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ISOLATION AND ANTIMICROBIAL SUSCEPTIBILITIES OF NONTUBERCULOUS MYCOBACTERIA FROM WILDLIFE IN JAPAN

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ABSTRACT: Nontuberculous mycobacteria (NTM) are opportunistic pathogens of humans and animals and are transmitted among the environment, wildlife, livestock, and humans. The aim of this study was to investigate the rate of isolation and antimicrobial susceptibility of NTM in wildlife. In total, 178 samples of feces (n=131) and tissues (n=47) were collected from 11 wildlife species in Gifu Prefecture and Mie Prefecture, Japan, between June 2016 and October 2018. We isolated NTM from 15.3% (20/131) of fecal samples using Ogawa medium, and isolates were identified by sequencing the *rpoB* and *hsp*65 genes. The *rpoB* sequences were compared with those from other strains of human and environmental origin. The NTM isolates were obtained from sika deer (*Cervus nippon*), wild boar (*Sus scrofa*), Japanese monkey (*Macaca fuscata*), raccoon dog (*Nyctereutes procyonoides*), masked palm civet (*Paguma larvata*), and Japanese weasel (*Mustela itatsi*) and were classified as rapidly growing mycobacteria (RGM) and slowly growing mycobacteria (SGM). The 12 RGM identified were *Mycolicibacterium peregrinum* (n=5), *Mycolicibacterium fortuitum* (n=3), *Mycolicibacterium septicum* (n=3), and *Mycolicibacterium thermoresistibile* (n=1), and the eight SGM were *Mycobacterium paraense* (n=4), *Mycolicibacter arupensis* (n=2), *Mycolicibacter virginiensis* (n=1), and *Mycobacterium nebraskense* (n=1). The NTM from wildlife showed ≥99% similarity with strains from different sources including humans. The RGM were susceptible to the antimicrobial agents tested except for *M. fortuitum*, which was resistant to azithromycin and clarithromycin. The SGM showed multiple drug resistance qualities but were susceptible to amikacin, clarithromycin, and rifabutin. These results indicate that wildlife may be reservoir hosts of NTM in Japan. The presence of antimicrobial-resistant NTM in wildlife suggests that the trends of NTM antimicrobial susceptibility in wildlife should be monitored.

Key words: Antimicrobial resistance, fecal sample, nontuberculous mycobacteria, wildlife.

INTRODUCTION

Nontuberculous mycobacteria (NTM) are organisms residing in natural environments to which humans and animals are exposed (Falkinham 1996). Although they do not cause tuberculosis or leprosy, they are known to be opportunistic pathogens of humans and animals (Biet et al. 2005). Among NTM, *Mycobacterium avium–Mycobacterium intracellulare* complex (MAC) is the most important etiologic agent to humans and animals (Primm et al. 2004). In fact, NTM have been isolated from humans and livestock, but more attention has been given to MAC, since it is frequently associated with infections in humans (Nishiuchi et al. 2009). The importance of NTM, other than MAC, as an opportunistic infection in humans and animals has also been increasing (Donohue 2018). For example, there have been recent reports of the isolation of *Mycolicibacterium thermoresistibile* from feline panniculitis and isolation of *Mycolicibacterium peregrinum* and *Mycobacterium genavense* from migratory birds (Biet and Boschiroli 2014; Patiño et al. 2018).

Based on a recent comparative and phylogenomic study of the genus *Mycobacterium*, the division of the genus into five distinct monophyletic clades has been proposed. The

suggested modifications include an emended genus *Mycobacterium* (Tuberculosis-Simiae clade) and four other new genera, *Mycolicibacterium* gen. nov. (Fortuitum-Vaccae clade), *Mycolicibacter* gen. nov. (Terrae clade), *Mycolicibacillus* gen. nov. (Triviale clade), and *Mycobacteroides* gen. nov. (Abscessus-Chelonae clade; Gupta et al. 2018). This comparative analysis has improved our understanding of NTM and clearly places all the rapidly growing mycobacteria (RGM) and the slowly growing mycobacteria (SGM) in distinct clades.

To provide more information on the ecology and etiology of NTM, we previously presented the genome sequences of *Mycolicibacter virginiensis*, *Mycolicibacter senuensis*, and *Mycobacterium colombiense* isolated from the environment surrounding pigs in Japan (Ito et al. 2018a, b). In addition, we analyzed the genome sequences of *Mycolicibacterium peregrinum* isolated from the lymph nodes of a pig and from the surrounding soil by in silico DNA-DNA hybridization, and the results showed that the two isolates were 100% identical (Komatsu et al. 2019), implying circulation of NTM between the animal and the surrounding environment. Although NTM cases have been recorded in both humans and animals, the focus has been on humans more than on animals, especially wildlife.

