 and 2004 surveys, in which there were 30 deer isolates tested in 1999 and 28 deer isolates tested in 2004 [8].

4. Discussion

It was expected that all deer *M. bovis* isolates would be resistant to pyrazinamide. Historically, *M. bovis* isolates are resistant to pyrazinamide because the organism does not produce the enzyme pyrazinamidase which is needed to convert pyrazinamide into pyrazinic acid, the active form of the antimicrobial agent [14]. This resistance is one of the basic features which can be used to distinguish isolates of *M. bovis* (universally resistant to pyrazinamide) from *M. tuberculosis* (commonly susceptible).

The fact that no detectable emerging antimicrobial resistant *M. bovis* strains were found is good news, especially for the rare individual who is infected with this *M. bovis* strain and must undergo therapy. It is most likely explained by the fact that wild deer and other wild spill-over hosts are commonly found to be infected with *M. bovis* only after they have been harvested by hunters or trappers. Likewise, when either domestic cattle or captive cervids in Michigan are detected as TB suspects or reactors on antemortem surveillance, they are routinely slaughtered and sent for full tuberculosis testing which includes culture and PCR assays. In either circumstance, there is no antibacterial treatment applied to infected or possibly infected individual animals, which means there is no selection pressure to favor the development of drug-resistant strains. Furthermore, the antimicrobial susceptibility surveys conducted at 50-year intervals give us a broad overview of resistance development since the disease was first recognized as endemic in Michigan deer 16 years ago.

Unlike the two previous susceptibility surveys conducted in 1999 and 2004, this survey was limited to wild deer, the primary reservoir host of *M. bovis* in Michigan. This is due to changes in the processing of tuberculosis surveillance samples. In earlier years, all wild animal samples including the spill-over hosts comprised by various wild carnivores and omnivores, used to run through the state diagnostic laboratory and the Tuberculosis Laboratory, Michigan Department of Community Health. Starting in 2005, personnel from the Wildlife Services, United Sates Department of Agriculture, took over the responsibility for nondeer wildlife surveillance, and those samples are shipped to another state for testing. Likewise, during the earlier years of the tuberculosis endemic the suspect cattle which were processed at the state diagnostic laboratory had duplicate samples collected for culture both at the Diagnostic Bacteriology Laboratory, National Veterinary Services Laboratory (NVSL), Ames, Iowa, as well as at the local Michigan Department of Community Health Laboratory. However, in recent years, samples have only been collected for NVSL, as this is a program disease for which the USDA has primary authority, and the fact that duplicate processing was considered redundant and too expensive for current fiscal budgets. Since the wild deer are considered the principal reservoir host, we feel that the current antimicrobial susceptibility survey is still valid in spite of the absence of the other wildlife and domestic cattle isolates.

While the lack of any detectable antimicrobial resistance development is good news, there remains reason for caution and continued regular surveys of this type. In July, 2009, the USDA, Animal and Plant Health Inspection Service produced a concept paper for new approaches to managing bovine tuberculosis which are currently being implemented [15]. One of their recommendations was to begin applying whole-herd depopulation judiciously and developing alternative control strategies including test and cull of individual suspect animals. This is a reasonable response to the enormous costs that USDA and state departments of agriculture must expend in order to depopulate and indemnify ever larger cattle herds, many with valuable individual breeding animals. However, we should remember that the reason why drug-resistant strains of *M. bovis* are not emerging in animals nearly as rapidly as in human populations is because of the lack of treatment-related selection pressures. No one is suggesting that individual cattle or captive cervids which are identified as tuberculosis suspects should start to receive antimicrobial therapy as we do with infected people. When whole-herd test and individual animal culling practices are employed, there is the very real opportunity for other animals in the same herd which may be harboring tuberculosis to receive limited antibacterial therapy for other conditions, such as respiratory disease, mastitis, or other localized infections. Alternatively, whole beef cattle herds may be treated with long-term low-level antibacterial agents as growth promoters. In this type of environment, asymptomatic and undetected tuberculosis carriers will be subjected to similar selection pressures that have produced the current worldwide emergence of MDR and XDR tuberculosis strains in humans. This is not meant

to find fault with newer management procedures for bovine tuberculosis but only to point out that selection pressures which promote antimicrobial resistance development may well be increasing, at least in our domestic and captive animal species. Therefore, continued surveillance for drug-resistant strains of *M. bovis* will be even more important as we move forward to ensure the safety of our milk, beef, and venison sources for human consumption.

Acknowledgments

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