

The water environment as a source of potentially pathogenic mycobacteria

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ABSTRACT

Nontuberculous mycobacteria (NTM) are ubiquitous organisms of a wide variety of environmental reservoirs, including natural and municipal water, soil, aerosols, protozoans, animals and humans. Several of these species are potential pathogens which affect human health. The aim of this study was to determine the occurrence of NTM in the water environment. Samples were taken from 13 water-related facilities including fish ponds, storage ponds, drinking water reservoirs and an experimental recirculation system. Altogether, 396 samples of water, sediment and aquatic plants were collected and analysed. All samples were examined using conventional culture methods. Suspected microbial isolates were subjected to polymerase chain reaction analysis and identified using partial sequence analysis of the *16S rDNA* gene. The culture revealed 94/396 samples (23.7%) that contained mycobacteria. Among known NTM we identified potentially pathogenic mycobacteria isolated from the fresh water environment for the first time: *Mycobacterium asiaticum*, *M. chimaera*, *M. interjectum*, *M. kumamotoense*, *M. lentiflavum*, *M. montefiorensis*, *M. nebraskense*, *M. paraffinicum* and *M. simiae*. Epidemiologic studies suggest that the natural water environment is the principal source of human exposure. Our results indicate that besides the well-known potentially pathogenic mycobacteria it is important to observe occurrence, proliferation and persistence of newly discovered mycobacterial species.

Key words | nontuberculous mycobacteria, opportunistic pathogens, water environment

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INTRODUCTION

Nontuberculous mycobacteria (NTM) are ubiquitous organisms in all natural ecosystems, including water, soil, food, dust and aerosols (Falkinham 1996; Covert *et al.* 1999; Dailloy *et al.* 1999). About one-third of NTM species are potentially pathogenic and have been associated with human diseases, e.g., *Mycobacterium avium*, *M. intracellulare*, *M. kansasii*, *M. simiae*, *M. interjectum*, *M. szulgai*, *M. fortuitum*, *M. chelonae*, *M. septicum* (Katoch 2004; Primm *et al.* 2004; Kazda *et al.* 2009).

Surface water plays an important role in the circulation of NTM in the environment (Kazda *et al.* 2009). NTM occur in different elements of the water environment – water, sediment and biofilm on different surfaces (Parsek & Fuqua 2004).

The most frequently isolated NTM from fish and aquatic environments are various members of the *M. avium*

complex (MAC), *M. abscessus*, *M. aurum*, *M. flavescens*, *M. fortuitum*, *M. gordonae*, *M. chelonae*, *M. kansasii*, *M. marinum* and *M. triplex* (Beran *et al.* 2006; Gauthier & Rhodes 2009; Mrlik *et al.* 2012).

In humans with chronic obstructive pulmonary disease or immunodeficiency due to HIV infection, cancer or chemotherapy or skin lesions exposed to water contaminated with NTM, acute disorders can develop (Marras & Daley 2002; Kim *et al.* 2005). The most common clinical manifestation of NTM disease is lung disease, but lymphatic, skin/soft tissue and disseminated disease are also observed (Katoch 2004). Because NTM are widespread in the environment and transmission of infection between people is not common, it is assumed that NTM infections are derived from water, food, the environment or contact with animals

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(Nichols *et al.* 2004). Infections with NTM are caused by ingestion or inhalation of contaminated food or aerosol, respectively, and through injured skin, which represents a significant entry gate of NTM to a host organism from environmental sources such as water (Falkinham 1996; Kazda *et al.* 2009).

Environmental studies are limited by their detection and identification methods. Moreover, the isolation of NTM from water may be affected by decontamination procedures, selection and enrichment of media, incubation temperatures, as well as the duration of incubation (Neumann *et al.* 1997). The use of direct polymerase chain reaction (PCR) is technically more demanding, although it may offer a more complete scope of the presence of NTM in environmental samples (Mendum *et al.* 2000). Historically, many studies have focused on specific species, generally *MAC*, applying detection and identification tools specifically developed for these species (Kirschner *et al.* 1992; von Reyn *et al.* 1993). However, the application of such tools can lead to the underestimation of NTM species diversity in environmental samples as it disregards unidentifiable and not yet described species (Kazda *et al.* 2009).