Antimicrobial resistance in NTM from wildlife is critical, since there is the possibility of spreading to humans and livestock. However, the antimicrobial resistance pattern varies according to the species of NTM and often hinders the efficacy of therapeutic regimens (van Ingen et al. 2012). In Japan, antimicrobial resistance has been observed in clinical NTM isolates (Hatakeyama et al. 2017), but antimicrobial susceptibility testing of NTM from wildlife has not been reported.

Wildlife can acquire environmental mycobacteria through contaminated water and food (Kazda et al. 2009). The bacteria then inhabit favorable niches within the animals and can be transmitted through their feces to the environment (Pavlik et al. 2009). We investigated the rate of isolation and antimicrobial susceptibility of NTM from wildlife in two prefectures (Gifu and Mie) in Japan and examined the genetic relationship between NTM isolated from wildlife and other sources.

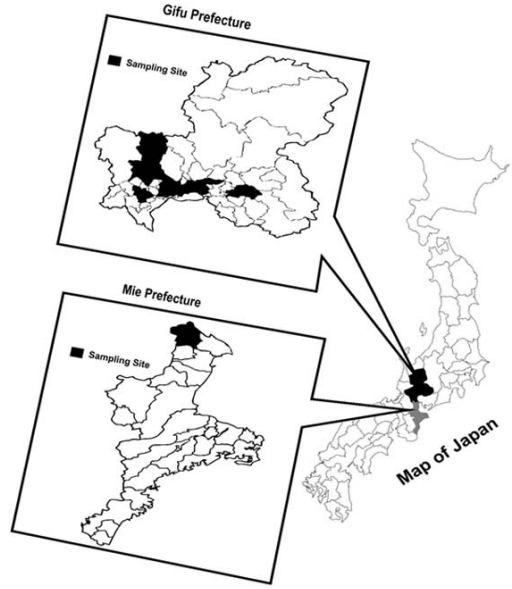

FIGURE 1. The samples were collected from wildlife in Gifu Prefecture and Mie Prefecture, located in central Japan. The two prefectures provide habitats for various wildlife species. The animals were hunted with traps by authorized hunters between June 2016 and October 2018. The study sites were selected according to the population density of wildlife, with a focus on mammals as potential reservoir hosts in proximity to the human environment. The dark shaded portion of the map represents the location of wildlife species from which samples were used.

MATERIALS AND METHODS

Study area and design

The samples were collected from wildlife (Table 1) in Gifu Prefecture and Mie Prefecture, located in central Japan (Fig. 1). The two prefectures provide habitats for various wildlife species. The study sites were selected according to the population density of wildlife, with a focus on mammals as potential reservoir hosts in proximity to the human environment. Some wildlife species were annually hunted as part of culling practices in the fields, forests, and rivers in the sampling areas (Table 2). Other animals (e.g., fox and some weasels) were found as roadkill or had died of natural causes. The animals were hunted with traps by authorized hunters between June 2016 and October 2018 in Gifu Prefecture and Mie Prefecture, Japan, with adherence to the Protection and Control of Wild

TABLE 1. Rate of isolation of nontuberculous mycobacteria (NTM) from wildlife species included in this study in Japan. The animals were hunted with traps by authorized hunters between June 2016 and October 2018 in Gifu Prefecture and Mie Prefecture, Japan. Fecal and tissue samples were collected at a game-handling establishment during gralloching (removal of the gut) and carcass inspection and subjected to NTM isolation using 2% Ogawa slant culture tube.

Order	Family	English name	Scientific name	No. samples		No. positive for NTM (%)	
				Feces	Tissue	Feces	Tissue
Primates	Cercopithecidae	Japanese monkey	*Macaca fuscata*	31	10	1 (3)	0
Rodentia	Myocastoridae	Nutria	*Myocastor coypus*	1	0	0	0
Carnivora	Canidae	Red fox	*Vulpes vulpes*	1	0	0	0
		Raccoon dog	*Nyctereutes viverrinus*	22	13	1 (5)	0
	Procyonidae	Raccoon	*Procyon lotor*	6	10	0	0
	Mustelidae	Japanese weasel	*Mustela itatsi*	2	0	1 (50)	0
		Siberian weasel	*Mustela sibirica*	4	0	0	0
	Viverridae	Masked palm civet	*Paguma larvata*	9	6	1 (11)	0
Artiodactyla	Cervidae	Sika deer	*Cervus nippon*	41	8	13 (32)	0
	Suidae	Wild boar	*Sus scrofa*	12	0	3 (25)	0
Anseriformes	Anatidae	Wild duck (mallard)	*Anas platyrhynchos*	2	0	0	0
Total				131	47	20 (15.3)	0

Birds and Mammals and Hunting Management Law (law no. 88, 2002). Samples used in this study were collected at a game-handling establishment during gralloching (removal of the gut) and carcass inspection. Because animals were not killed specifically for this study, no ethical approval was required from the Gifu University ethical committee. However, the Protection and Control of Wild Birds and Mammals Hunting Management Law (law no. 88, 2002) was followed during sample collection. The sample size was dependent on availability and the hunting period.

