

***Mycobacterium arupense* among the isolates of non-tuberculous mycobacteria from human, animal and environmental samples**

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ABSTRACT: *Mycobacterium arupense* is a non-tuberculous, potentially pathogenic species rarely isolated from humans. The aim of the study was to ascertain the spectrum of non-tuberculous mycobacteria within 271 sequenced mycobacterial isolates not belonging to *M. tuberculosis* and *M. avium* complexes. Isolates were collected between 2004 and 2009 in the Czech Republic and were examined within the framework of ecological studies carried out in animal populations infected with mycobacteria. A total of thirty-three mycobacterial species were identified. This report describes the isolation of *M. arupense* from the sputum of three human patients and seven different animal and environmental samples collected in the last six years in the Czech Republic: one isolate from leftover refrigerated organic dog food, two isolates from urine and clay collected from an okapi (*Okapia johnstoni*) and antelope bongo (*Tragelaphus eurycerus*) enclosure in a zoological garden, one isolate from the soil in an eagle's nest (*Haliaeetus albicilla*) and two isolates from two common vole (*Microtus arvalis*) livers from one cattle farm. All isolates were identified by biochemical tests, morphology and 16S rDNA sequencing. Also, retrospective screening for *M. arupense* occurrence within the collected isolates is presented.

Keywords: 16S rDNA sequencing; non-tuberculous mycobacteria; ecology

Non-tuberculous mycobacteria (NTM) are ubiquitous in the environment and are responsible for several diseases in humans and/or animals known as mycobacterioses (Han et al., 2007; Shitaye et al., 2009). More than 140 mycobacterial species are currently described; moreover, the number of NTM species is increasing (Tortoli, 2006; Kazda et al., 2009). NTM infections have been increasing in number over the past decades, especially in immunocompromised and HIV/AIDS patients (Sack, 1990; Echevarria et al., 1994; Tortoli et al., 2000) and in domestic and free living animals (Pavlik et al., 2007; Moravkova et al., 2008a; Shitaye et al., 2008a,b; Trckova et al., 2009; Slany et al., 2010).

According to the commonly used Runyon classification scheme, NTM are categorized by growth rate and pigmentation. Fast-growers require less than seven days for production of visible colony forming units (CFU) on solid agar, whilst slow-growers may require weeks to months to achieve comparable growth of CFU (Runyon et al., 1959). Mycobacteria are capable of producing biofilms (Williams et al., 2009). Mycobacteria in these biofilms probably become the major environmental reservoir for rapidly-growing mycobacteria such as *Mycobacterium simiae*, *M. xenopi* and *M. goodii* (Schulze-Robbecke et al., 1992; Tortoli, 2006; Krizova et al., 2010). Slowly-growing my-

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cobacteria such as *M. marinum*, *M. kansasii* or members of the *M. avium* complex are also able to produce biofilms (Hall-Stoodley et al., 2006; Williams et al., 2009).

M. arupense, first described in the USA in 2006, is of those NTM which grow rapidly at 30°C and slowly at 37°C (Cloud et al., 2006). It has been occasionally found in human sputum (Masaki et al., 2006) and tenosynovitis (Cloud et al., 2006; Tsai et al., 2008). *M. arupense* has also been isolated from small terrestrial mammals, i.e., *Mus musculus* and *Cricetomys gambianus* in Tanzania (Cloud et al., 2006; Durnez et al., 2008; Tsai et al., 2008) and from surface water in Korea (Lee et al., 2008).

The aim of the study was to ascertain the spectrum of NTM within 271 mycobacterial isolates not belonging to *M. tuberculosis* and *M. avium* complexes, which were obtained from human, animal and environmental sources between 2004 and 2009 in the Czech Republic. Also, screening for *M. arupense* occurrence within collected isolates is presented.