The aim of our study was to survey the occurrence and species diversity of mycobacteria in different fresh water environments: natural water sources (fish ponds, water reservoirs) and man-made sources (an experimental recirculation system and storage ponds) located in the Czech Republic.

METHODS

Collection of samples from the water environment

A total of 396 samples were collected during spring from four different types of water environment (Table 1), which included fish ponds (natural systems with standing water), drinking water reservoirs (created in river valleys by the construction of dams), storage ponds (man-made systems, designed for commercial fish breeding), and an experimental recirculation system (farming of fish under controlled conditions). The experimental recirculation system consists of eighteen 800 L tanks and twelve 200 L tanks that are linked in a temperature-controlled water recirculation

Table 1 | Characteristics of four water sources

Water sources	Temperature (°C)	pH	Depth (m)	Water area (km ²)
Fish ponds	17–20	7.5–8.5	0.5–3.0	0.04–0.3
Drinking water reservoirs ^a	19–22	6.0–7.0	47.5–65.6	2.1–16.0
Storage ponds	6–8	7.5–8.5	0.7–1.2	–
Experimental recirculation system ^a	20–23	6.0–8.0	0.5–0.8	–

^aData kindly provided by local authorities of water sources.

system. The day feeding ration is 1–2% of the weight of the fish stock. The daily water supply is approximately 1–2% of the total volume. Water quality is maintained by recirculation through a bacteriological filter at acceptable levels. The total volume of water is 36 m³ (Table 1). Water and aquatic plants were sampled from the surface and sediment near the fish pond side. Water from drinking water reservoirs was sampled from deep strata and sediment from the deepest point near the dam using a gravitational corer (Hruska 1986). Matrices (1 L of water or 50 g of sediment or aquatic plant) were collected in a sterile glass bottle or sterile plastic bag. Samples were transported to the laboratory at 4 °C and processed within 48 h of collection.

Sample processing and culture

Environmental samples (sediment and aquatic plant) were homogenised, and the water samples were centrifuged (3,500 g/20 min) and decontaminated using HCl-NaOH according to a procedure described previously (Fischer *et al.* 2000; Klanicova *et al.* 2013). Each decontaminated sample (0.1 mL) was inoculated onto Herrold egg yolk medium, Stonebrink, Löwenstein-Jensen and liquid medium according to Sula (1947) in duplicate and then incubated at 25 °C, 30 °C and 37 °C. Media were checked for bacterial growth after 7, 14, 30 and 60 days of incubation.

Identification of mycobacterial isolates

Colonies growing on primary culture media were preliminarily identified as mycobacteria based on the detection of acid-fast rods after Ziehl-Neelsen (ZN) staining and

determination of colony morphology. Suspected colonies were subcultured on the same culture medium and obtained subcultures were again checked using ZN. A single colony obtained from mycobacterial culture was scraped from the solid media and resuspended in 100 µL TE-buffer. The mycobacterial cells were lysed by incubation at 95 °C for 20 min and centrifuged at 18,000 × g for 5 min. The supernatant was used as the DNA template for a PCR reaction. All isolates were analysed with two independent PCR assays previously described by Moravkova et al. (2008). The first assay enables identification of the genus *Mycobacterium* and the second assay distinguishes between *M. avium* species. The identification of isolates not belonging to *M. avium* species was carried out using 16S rDNA PCR, followed by sequence analysis of a 921 bp long region according to a previously described method (Harmsen et al. 2003). Mycobacterial isolates were identified by their growth characteristics, morphology and sequence analysis data. Experimental sequences were aligned with the available database entries using the 'blastn algorithm' (EZ Taxon: <http://www.eztaxon-e.org/>). The cut-off value for the identity scores of the 16S rDNA gene was set at 99%. Another applied criterion was a difference of less than 3 bp from a reference species.

Statistical evaluation

Data analysis was performed using GraphPad Prism 5.04 (GraphPad, Inc., San Diego, CA, USA). Proportions of mycobacteria-positive samples in respective matrices and the water environment were statistically evaluated and compared using Fisher's exact test. *P*-values of post-hoc tests were adjusted according to the method of Benjamini–Hochberg (Benjamini & Hochberg 1995). *P*-values lower than 0.05 were considered statistically significant. In cases of significantly different proportions of positive samples, odds ratios (OR) were calculated (Mosteller 1968).