Sample collection

In total, 178 samples were investigated, which included 131 fecal samples from the large intestine and 47 tissue samples of the lymph node (n=11), lung (n=9), tonsil (n=9), liver (n=9), spleen (n=6), and kidney (n=3). The samples were collected from Japanese monkey (*Macaca fuscata*), nutria (*Myocastor coypus*), red fox (*Vulpes vulpes*), raccoon dog (*Nyctereutes viverrinus*), raccoon (*Procyon lotor*), Japanese weasel (*Mustela itatsi*), Siberian weasel (*Mustela sibirica*), masked palm civet (*Paguma larvata*), sika deer (*Cervus nippon*), wild boar (*Sus scrofa*), and mallard (*Anas platyrhynchos*) as shown in Table 1. Classification and scientific names of the wildlife species were attributed according to previous literature (Ohdachi et al. 2015).

Isolation of bacteria

Sample collection was conducted by veterinary wildlife specialists to avoid contamination of samples during transportation and at the laboratory (Pate et al. 2016). The fecal and tissue samples were immediately processed using 2% sodium hydroxide (Hibiya et al. 2010; Ito et al. 2018b). The inocula obtained from both tissues and feces were inoculated onto a 2% Ogawa slant culture tube (Kyokuto Pharmacy, Tokyo, Japan) and into BBL™ (Difco Laboratories, Sparks, Maryland, USA) mycobacterium growth indicator tube liquid media supplemented with polymyxin B, amphotericin B, nalidixic acid, trimethoprim, and azlocillin (Becton Dickinson, Franklin Lakes, New Jersey, USA) and incubated at 37 C for 4 wk. Each single colony formed on the slant tube was subcultured on Middlebrook 7H11 agar (Difco) to obtain distinct colonies (Ito et al. 2018b).

DNA extraction, sequencing, and phylogenetic analysis

We extracted DNA using the boiling method (Iwamoto et al. 2012). The first round of PCR was performed to determine the samples that were positive for mycobacteria (Chen et al. 1996). To identify the isolated mycobacteria, sequencing of the *rpoB* and *hsp*65 genes (Telenti et al. 1993; Adekambi et al. 2003) was performed. Because of its high discrimination ability, the partial sequence of the *rpoB* gene was used to compare

TABLE 2. Species of nontuberculous mycobacteria (NTM) identified from wildlife in Japan. Bacteria identification was achieved by partial sequencing of *rpoB* and *hsp*65 gene (Telenti et al. 1993; Adekambi et al. 2003). Except when otherwise stated, all of the isolates were made from samples from animals from Gifu Prefecture, Japan.

Wildlife species	Identified NTM	Isolate ID	City	GenBank accession no.	
				rpoB	*hsp*65
Sika deer	*Mycolicibacterium peregrinum*	wg41	Ibigawa	MK341497	MK341516
	Mycolicibacterium septicum	wg56	Ibigawa	MK341503	MK341522
		wg60	Ogaki	MK341504	MK341523
	Mycolicibacterium fortuitum	wg7	Ibigawa	MK341491	MK341511
		wg21	Ibigawa	MK341494	MK341514
		wg86	Ibigawa	MK341508	MK341527
	Mycolicibacterium thermoresistibile	wg48(i)	Motosu	MK341499	MK341518
	Mycolicibacter arupensis	wg48(ii)	Motosu	MK341500	MK341519
		wg68	Ibigawa	MK341506	MK341525
	Mycobacterium nebraskense	wg8	Ibigawa	MK341492	MK341512
	Mycobacterium paraense	wg4	Ibigawa	MK341490	MK341510
		wg42	Ibigawa	MK341498	MK341517
		wg54	Ibigawa	MK341502	MK341521
Wild boar	*Mycolicibacterium peregrinum*	wg18	Ibigawa	MK341493	MK341513
	Mycobacterium paraense	wg29	Motosu	MK341495	MK341515
	Mycolicibacter virginiensis	wg30	Motosu	MK341496	MK587449
Japanese monkey	*Mycolicibacterium peregrinum*	wg51	Ibigawa	MK341501	MK341520
Raccoon dog	*Mycolicibacterium peregrinum*	wg90	Ibigawa	MK341509	MK341528
Masked palm civet	*Mycolicibacterium septicum*	wg72[a]	Miegun	MK341507	MK341526
Japanese weasel	*Mycolicibacterium peregrinum*	wg62	Ibigawa	MK341505	MK341524

[a] Isolate was obtained from an animal from Mie Prefecture.

the isolates obtained from wildlife (Table 2) with previously characterized NTM sequences downloaded from GenBank; isolates with ≥99% identities were considered closely related (de Zwaan et al. 2014). The obtained sequences were analyzed using nucleotide BLAST (National Center for Biotechnology Information 2018), and the sequences with a similarity percentage above 98% were downloaded for phylogenetic analysis. The phylogenetic analysis was conducted according to the neighbour-joining method (Saitou and Nei 1987) under the total gap removal and Kimura's two-parameter substitution model (Kimura 1980), and it was evaluated by bootstrap analysis based on 1,000 replicates using MEGA software version 7 (Kumar et al. 2016). The tree was rooted using *Rhodococcus equi* (GenBank no. AY492285) as the outgroup.