MATERIAL AND METHODS

Origin of samples

A total of 1330 environmental, animal and human isolates (collected by our laboratory over the last six years) were identified: 1003 isolates were members of the *M. avium* complex, 271 isolates were identified as NTM and 56 isolates were identified as non-mycobacterial strains.

For the purposes of this study, members of the *M. avium* complex were not included in the NTM group because generally, mycobacterial species belonging to the *M. avium* complex were identified by a previously described method and no sequence analysis was performed (Moravkova et al., 2008b).

NTM isolates originating from human patients ($n = 26$; sputum and skin biopsies), animals ($n = 63$; tissues and/or faeces) and the environment ($n = 182$; soil, dust, organic food refusals, biofilms, urine, clay, surface and aquarium water) were subjected to sequencing analysis. The environmental samples were examined within the framework of ecological studies carried out in animal populations infected with mycobacteria (cattle, dogs, domestic pigs, pigeons, pheasants, small terrestrial mammals, freshwater and ornamental fish).

Sample processing

Human sputa or skin biopsies were subjected to specific mycobacterial cultivation at 37°C. Clinical isolates were grown on Löwenstein-Jensen and Middlebrook 7H9 agar media (Becton Dickinson, Sparks, USA) supplemented with antibiotics (BD PANTA antibiotic mixture, Becton Dickinson, USA). An automatic system BACTEC MGIT 960 (Becton Dickinson, USA) was used.

Animal and environmental samples were homogenized and decontaminated according to a procedure described previously (Fischer et al., 2000; Matlova et al., 2003). For isolation, Stonebrink and Herrold egg yolk media were used. Samples were grown in the presence or absence of Mycobactin J (Allied Monitor, USA) at 25°C and 37°C.

Identification of mycobacterial isolates

The following phenotypic tests were performed in the laboratory: experiments to identify temperature preference, growth rate, pigment production in the dark and on exposure to light, and growth in the presence of 5% NaCl. In addition, clinical isolates were tested for nitrate reduction, aryl sulphatase activity and iron uptake. After Ziehl-Neelsen staining, all microscopically positive isolates were subjected to PCR and sequence analysis. The pellet obtained from mycobacterial cultures was resuspended in 100 µl elution buffer (50mM Tris HCl, pH 8.5, 1mM EDTA). The mixture was incubated at 95°C for 20 min and centrifuged at 9000 × G for 5 min.

The prepared supernatant was used as a DNA template for a PCR reaction. All isolates were analysed according to a previously described duplex and M4D PCR, which enables identification of the genus *Mycobacterium* and distinguishes between *M. avium* species (Moravkova et al., 2008b). Isolate identification was carried out using 16S ribosomal DNA (16S rDNA) PCR, followed by sequence analysis of a 921 bp long region according to a previously described method (Harmsen et al., 2003). PCR products for all isolates were sequenced in both directions using the broad-range primers 16S27f (5'-AGA GTT TGA TCM TGG CTC AG-3') and 16S907r (5'-CCG TCA ATT CMT TTR AGT TT-3'). Mycobacterial isolates were identified by their biochemical properties, morphology and sequence analysis data. Experimental sequences were aligned

with the available database entries using the “blastn algorithm” (EZ Taxon database, <http://www.eztaxon-e.org>). The cut-off value for the identity scores of the 16S rDNA was set as 99%. Another applied criterion was a difference of less than three bp from a reference species.

Screening for *M. arupense* occurrence within analysed NTM isolates

Retrospective screening for *M. arupense* presence within the group of analysed NTM strains collected before its characterisation in 2006 is also presented in this report. All NTM strains previously identified by sequence analysis only to the species level were realigned with a known 16S rDNA sequence originating from *M. arupense* (Accession Number DQ157760).

RESULTS

Analysis of 271 mycobacterial isolates subjected to 16S rDNA sequencing revealed the presence of 32 different NTM (Table 1). Simultaneously, the retrospective screening for *M. arupense* occurrence within the analysed group revealed three isolates from human patients, two isolates from clinically healthy animals and five isolates from the environment (Table 1).