RESULTS AND DISCUSSION

From a total of 396 examined samples, 94 (23.7%) were positive for the presence of culturable *Mycobacterium* species (Table 2). Generally, the most frequently detected isolates

identified belonged to the *M. peregrinum/septicum* group (18.1%) which was found in all water environments except fish ponds. *M. gordonae* was the only single species present in all monitored water environments. Mycobacterial strains isolated from 18 samples (19.2%) were not identified to the species level owing to not meeting the criteria (low similarity in analysed 16S rDNA region). Some of the potentially pathogenic mycobacteria identified (*M. asiaticum*, *M. chimaera*, *M. interjectum*, *M. kumamotonense*, *M. lentiflavum*, *M. montefiorensis*, *M. nebraskense*, *M. paraffinicum* and *M. simiae*) were isolated from fresh water environments for the first time.

Total proportion of mycobacteria-positive samples related to matrix and water environment

The total proportion of mycobacteria-positive samples was significantly associated with the type of matrix. The highest proportion of mycobacteria-positive samples was found in sediment (35.1%), the lowest in water (11.1%). Statistically significant differences were observed between water and sediment samples (OR = 4.3), and aquatic plant and sediment samples (OR = 3.5, Table 2). The interpretation of the OR = 4.3 means that the odds that the sample of the sediment will be mycobacteria-positive are 4.3 times higher than the water sample. The total proportion of mycobacteria-positive samples was also significantly associated with the type of locality. The highest proportion of mycobacteria-positive samples was found in samples obtained from the experimental recirculation system (49.3%), the lowest in storage pond samples (8.2%). Statistically significant differences were registered between fish ponds and drinking water reservoirs (OR = 4.3), fish ponds and the experimental recirculation system (OR = 7.8), drinking water reservoirs and storage ponds (OR = 6.0) and storage ponds and the experimental recirculation system (OR = 6.0).

Mycobacteria isolated from fish ponds

Mycobacteria were isolated from more than 10% of samples obtained from fish ponds (Table 2). The highest proportion of mycobacteria-positive samples collected from fish ponds was found in water samples (15.6%) and the lowest in sediment samples (9.6%). Proportions of mycobacteria-positive

Table 2 | Numbers of mycobacterial isolates originating from the water environment

Mycobacterial species ^a	Fish ponds				Drinking water reservoirs				Storage ponds				Experimental recirculation system			Subtotals				Prop %
	W	AP	S	Tot	W	AP	S	Tot	W	AP	S	Tot	W	S	Tot	W	AP	S	Tot	
<i>M. arupense</i>	0	0	0	0	0	0	1	1	0	0	0	0	0	0	0	0	0	1	1	1.1
<i>M. asiaticum</i>	1	0	0	1	0	0	0	0	0	0	0	0	0	0	0	1	0	0	1	1.1
<i>M. avium</i> subsp. <i>avium</i>	0	0	0	0	0	1	3	4	0	0	0	0	0	2	2	0	1	5	6	6.4
<i>M. chelonae/abscessus</i> ^b	0	0	0	0	0	0	0	0	0	0	0	0	1	2	3	1	0	2	3	3.2
<i>M. chimaera</i>	1	0	0	1	0	0	0	0	0	0	0	0	0	0	0	1	0	0	1	1.1
<i>M. fortuitum</i>	0	1	1	2	0	0	2	2	0	0	0	0	0	1	1	0	1	4	5	5.3
<i>M. gordonae</i>	0	2	0	2	0	0	1	1	0	0	1	1	1	3	4	1	2	5	8	8.5
<i>M. hiberniae</i>	0	0	0	0	0	0	1	1	0	0	0	0	0	0	0	0	0	1	1	1.1
<i>M. interjectum</i>	0	0	0	0	0	0	0	0	0	0	0	0	0	1	1	0	0	1	1	1.1
<i>M. intracellulare</i>	0	0	0	0	0	0	0	0	1	1	0	2	0	0	0	1	1	0	2	2.1
<i>M. kansasii</i>	0	0	0	0	0	0	0	0	0	0	0	0	0	1	1	0	0	1	1	1.1
<i>M. kumamotonense</i>	1	0	0	1	0	0	0	0	0	0	0	0	0	0	0	1	0	0	1	1.1
<i>M. lentiflavum</i>	0	0	0	0	0	0	5	5	0	0	0	0	0	0	0	0	0	5	5	5.3
<i>M. montefiorensis</i>	1	1	1	3	0	0	2	2	0	0	0	0	0	0	0	1	1	3	5	5.3
<i>M. nebraskense</i>	1	0	1	2	0	0	0	0	0	1	0	1	0	0	0	1	1	1	3	3.2
<i>M. nonchromogenicum</i>	0	0	0	0	0	0	3	3	0	0	0	0	0	0	0	0	0	3	3	3.2
<i>M. paraffinicum</i>	0	0	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	1	1	1.1
<i>M. peregrinum/septicum</i> ^b	0	0	0	0	0	0	1	1	1	0	1	2	3	11	14	4	0	13	17	18.1
<i>M. simiae</i>	0	0	0	0	1	0	0	1	0	0	0	0	0	0	0	1	0	0	1	1.1
<i>M. szulgai/intermedium</i> ^b	0	0	0	0	0	0	0	0	0	0	0	0	2	2	4	2	0	2	4	4.3
<i>M. triplex</i>	0	0	1	1	0	0	5	5	0	0	0	0	0	0	0	0	0	6	6	6.4
<i>M. sp.</i> ^c	0	0	2	2	0	1	11	12	0	0	0	0	0	4	4	0	1	17	18	19.2
No. of samples	32	40	73	145	36	10	63	109	42	9	22	73	25	44	69	135	59	202	396	
No. of positive samples	5	4	7	16	1	2	35	38	2	2	2	6	7	27	34	15	8	71	94	
% of positive samples	15.6	10.0	9.6	11.0	2.8	20.0	55.6	34.9	4.8	22.2	9.1	8.2	28.0	61.4	49.3	11.1	13.6	35.1	23.7	