Antimicrobial susceptibility testing of RGM

We used broth microdilution plates containing antimicrobial agents and cation-supplemented Müeller-Hinton broth (Eiken Chemical Co., Ltd., Tokyo, Japan). The final concentrations of the antimicrobial agents evaluated for RGM isolates were: clarithromycin (CAM) 0.0015 to 64 μg/mL, azithromycin (AZM) 0.0015 to 64 μg/mL, sitafloxacin 0.0015 to 64 μg/mL, moxifloxacin 0.0015 to 64 μg/mL, ciprofloxacin 0.0015 to 64 μg/m, imipenem 1 to 64 μg/mL, meropenem 1 to 64 μg/mL, amikacin (AMK) 2 to 64 μg/mL, and linezolid 2 to 64 μg/mL. An inoculum with a final concentration ranging from 1×10^5 to 5×10^5 colony-forming units/mL was prepared according to the Clinical and Laboratory Standards Institute (CLSI) guidelines (2011). The plates were inoculated and incubated at 37 C. The minimum inhibitory concentration (MIC) was determined on day 3, 4, or 5 (Hatakeyama et al. 2017). However, to detect inducible resistance in the case of CAM, the incubation period was extended, and the final MIC was determined on day 14 (Brown-Elliott et al. 2012). The MIC breakpoints were interpreted according to CLSI criteria (CLSI 2011) with the exception of sitafloxacin, which was interpreted using the breakpoint proposed by O'Grady et al. (2001).

Antimicrobial susceptibility testing of SGM

The BrothMIC NTM system (Kyokuto Pharmaceutical Industrial Co. Ltd., Tokyo, Japan) was used to evaluate the susceptibility of SGM to streptomycin 0.06 to 128 μg/mL, ethambutol (EB) 0.06 to 128 μg/mL, kanamycin 0.06 to 128 μg/mL, rifampicin 0.06 to 32 μg/mL, rifabutin (RBT) 0.06 to 16 μg/mL, levofloxacin 0.06 to 32 μg/mL, CAM 0.06 to 32 μg/mL, ethionamide 0.06 to 16 μg/mL, and AMK 0.5 to 16 μg/mL, based on the manufacturer's guidelines, which were in accordance with the standard protocol of CLSI. The suspension for inoculation was prepared as described elsewhere (Pang et al. 2017). The plates were incubated at 37 C for 7 d, and the growth end point was determined visually. The MIC breakpoints were interpreted following the guidelines of CLSI (2011) and of the World Health Organization (2008).

RESULTS

Rate of isolation of NTM in wildlife species

Twenty NTM were isolated from fecal samples of six wildlife species, sika deer (32%, 13/41), wild boar (25%, 3/12), Japanese monkey (3%, 1/31), raccoon dog (5%, 1/22), masked palm civet (11%, 1/9), and Japanese weasel (50%, 1/2), resulting in an estimated isolation rate of 15.3% (Table 1). The isolated NTM included four RGM, *Mycolicibacterium peregrinum* (*n*=5), *Mycolicibacterium fortuitum* (*n*=3), *Mycolicibacterium septicum* (*n*=3), and *Mycolicibacterium thermoresistibile* (*n*=1), and four SGM, *Mycobacterium paraense* (*n*=4), *Mycolicibacter arupensis* (*n*=2), *Mycolicibacter virginiensis* (*n*=1), and *Mycobacterium nebraskense* (*n*=1), as shown in Figures 2 and 3.

The phylogenetic tree of the NTM identified was based on the 430-base pair *hsp*65 gene (Fig. 3). The *hsp*65 gene could not clearly differentiate between some members of the *Mycolicibacter terrae* clade. The sequences of both *rpoB* and *hsp*65 were deposited in GenBank (accession numbers shown in Table 2).

Comparison of NTM isolated from wildlife with NTM from other sources—*Fortuitum-Vaccae* clade

The NTM from wildlife were compared with those from other sources to investigate their genetic relatedness. We found similarity among all the NTM from different sources (Fig. 4). The isolates of *Mycolicibacterium peregrinum* were 100% identical to each other and were also 100% identical to the reference strain *Mycolicibacterium peregrinum* strain Y7 (KU861853) isolated from water in Iran. Similarly, the isolates from wildlife identified in this study as *Mycolicibacterium fortuitum* were 100% identical to each other and to the *Mycolicibacterium fortuitum* strain garg (KC242461) isolated from water in India. The *Mycolicibacterium septicum* isolated from wildlife and the reference strain *Mycolicibacterium septicum* strain 14MF (KY400670) isolated from soil in China were 99% similar. The *Mycolicibacterium thermoresistibile* isolated from wildlife was 100% identical to isolates from humans (France) and soil (UK).