Human isolates

A total of 11 different mycobacterial species were detected in 26 human isolates from sputum or skin biopsies (Table 1). The most frequent human isolate belonging to the NTM was *M. xenopi* (38.5%). Three out of 26 patients showed positivity for *M. arupense* in sputum (Tables 1 and 2).

The first patient, a 55-year-old woman with diabetes mellitus treated in childhood for human tuberculosis, was admitted to medical care with respiratory complications, increased temperature and fatigue. Physical examination showed abnormalities in the X-ray findings when compared to images from previous years. Complications were diagnosed as bronchitis, and treated with foradil (Novartis, Switzerland). Two consecutive sputum specimens collected within a period of two weeks were culture-positive for non-pigmented mycobacteria after six weeks of cultivation on Löwenstein-Jensen medium. Based on the 16S rDNA sequence

homology and biochemical properties, isolates were identified as *M. arupense*. No additional antibiotic treatment was applied. One month after the initial isolation of *M. arupense*, cultures of the sputum were negative for mycobacteria. The patient was asymptomatic and no recurrence was detected.

The second patient was a 66-year-old male with an intensive cough giving rise to yellow or pink sputum, increased temperature and fatigue. During a hospital health inspection, chronic gastritis was diagnosed. Radiography of the chest revealed a round shadow in the right lung. Laboratory testing detected elevation of inflammatory markers; therefore a 12-day course of 625 mg augmentin (SmithKline Beecham Pharmaceuticals, Brentford, United Kingdom) was applied twice per day. Mycobacterial culture of five consecutive sputa revealed the presence of *M. arupense* only in the first sample analysed before the medication was taken. The sputum specimen was culture positive for a non-pigmented mycobacterial isolate after 35 days of cultivation on Middlebrook 7H9 medium identified as *M. arupense*. Sputum cultures were negative for mycobacteria one month after the initial isolation of *M. arupense*. The radiography examination showed partial regression in the right lung.

The third patient was a 77-year-old woman with no health complications and a clean X-ray screen. She was tested for mycobacteria because of her close contact with a patient diagnosed with open pulmonary tuberculosis caused by *M. tuberculosis*. The initial and the last two of six consecutive sputum samples showed growth of mycobacterial strains identified as *M. arupense*. The patient has not demonstrated any health complications and is still without histological findings, though the last two sputum samples analysed eight months after initial testing were culture positive for *M. arupense*.

Animal isolates

A total of 16 different mycobacterial species were detected among 63 animal isolates from tissues and/or faeces (Table 1). The most frequent environmental NTM isolates were *M. marinum* (14.3%) and *M. chelonae* (14.3%). Mycobacterial strains were mainly observed in pigs: *M. chelonae*, *M. xenopi*, *M. kansasii*, *M. nonchromogenicum*; in fish: *M. marinum*, *M. fortuitum*, *M. peregrinum/septicum*, *M. szulgai*, *M. nonchromogenicum* and in mice: *M. arupense*, *M. triviale*, *M. fortuitum*, *M. terrae* and *M. nonchromogenicum*.