^aMycobacterial isolates were identified by their growth characteristics, morphology and sequence analysis data of 16S rDNA.

^bIdentical region of analysed 16S rDNA gene. Not distinguished in this study.

^cIsolates identified only to the species level. Sequencing data are below defined criteria.

W, water; AP, aquatic plant; S, sediment; Tot, total; Prop, proportion of *M. species* (100% ~ 94).

samples originating from fish ponds were not associated with the type of matrix ($P > 0.05$).

The majority of mycobacterial species derived from the water environment of fish ponds were first isolated and described as the aetiologic agents of human diseases. The exceptions are *M. montefiorensis* described in granulomatous skin infection in moray eels (Levi et al. 2003) and *M. paraffinicum* first isolated from soil (Davis et al. 1956) and described in patients with pulmonary symptoms (Wang et al. 2009). All mycobacteria isolated from fish ponds except *M. fortuitum* and *M. gordonae* have not been connected with fresh water until now. From the fish pond environment three mycobacteria, which were first isolated from the respiratory tract and are known as agents of pulmonary diseases in humans, were isolated: *M. chimaera* (Tortoli et al. 2004; Schweickert et al. 2008), *M. nebraskense* (Mohamed et al. 2004; Iwen et al. 2006) and *M. asiaticum* (Grech et al. 2010). The other isolated NTM species – *M. triplex* and *M. kumamotonense* – belong to the potentially pathogenic organisms and were previously isolated from, among others, the lymph nodes of human patients with weakened immune systems (Hoff et al. 2001; Zeller et al. 2003; Masaki et al. 2006; Grech et al. 2010). The first occurrence of both these mycobacterial species in freshwater fish was described by Mrlik et al. (2012). Further studies of the epizootiology and pathogenesis of the mentioned mycobacterial species in fish, aquatic mammals and humans will be critical in the elucidation of the impact of newly detected potential pathogens on ecosystems, as well as human health. Fish ponds were also the source of other water- or soil-related mycobacteria – *M. gordonae*, *M. fortuitum* (Goslee & Wolinsky 1976; Wolinsky 1979) and the above-mentioned *M. paraffinicum*. *M. gordonae* is generally viewed as a contaminant when isolated from clinical specimens (Arnou et al. 2000). *M. fortuitum* can cause chronic infections of the skin and soft tissues, often associated with trauma, injection or surgery (Brown-Elliott & Wallace 2002; Uslan et al. 2006).