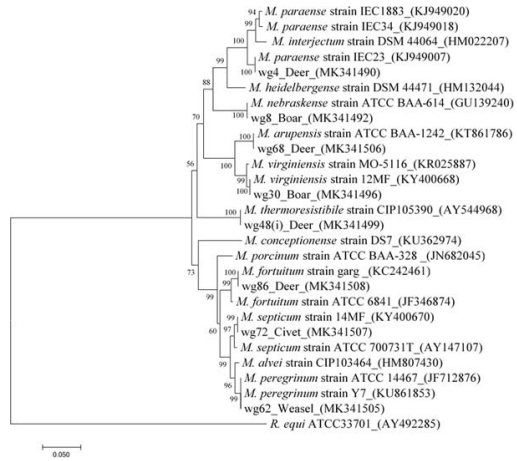

FIGURE 2. Neighbor-joining phylogenetic tree of nontuberculous mycobacteria (NTM) isolates based on the alignment of partial nucleotide sequence (720-base pair) of *rpoB* gene sequenced using primers described by Adekambi et al. (2003). The representative isolates of NTM identified in this study are preceded with the letters wg, and the source is indicated with the obtained accession numbers in parentheses. The reference NTM strains downloaded from the National Center for Biotechnology Information are in italics, with their respective accession numbers in parentheses. Branching with greater than 55% support from 1,000 bootstrap replicates is shown at the nodes. Horizontal distances indicate genetic distance, and the scale bar represents nucleotide substitution per site.

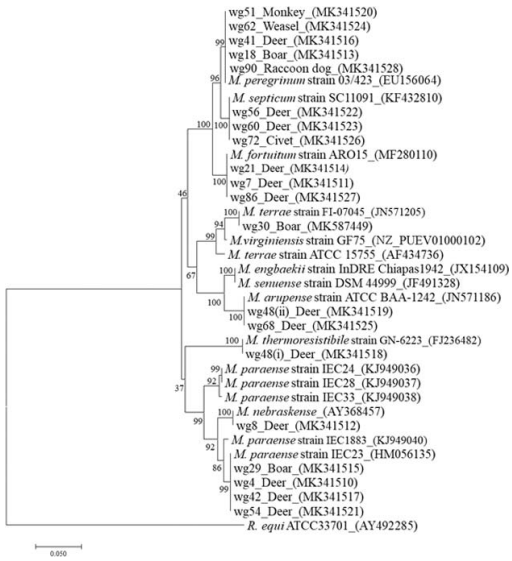

FIGURE 3. Neighbor-joining phylogenetic tree of nontuberculous mycobacteria (NTM) isolates based on the alignment of partial nucleotide sequence (430-base pair) of *hsp*65 gene sequenced using primers described by Telenti et al. (1993). The isolates of NTM identified in this study are preceded with the letters wg, and the source is indicated with the obtained accession numbers in parentheses. The reference NTM strains downloaded from the National Center for Biotechnology Information are in italics, with their respective accession numbers in parentheses. Horizontal distances indicate genetic distance, and the scale bar represents nucleotide substitution per site.

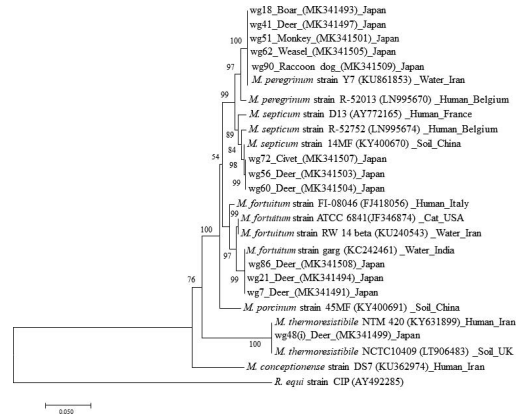

FIGURE 4. Comparison of the nontuberculous mycobacteria (NTM) from the Fortuitum-Vaccae clade isolated from wildlife (this study) and other sources, including human, soil, and water based on nucleotide sequence (720-base pair) of *rpoB* gene (Adekambi et al. 2003). The isolates of NTM identified in this study are preceded with the letters wg, and the source is indicated with the obtained accession numbers in parentheses. The reference NTM strains downloaded from the National Center for Biotechnology Information are in italics, with their respective source and accession numbers in parentheses.

