Isolation of multidrug-resistant (MDR) *Mycobacterium bovis* from a dog in Korea

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ABSTRACT. A 3-year-old female Miniature Schnauzer dog with a week-long history of generalized intention tremor and progressive weight loss for several months was admitted. Mild anemia, fever, splenomegaly, aseptic cerebral meningitis and systemic lymph nodes enlargement were examined through erythrogram, ultrasonography, computed tomography and magnetic resonance imaging. *Mycobacterium bovis* was identified via molecular microbiology having the same molecular type as isolates from a cattle farm previously identified. However, the dog was raised in a city. The *M. bovis* had multidrug resistance (MDR)-bearing mutations in both *katG* and *rpoB* genes toward first-line antibiotics. To the best of our knowledge, this is the first report describing an MDR *M. bovis* infection of a dog in Korea.

KEYWORDS: BCG-vaccinated, canine tuberculosis, multi-drug resistant, *Mycobacterium bovis*, one health

Tuberculosis (TB) remains one of the leading devastating infections globally, claiming millions of lives every year [33]. Despite unquestionable progress in the fight against human and bovine TB, human TB is still very prevalent in certain areas, including Middle Africa and Southeast Asia, areas with dense populations and poor social conditions [18]. Although, *Mycobacterium (M.) tuberculosis* is recognized as the primary cause of human TB worldwide, *M. bovis* has become the most common cause of zoonotic TB globally. In 2019, an estimated 140,000 (1.4%, range 69,800–235,000) new cases of zoonotic TB occurred globally, and of those cases, approximately 11,000 (8.1%, range 4,470–21,000) resulted in death [33].

TB in dogs and cats is commonly reported in BCG non-universal-vaccinated countries, such as the US, UK and other European countries [18, 22]. Meanwhile, cases of tuberculosis in companion animals are rarely reported in general BCG vaccinated countries [26] including Korea. In Korea, many TB cases have been reported only in farm animals, however, this is the first companion animal mycobacteriosis by *M. bovis*. This could be explained by the fact that routine diagnostics in veterinary clinics do not cover testing for TB [12]. TB in animals is classified as a category B in European countries [17], list B by OIE [23] and FAO [10] and class II legal infectious disease in Korea. Livestock animals diagnosed with TB are culled with compensation. However, in case of companion animals being diagnosed with TB, there is no mandated course of action and/or standardized measure. Treatment is not often recommended for companion animals, rather euthanasia should be considered for affected companion animals in close contact with people due to public health concerns.

A 3-year-old female Miniature Schnauzer dog with a week-long history of generalized tremor and progressive weight loss for several months was admitted to a veterinary hospital in Korea. Upon neurological examination, the dog showed generalized intention tremor, but cranial and spinal nerve reflexes were unremarkable. All superficial lymph nodes (LN) were enlarged on physical examination. This LN enlargement developed a year prior to admission, and they recently increased in size, according to the owner. The dog was alert and vital signs were stable. An erythrogram revealed mild anemia (HCT 30.1%; reference range 37.0–55.0%) and thrombocytopenia (PLT 85 × 10³/µL; reference range 175–500 × 10³/µL). The dog’s blood biochemistry profiles were within normal limits. Ultrasonography and computed tomography revealed splenomegaly and systemic LN enlargement, and mild high signals of cerebral meninges, not distinguishable from normal variation, were noted on magnetic resonance imaging (MRI). The results of cerebrospinal fluid examination were unremarkable. The dog was hospitalized at the intensive care unit and treated with ciprofloxacin, doxycycline and rifampin for mycobacteriosis, and prednisolone, methocarbamol and zonisamide for aseptic meningitis which was diagnosed tentatively, since the next day of presentation. The intensity of tremor was gradually decreased. Despite of treatments, its overall condition was rapidly deteriorated, and the dog was euthanized 4 days after presentation.

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On cytological examination of biopsy specimens from the submandibular and popliteal lymph nodes, the presence of acid-fast bacilli was revealed by Ziehl–Neelsen staining, indicating Mycobacteria. Since acid-fast bacilli were found, an M. tuberculosis complex (MTC) specific PCR was performed with the tissue sample using Anyplex MTB/NTM Real-time Detection (Seegene, Seoul, Korea) according to the manufacturer’s instruction, and resulted in positive for MTB.