Mycobacteria in drinking water reservoirs

The occurrence of mycobacteria in drinking water reservoirs was high; more than one-third of samples collected from drinking water reservoirs were positive for NTM (Table 2). The proportions of mycobacteria-positive samples were

significantly associated with the type of matrix; statistically significant differences were observed between samples of water and sediment (OR = 43.8) only.

Besides the isolation of *M. fortuitum* ($n = 2$), *M. gordonae* ($n = 1$), *M. montefiorensis* ($n = 2$), *M. triplex* ($n = 5$) and non-specified mycobacteria ($n = 12$), two mycobacterial species originating from the environment were obtained: *M. nonchromogenicum*, first found in soil (Tsukamura 1965) and *M. hiberniae*, isolated from sphagnum vegetation, true moss and soil (Kazda et al. 1993; Cooney et al. 1997). *M. nonchromogenicum* can cause infection in humans (Sawai et al. 2006). In the case of *M. hiberniae*, there have been no further references regarding environmental or clinical occurrence until now. Both mycobacterial species were isolated from the water environment of drinking water reservoirs only. In the water environment, *M. avium* subsp. *avium* (MAA) and *M. simiae* – a mycobacterium originally isolated from animals (Chester 1901; Thorel et al. 1990; Karassova et al. 1965) – were detected.

Unlike other members of the MAC, MAA is not generally ubiquitous in the environment (Thorel et al. 1997). It has been recovered from different water sources (surface water, drinking water distribution system or hospital hot water system). In many cases a link between MAA presence in these systems and human infections has been proven (von Reyn et al. 1993; Falkinham et al. 2001; Falkinham 2010). MAA is known as a zoonotic pathogen that causes disseminated infections, lung diseases or osteomyelitis in immunocompromised or HIV/AIDS patients (Primm et al. 2004; Griffith et al. 2007). MAA is the causative agent of avian tuberculosis – a chronic wasting disease affecting many species of birds, and domestic and wild animals (Thegerstrom et al. 2005). The reasons why other members of MAC were not identified could be caused by the selected method of decontamination, the low amount of the MAC members in the sample, which was below the level of culture detection, or unculturable forms of the other MAC members that might be present in the water environment. *M. simiae* causes localised or disseminated opportunistic infections in humans (Narang et al. 2010; Baghaei et al. 2012). It has also been detected in water from a hospital and patients' home showers (Conger et al. 2004), but no information about its occurrence in surface water has yet been described. Furthermore, mycobacteria like *M. lentiflavum* (Springer et al. 1996; Torvinen et al. 2004; Lee et al. 2008), *M. arupense* and

M. peregrinum/septicum (Brown-Elliott & Wallace 2002; Cloud et al. 2006; van Ingen et al. 2010) isolated in this study and associated with human diseases can occur in water distribution systems, which suggests that potable water can be a possible source of infection in humans. *M. arupense* and *M. peregrinum/septicum* were also previously associated with natural water and the environment (Lee et al. 2008; Slany et al. 2010) while *M. lentiflavum* was not described in the natural water environment until now.

Mycobacteria in storage ponds

Mycobacteria were detected in less than 10% of samples taken from storage ponds, which is the lowest prevalence in the monitored water systems (Table 2). The highest incidence of mycobacteria was identified in samples from aquatic plants (22.2%) and the lowest in water samples (4.8%). Proportions of mycobacteria-positive samples originating from storage ponds were not associated with the type of matrix ($P > 0.05$). Besides mycobacteria detected in other water environments, *M. nebraskense* ($n = 1$), *M. gordonae* ($n = 1$) and *M. peregrinum/septicum* ($n = 2$), two isolates of *M. intracellulare* were found in storage ponds exclusively. *M. intracellulare* has been found in soil and water several times, including drinking water distribution systems (Brooks et al. 1984; Falkinham et al. 2001). In connection with human health, there are multiple reports regarding the isolation of *M. intracellulare* from patients with pulmonary infections with or without predisposing conditions such as acquired immunodeficiency (Hawkins et al. 1986; Prince et al. 1989; Schweickert et al. 2008).