Comparison of NTM isolated from wildlife and NTM from other sources—*Terrae* and *Tuberculosis-Simiae* clades

Mycobacterium paraense and *Mycobacterium nebraskense* are members of the Tuberculosis-Simiae clade, while the Terrae clade includes the *Mycolicibacter arupensis* and *Mycolicibacter virginiensis* isolates identified in this study. Five isolates were members of the Tuberculosis-Simiae, and three were members of the Terrae clade. The two clades were analyzed together because of the relatively small number of each. *Mycobacterium paraense* isolated in this study was 100% identical to *Mycobacterium paraense* strain IEC23 isolated from humans in Brazil. *Mycobacterium nebraskense* was 100% identical to *Mycobacterium nebraskense* isolated from humans in the US. *Mycolicibacter arupensis* and *Mycolicibacter virginiensis* were also 100% identical to the *Mycolicibacter arupensis* strain FI-06239 isolated from a human in Italy and the *Mycolicibacter virginiensis* strain 12MF isolated from soil in China (Fig. 5).

Antimicrobial susceptibility testing of RGM and SGM

Most of the RGM isolated from wildlife were susceptible to all nine antimicrobial agents used in this study (Table 3); the exception was *Mycolicibacterium fortuitum*, which showed resistance to CAM and AZM. Table 4 shows the MIC and the resistance pattern of the SGM to the antimicrobial agents tested. All the SGM investigated were resistant to more than two antimicrobial agents but susceptible to CAM, RBT, and AMK.

DISCUSSION

In this study, we successfully isolated 20 NTM from the large intestine of six wildlife

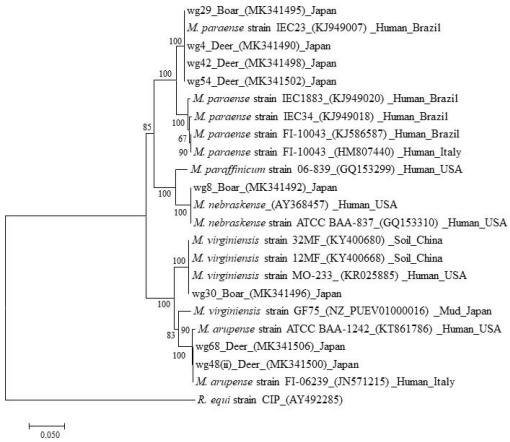

FIGURE 5. Comparison of the nontuberculous mycobacteria (NTM) belonging to Terrae and Tuberculosis-Simiae clades isolated from wildlife (this study) and other sources, including human, soil, and water, based on the nucleotide sequence (720-base pair) of the *rpoB* gene (Adekambi et al. 2003). The isolates of NTM identified in this study are preceded with the letters wg, and the source is indicated with the obtained accession numbers in parentheses. The reference NTM strains downloaded from the National Center for Biotechnology Information are in italics, with their respective source and accession numbers in parentheses.

species. The isolated NTM included four RGM (*Mycolicibacterium peregrinum*, *Mycolicibacterium fortuitum*, *Mycolicibacterium septicum*, and *Mycolicibacterium thermoresistibile*) and four SGM (*Mycobacterium paraense*, *Mycobacterium nebraskense*, *Mycolicibacter virginiensis*, and *Mycolicibacter arupensis*). The isolation rate of NTM in feces in this study was 15.3%, which is higher than the rates of 11.8% and 8.4% observed in Slovenia (Pate et al. 2016) and Hungary (Ronai et al. 2016), respectively. The occurrence of NTM in wildlife may be due to their extensive interaction with the environment, indicating that these animals may be reservoir hosts for NTM.

Mycolicibacterium peregrinum was the most frequently isolated RGM from the different wildlife species—sika deer, wild boar, Japanese monkey, Japanese weasel, masked palm civet, and raccoon dog—which strongly suggested that wildlife may acquire *Mycolicibacterium peregrinum* from their environment. Studies conducted in Japan revealed that the draft genome sequence of *Mycolicibacterium peregrinum* isolated from a pig affected with lymphadenitis shares close identity with that of *Mycolicibacterium peregrinum* isolated from the soil of the pig's environment (Komatsu et al. 2019). Moreover, the fact that *Mycolicibacterium peregrinum* was isolated from other animals (e.g., lion; *Panthera leo*), and from drinking water (Gcebe and Hlokwe 2017; Mohajeri et al. 2017) indicates that *Mycolicibacterium peregrinum* can inhabit several environments. In our study, the *rpoB* gene of the *Mycolicibacterium peregrinum* isolated from wildlife showed 100% similarity to that of *Mycolicibacterium peregrinum* isolated from drinking water (Mohajeri et al. 2017).