At necropsy, scattered tan granulomas were found in lungs, mostly dorsal lung fields. Disseminated lymph node (LN) enlargements were noticed. Representative popliteal LN was shown in Fig. 1, and its cut surface revealed thick yellow caseous material. Tissue samples were fixed in 10% neutral buffered formalin, sections were stained with hematoxylin and eosin (H&E) and Ziehl–Neelsen stain, and then subjected to a microscopic examination. Histopathology of the popliteal LN sections revealed diffuse granulomatous lymphadenitis with extensive infiltration of macrophages (Fig. 2A), and the numbers of acid-fast bacilli were dispersed (Fig. 2B). The enlarged spleen was found to have similar histopathological finding with the LN, and the brain lesions that showed a mild high signal on MRI and responded to corticosteroid treatment were revealed to have inflammatory cell infiltration around meninges (data-not-shown). Bacterial culture was performed using a Mycobacteria Growth Indicator Tube (BD MGIT™, BD, Berkshire, UK) according to the manufacturer’s instruction. Briefly, the lymph node sample was digested and decontaminated with N-acetyl-L-cysteine sodium hydroxide (NALC-NaOH) using the BBL® MycoPrep™ Specimen Digestion/Decontamination Kit (Becton Dickinson, Sparks, MD, USA), following the manufacturer’s protocol. Bacteria samples were concentrated by centrifugation for 20 min at 3,000 × g and sediments were resuspended in 2 mL phosphate buffer before further testing. NALC-NaOH sediments were inoculated into one tube of liquid medium of the BD BACTEC™ MGIT™ 960 System for Mycobacteria Culture (Becton Dickinson), and incubated for up to 8 weeks at 37°C or until the growth was detected. For molecular confirmation and identification of the bacterial growth in the MGIT
culture medium, bacterial cells were collected by centrifugation at 10,000 × g, resuspended in distilled water, and boiled for 10 min. Cell debris was sedimented by centrifugation for 2 min at 10,000 × g, and the resultant DNA concentration was measured using a spectrophotometer (260 nm, Amersham Pharmacia Biotech Inc., Piscataway, NJ, USA). DNA was confirmed as MTC using Anyplex MTB/NTM Real-time Detection (Seegene, Seoul, Korea) according to the manufacturer’s instruction. After identification of the MTC, the species level was determined using the RD1 (ESAT-6) and RD8 sequences as described elsewhere [15].

Subsequently, *M. bovis* was isolated and identified (Fig. 3). Then, the further molecular type was determined using spoligotyping and the mycobacterial interspersed repetitive unit-variable number tandem repeat (MIRU-VNTR) methods [5, 12, 14]. Spoligotyping used for genotyping of our isolates is a PCR-based method allowing us to analyze strain-dependent polymorphisms observed in spacer sequences present within the direct repeat (DR) genomic region of MTC bacteria [5, 14]. It allowed to classify *M. bovis* into spoligotype families and subfamilies. The spoligotyping patterns obtained were compared by visual examination and by analyzing the results in BioNumerics software, version 3.5 (Applied Maths, Kortrijk, Belgium). All the obtained spoligotype signatures were compared with those available in SpolDB4.0, the international spoligotyping database [5]. Additionally, spoligotype signatures were entered into the *M. bovis* spoligotype database (http://www.mbovis.org/spoligodatabase/singlepattern.php). The spoligotype was determined to be SB0140, and the MIRU-VNTR allelic profile was 4-2-5-3-2-7-5-4-3-4-3-4-3. This spoligotype and MIRU-VNTR type has been identified in farmed cattle in Korea [14].

Since tuberculosis in veterinary clinical settings is not basically recommended for treatment, antibiotic resistance tests were conducted on the isolated strain out of curiosity. The isolates were subjected to investigation of drug-resistance via the Anyplex II MTB/MDR/XDR detection assay (Seegene) according to the manufacturer’s instruction [6]. The results suggested that *M. bovis* isolated in this case was associated with MDR-bearing mutations in *katG* (isoniazid) and *rpoB* (rifampicin) genes, conferring resistance toward first-line antibiotics for human use. As recommended by the United States Centers for Disease Control and Prevention, treatment of disease due to *M. bovis* usually consists of rifampicin, isoniazid and ethambutol [1]. Therefore, it is considered to be of great importance from the point of view of public health between companion animals and humans.