Mycobacteria in experimental recirculation system

We observed a very high positivity for mycobacteria in samples originating from the experimental recirculation system: almost half of the samples were positive (Table 2). The proportion of positive samples of water (28.0%) and sediment (61.4%) showed a statistically significant difference (OR = 4.1). The most frequently isolated mycobacterium in samples was *M. peregrinum/septicum* ($n = 14$). Furthermore, *M. gordonae* ($n = 4$), *MAA* ($n = 2$), *M. fortuitum* ($n = 1$) and *M. sp.* ($n = 4$) were detected.

Experimental recirculation systems have been described to be the source of mycobacteria originally isolated from humans, and related to human diseases both in immunocompromised and immunocompetent people and also associated with the water environment. The most significant finding is *M. kansasii* and *M. interjectum* isolation.

M. kansasii is often considered to be the most virulent of the NTM and usually causes lung disease that is clinically indistinguishable from tuberculosis (Marras & Daley 2002; Taillard et al. 2003; Arend et al. 2004). Tap water has been described to be the major reservoir for human infection with *M. kansasii* (O'Brien et al. 1987), and no other environmental source (water or soil) of *M. kansasii* has been identified (Griffith 2002). The other isolated mycobacterium which affects human health is *M. interjectum* (de Baere et al. 2001; Fukuoka et al. 2008; Bagley & Gujral 2010), which was also detected in fish (Rhodes et al. 2004); we can, therefore, assume its presence in water.

The other obtained isolates were classified in the *M. szulgai/intermedium* group and *M. chelonae/abscessus* group. Both *M. szulgai* and *M. intermedium* are associated with pulmonary diseases or extrapulmonary infections (Meier et al. 1993; Edson et al. 2006; Meyer & Gelman 2008). *M. intermedium* causing granulomatous dermatitis was associated with home hot tub exposure (Edson et al. 2006). *M. szulgai* was previously isolated from the water of a swimming pool, but in the natural environment it is quite rare (Tortoli et al. 1998). *M. abscessus* causes lung infections or subcutaneous abscess-like lesions and is one of the most dangerous and multidrug-resistant mycobacteria (Griffith et al. 2007). This mycobacterium was detected in the environment of aquaria and fish ponds (Beran et al. 2006). *M. chelonae* has a similar range of clinical manifestations as *M. abscessus*, but treatment outcomes are more successful (Griffith et al. 2007). *M. chelonae* is a ubiquitous environmental organism found in soil, water and dust (Brown-Elliott & Wallace 2004).

CONCLUSION

In the present study, a survey of mycobacterial presence in different elements of the water environment located in the Czech Republic was performed. Altogether, we were able to identify 22 mycobacterial species (subspecies, complexes).

The results of our study suggest that fish ponds and storage ponds are relatively mycobacteria-free compared to other water environments. This finding allows us to be optimistic with respect to the health of fish and consequently with regard to the safety of food made from fish. Fish ponds are surrounded by forests and meadows and have no permanent water supply. We assume that the occurrence of mycobacteria in fish ponds is because they are fertilised with manure or slurry. These interventions lead to increased eutrophication of fish ponds. Low incidence of mycobacteria in storage ponds is probably due to the low temperature of water. Also, constant inflow and outflow of water prevents the formation of bacteria in sediment.

The relatively high occurrence of mycobacteria-positive samples in drinking water reservoirs could be connected with the higher rate of agriculture at such locations.

The highest detection of mycobacteria (49.3% of positive samples) was observed in the experimental recirculation system. We can speculate that the reason is relatively high and constant water temperature.

A wide range of mycobacteria with pathogenic potential (*M. asiaticum*, *M. chimaera*, *M. interjectum*, *M. kumamotoense*, *M. lentiflavum*, *M. montefiorensis*, *M. nebraskense*, *M. paraffinicum* and *M. simiae*) were here isolated from the fresh water environment for the first time. The presence of *M. kansasii* and *M. interjectum* as significant PPM is quite alarming because the close contact of people working with untreated water may pose a risk of infection especially in immunocompromised hosts.

All the information gathered in this study represents a significant resource for facilitating risk assessment in connection with the water environment and protection of human health. The identification of mycobacteria still suffers from the limitations of the detection methods used. Altogether, 18 samples in this study were classified as *Mycobacterium* sp., which suggests that the spectrum of unidentified mycobacteria with unknown features is still high and their impact on human and animal health remains to be determined.

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