All the *Mycolicibacterium fortuitum* isolates in this study were from deer fecal samples, although *Mycolicibacterium fortuitum* was isolated from other animals, including monkey, in previous studies (Gcebe and Hlokwe 2017). The isolates of *Mycolicibacterium fortuitum* from wildlife in this study were identical to *Mycolicibacterium fortuitum* isolated from water (Fig. 4) and formed a cluster with other *Mycolicibacterium fortuitum* strains isolated from cats (Higgins et al. 2011) and hospital water (Khosravi et al. 2016), which suggests that, regardless of the location, NTM, in particular *Mycolicibacterium fortuitum*, were widespread in the environment, including among wildlife (Honda et al. 2018). *Mycolicibacterium septicum* and *Mycolicibacterium thermoresistibile* isolated from wildlife were 100% identical to other isolates from different sources, implying high relatedness among the isolates. In Japan, *Mycolicibacterium thermoresistibile* isolated from dust was not associated with infection (Tsukamura et al. 1985). However, both *Mycolicibacterium septicum* and *Mycolicibacterium thermoresistibile* have been reported in human and veterinary infection in other countries (Schinsky et al. 2000; Vishkautsan et al. 2016).

Among the wildlife fecal samples observed, diverse NTM were isolated from sika deer. Because NTM are known to inhabit the roots

TABLE 3. Results of the antimicrobial susceptibility test for rapidly growing mycobacteria (RGM). Minimum inhibitory concentration (MIC) range values were recorded along with the resistance percentage of the various RGM tested. Antimicrobial susceptibility testing of RGM was performed by broth microdilution in accordance with Clinical and Laboratory Standards Institute guidelines (CLSI 2011). Except where indicated, MIC values were interpreted based on breakpoints by CLSI (2011).

		MIC (μg/mL) range RGM isolates (no.)							
		Mycolicibacterium peregrinum (5)		*Mycolicibacterium fortuitum* (3)		*Mycolicibacterium septicum* (3)		*Mycolicibacterium thermoresistibile* (1)	
Antibiotic[a]	Reference MIC (μg/L)	MIC	% Resistant	MIC	% Resistant	MIC	% Resistant	MIC	% Resistant
CAM	≥8[b]	0.03–2	0	8	100	0.06–0.09	0	0.03	0
AZM	≥8[b]	0.06–4	0	8	100	0.16–0.38	0	0.02	0
STFX	≥30[c]	0.03–0.25	0	0.02–0.03	0	0.02–0.04	0	0.02	0
MFLX	≥4[b]	0.02–0.5	0	0.03–0.12	0	0.06–0.25	0	0.03	0
CPFX	≥4[b]	0.03–2	0	0.06–1	0	0.06–0.25	0	0.03	0
IPM	≥32[b]	1–4	0	≤1	0	≤1	0	≤1	0
MEPM	≥32[b]	1–2	0	≤2	0	≤2	0	≤2	0
AMK	≥64[b]	≤2	0	≤2	0	≤2	0	≤2	0
LZD	≥32[b]	2–4	0	2–4	0	≤2	0	≤2	0

[a] CAM = clarithromycin; AZM = azithromycin; STFX = sitafloxacin; MFLX = moxifloxacin; CPFX = ciprofloxacin; IPM = imipenem; MEPM = meropenem; AMK = amikacin; LZD = linezolid.

[b] Resistance breakpoint (μg/mL) based on CLSI guidelines (2011).

[c] Resistance breakpoint based on study by O'Grady et al. (2001).

and surface of leaves (Pavlik et al. 2009), it is possible that the high diversity of NTM detected in deer is related to the herbivorous feeding habits of the species. In addition, isolation of NTM, including *Mycolicibacterium fortuitum* and *Mycolicibacter arupensis*, from deer has been reported (de Lisle and Havill 1985; Mombeni et al. 2016). Three different NTM (*Mycolicibacterium peregrinum*, *Mycolicibacter virginiensis*, and *Mycobacterium paraense*) were isolated from wild boar samples because wild boar, as omnivores, can acquire NTM from infected carcasses they feed on as well as from the environment including plants (Pavlik et al. 2009). Moreover, *Mycobacterium paraense* was the most frequently isolated SGM and was isolated from sika deer, wild boar, and Japanese monkey. This finding is enlightening because most of the reported *Mycobacterium paraense* cases concern humans (Chin'ombe et al. 2016; Poonawala et al. 2017). *Mycobacterium paraense* was isolated from a human patient in Brazil and was described in 2015 as a novel species that is closely related to *Mycobacterium interjectum* (da Costa et al. 2015). Given the different sources of isolation in this study, it is possible that *Mycobacterium paraense* has a broad host range.

Mycolicibacter arupensis and *Mycolicibacter virginiensis* identified in this study were clustered together (Fig. 5), since they belong to the Terrae clade as described previously (Gupta et al. 2018). *Mycolicibacter arupensis* was isolated from sika deer fecal samples, while *Mycolicibacter virginiensis* was isolated from wild boar. The *Mycolicibacter virginiensis* from wildlife in our study was identical to the *Mycolicibacter virginiensis* of human and soil origin (Fig. 5). However, it was not identical to the *Mycolicibacter virginiensis* strain GF75 isolated in Japan (Ito et al. 2018a). The partial *rpoB* gene—with a region known to have high discrimination power (Higgins et al. 2011; de Zwaan et al. 2014)—was able to identify and highlight the diversity within NTM. The lack of MAC isolation could be due to its absence in the samples or to growth inhibition due to decontamination (Sattar et al. 2018). In this study, we could

TABLE 4. Results of antimicrobial susceptibility test for slowly growing mycobacteria (SGM). Minimum inhibitory concentration (MIC) range values were recorded along with the resistance percentage of the various SGM tested. Antimicrobial susceptibility testing of SGM was performed by broth microdilution in accordance with Clinical and Laboratory Standards Institute guidelines (CLSI 2011). Except where indicated, MIC values were interpreted based on breakpoints by CLSI (2011).