*M. bovis* strains isolated from bovines often carries resistance-related mutations for first- and second-line antibiotics. Resistance to isoniazid, fluoroquinolones and aminoglycosides are particularly worrisome considering the risk for emergence of MDR and XDR TB [2]. There is a significant underreporting of drug-related mutations among livestock and wildlife animals [29]. Indeed, several reports of *M. bovis* drug-resistant isolates recovered from humans, ranging from mono-, poly-resistant and MDR detected strains have been reported [2, 32]. *M. bovis* primarily affects cattle but can also infect humans (zoonotic transmission) and in some cases it might result in MDR and XDR TB [2, 32].

*M. bovis* is one of the nine member species of the MTC that are capable of causing TB across a broad range of social mammals, including but not limited to humans, lions, elephants and meerkats [4, 7, 19, 20]. Within this group of pathogens, *M. bovis* stands out as the least host restricted and thus has significant zoonotic potential [21, 25]. Subclinical and clinical canine and feline infections with *M. bovis* can commonly occur, especially in countries with a non-BCG-vaccination policy. Although dogs and cats do not function as *M. bovis* reservoirs, they may transiently shed infective organisms into the environment. For this reason, infected dogs and cats are suspected to be potential sources of *M. bovis* infections for susceptible cattle and other animals, and for people [24].

In dogs, the clinical signs of TB are nonspecific, including weakness, weight loss, opportunistic infection, fever, coughing, and/or neurological signs, such as those observed in our patient dog, depending on the organs affected and the severity of the infection [11]. However, TB is not generally included as a cause of a massively enlarged spleen or aseptic meningitis, rather could be involved in an autoimmune condition where the body’s immune system attacks blood vessels in the lining of the nervous system for an unknown reason. Defining the extent and progression of the disease is difficult, resulting in delayed diagnosis and treatment, which usually takes several months. Therefore, treatment of TB in dogs is generally not recommended. Moreover, the human TB treatment regimen is not directly adaptable to dogs because isoniazid causes neurological side effects, rifampicin is hepatotoxic, and streptomycin is reserved for human use only. In addition, *M. bovis* is resistant to pyrazinamide [16, 31].

![Fig. 3.](image)

Fig. 3. Multiplex PCR for *Mycobacterium tuberculosis*, *M. bovis* and the *M. bovis* Bacillus Calmette-Guérin (BCG) strain. Lanes M: 100 bp DNA marker; 1: negative control; 2: *M. bovis* BCG; 3: *M. bovis* AN5; 4: *M. tuberculosis* H37Rv; 5: sample.
In the present study, one canine TB case caused by *M. bovis* was described. Clinical, microbiological, pathological, and molecular techniques were devoted to uncover the exact etiology. The source of infection in this dog remains to be identified. Commonly known cattle or human infections was suspected, but owners of the dog were tested negative for TB. In this case, since the dog was living in the city where it would be difficult to come into contact with cattle, it is unlikely that the infection was due to direct contact with cattle or from contaminated livestock products. However, as the TB crossinfection between humans and cattle in Korea continues to be reported every year [13–15], it is estimated that there are various routes of infection for dogs that have not been identified.

The close contact between dogs and humans creates scenarios of interspecies transmission in which humans could act as a source of infection for dogs both for *M. bovis* and *M. tuberculosis*. Several case reports have suggested that *M. bovis* infections are generally associated with close contact to TB-infected herds or consumption of contaminated meat products [8], and *M. tuberculosis* from owner to dog [9]. The transmission of *M. tuberculosis* from experimentally infected dogs to uninfected animals kept in close contact has been demonstrated [3, 28]. Moreover, *M. tuberculosis* was isolated from body fluids of a dog with a generalized infection [27]. These findings suggest that dogs can shed mycobacteria in the environment and potentially act as a source of infection for other animals and humans. On that account, *M. bovis* or *M. tuberculosis* infections should be considered in dogs showing respiratory symptoms and living in areas in which mycobacterial infections are widespread in wildlife, farm animals or humans [24, 30]. Veterinary practitioners cannot be expected to know how to diagnose and/or rule out TB among compromising animals. In countries running universal BCG vaccination programs, humans defend against tuberculosis, but companion animals can get infected [18]. Although the dog’s owner couple tested negative in this case, it cannot be ruled out that they were protected by the BCG vaccine received in childhood. The emergence of MDR *M. bovis* is an important public health problem that jeopardizes the success of TB control programs in the region. To the best of our knowledge, this is the first report describing an MDR *M. bovis* infection of dogs in Korea.

CONFLICT OF INTEREST. The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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