		MIC (μg/mL) range SGM isolates (no.)							
		Mycobacterium paraense (4)		*Mycolicibacter arupensis* (2)		*Mycolicibacter virginiensis* (1)		*Mycobacterium nebraskense* (1)	
Antibiotic[a]	Reference MIC (μg/L)	MIC	% Resistant	MIC	% Resistant	MIC	% Resistant	MIC	% Resistant
SM	≥5[b]	16–64	100	64–128	100	128	100	16	100
EB	≥4[b]	32–128	100	0.5–1	0	2	0	16	100
KM	≥4[c]	2–8	25	128	100	128	100	32	100
RFP	≥1[b]	0.25–2	25	16–32	100	8	100	1	100
RBT	≥2[b]	0.25–1	0	0.25–1	0	0.5	0	1	0
LVFX	≥8[b]	1–4	0	32	100	32	100	2	0
CAM	≥32[b]	0.25–1	0	1	0	1	0	0.25	0
TH	≥5[c]	8–16	100	16	100	8	100	16	100
AMK	≥64[b]	2–8	0	16	0	16	0	16	0

[a] SM = streptomycin; EB = ethambutol; KM = kanamycin; RFP = rifampicin; RBT = rifabutin; LVFX = levofloxacin; CAM = clarithromycin; TH = ethionamide; AMK = amikacin.

[b] Resistance breakpoint (μg/mL) based on CLSI guidelines (2011).

[c] Resistance breakpoint (μg/mL) based on World Health Organization guidelines (2008).

not isolate NTM from wildlife tissue samples (liver, lungs, lymph node, tonsils, kidney, and spleen), unlike in other studies (Katale et al. 2014; Ghielmetti et al. 2018). This may have been due to the absence of lesions when the tissue samples were macroscopically observed. Considering that identical NTM were isolated from different wildlife species, there is a possibility of wildlife transmitting NTM to the environment of domestic animals. Since wildlife, humans, and other domestic animals can interact, the use of whole-genome analysis will provide additional evidence and deepen our understanding of the genetic relatedness and possible transfer of NTM among humans, domestic animals, and wildlife.

All the 12 RGM tested were susceptible to the fluoroquinolones, aminoglycosides, and carbapenems used in this study, which is in line with other reports indicating that aminoglycosides, such as amikacin, were effective against RGM (Li et al. 2013; Pang et al. 2015). The observed resistance of *Mycolicibacterium fortuitum* to CAM and AZM may be due to the presence of erythromycin ribosomal methylase (*erm*; Brown-Elliott et al. 2012). The *Mycolicibacterium peregrinum, Mycolicibacterium septicum*, and *Mycolicibacterium thermoresistibile* isolates in this study were susceptible to all the antimicrobial agents tested in vitro (Table 3); however, in vitro antimicrobial susceptibility is not entirely conclusive.

All the *Mycobacterium paraense* isolates identified in this study were resistant to streptomycin, EB, and ethionamide (Table 4), similar to the resistance pattern observed in *Mycobacterium paraense* isolated from humans (da Costa et al. 2015). *Mycolicibacter arupensis* and *Mycolicibacter virginiensis* showed a similar resistance pattern and were susceptible to EB, CAM, RBT, and AMK. This finding is in agreement with the results of other studies in which EB, CAM, and RBT were found to be effective against the species of the Terrae clade (Vasireddy et al. 2017).

The study has some limitations. More fecal samples than tissue samples were investigated. However, this does not alter the conclusions of this study. In addition, we did not examine

the mechanism underlying the resistance of NTM to the individual antimicrobial agents tested. Furthermore, the partial sequencing of *rpoB* and *hsp*65 genes for identification and comparison with other NTM species was not sufficient to establish a strong relationship among NTM from different sources; whole-genome sequences are required to establish this relationship. Thus, this study can be considered a preliminary study.

In conclusion, the isolation of diverse NTM from wildlife fecal samples may not be directly related to high exposure. However, these findings provide insight into the occurrence of NTM in wildlife and indicate that wildlife may be reservoir hosts. Notably, the presence of antimicrobial-resistant NTM is important with respect to human and veterinary medicine, since interaction with wildlife is plausible. Furthermore, the isolation of antimicrobial-resistant NTM in wildlife indicates that the trends of NTM antimicrobial susceptibility in wildlife should be carefully considered and monitored for emergence of resistant strains.

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