Table 1. Species identification of 271 non-tuberculous mycobacterial isolates

Species	Number	Isolate origin		
		human ¹	animal ²	environment ³
<i>M. abscessus</i>	1	0	1 (reptile)	0
<i>M. alvei</i>	1	0	1 (bird)	0
<i>M. arupense</i>	10	3	2 (mouse)	5
<i>M. bohemicum</i>	1	1	0	0
<i>M. celatum</i>	1	0	0	1
<i>M. chelonae</i>	33	2	1 (cattle) 9 (domestic pig)	18
<i>M. chitae</i>	4	1	0	3
<i>M. confluentis</i>	1	0	1 (brown bear)	0
<i>M. engbaekii</i>	3	0	0	3
<i>M. fortuitum</i>	23	0	2 (bird) 1 (mouse) 5 (fish)	15
<i>M. gastri</i>	1	0	0	1
<i>M. gordonae</i>	30	1	1 (gastropod)	28
<i>M. hiberniae</i>	1	0	0	1
<i>M. interjectum</i>	4	0	0	4
<i>M. intaracellulare</i>	11	0	0	11
<i>M. kansasii</i>	8	2	1 (domestic pig) 1 (gastropod)	3
<i>M. kumamotoense</i>	4	0	0	4
<i>M. malmoense</i>	1	1	0	0
<i>M. marinum</i>	13	0	9 (ornamental fish)	0
<i>M. mucogenicum</i>	1	0	0	1
<i>M. nebraskense</i>	1	0	0	1
<i>M. neoaurum</i>	1	1	0	0
<i>M. nonchromogenicum</i>	13	0	1 (mouse) 2 (domestic pig) 1 (fish)	9
<i>M. paraffinicum</i>	1	0	1 (gastropod)	0
<i>M. peregrinum/septicum</i> ⁴	34	0	2 (fish)	32
<i>M. porcinum</i>	2	0	0	2
<i>M. simie</i>	1	0	0	1
<i>M. szulgai</i>	3	0	1 (fish)	2
<i>M. terrae</i>	10	3	1 (mouse) 1 (reptile)	5
<i>M. triviale</i>	5	1	2 (mouse)	2
<i>M. vaccae</i>	1	0	0	1
<i>M. xenopi</i>	15	0	4 (domestic pig)	1
<i>M. spp</i> ⁵	40	0	12	28
Total	271	26	63	182

¹clinical isolates obtained by cultivation from sputum or skin biopsies²tissues and faeces are included in animal samples³water, soil, dust, biofilms and organic remains are included in environmental samples⁴identical sequenced region of the *16S rRNA* gene; not distinguished in this study⁵isolates identified only to the species level; sequencing data are outside the defined criteria

Table 2. *Mycobacterium arupense* detection in three clinical samples, two animals and five environmental samples¹

Host or place	Sample origin source (year)	Number of isolates	Reasons for culture examinations (anamnestic data)
Human sputum	55/f ² (2008)	1	abnormal lung X-ray shadows and bronchitis (lung tuberculosis in childhood)
	66/m ² (2008)	1	abnormal lung X-ray shadows and pneumonia
	77/f ² (2008, 2009)	1	close contact with open lung tuberculosis patient
Common vole ³ (liver tissue)	cattle farm (2004)	1	trapped on one cattle farm
	wet field (2004)	1	trapped on the field without contact with domestic animals
Environment	clay from enclosure for antelope bongo ⁴ (2007)	1	ecological study in one Zoo with lung mycobacteriosis of a bongo antelope ⁴ caused by <i>M. a. hominissuis</i>
	one urine and one clay sample from enclosure for okapi ⁵ (2007)	2	okapi in the same Zoo housed in the paddock near the bongo antelope ⁴
	fridge with dog food (2007)	1	working room of school caretaker with open lung tuberculosis
	soil in sea eagle's nest ⁶ (2007)	1	ecological study for the presence of mycobacteria transmitted by fresh water fish

¹all isolates were identified using biochemical properties, morphology and sequencing analysis data of the *16S rRNA* gene

²Age/sex: f = female, m = male

³*Microtus arvalis*; ⁴*Tragelaphus eurycerus*; ⁵*Okapia johnstoni*; ⁶*Haliaeetus albicilla*

M. arupense was isolated from liver tissue of two small terrestrial mammals (*Microtus arvalis*; Table 2).

Environmental isolates

A total of 24 different mycobacterial species were detected in 182 environmental isolates from water, soil, dust, biofilms or organic food refusals (Table 1). The most frequent environmental NTM isolates were *M. peregrinum/septicum* (17.6%), *M. gordonae* (15.4%), *M. chelonae* (9.9%) and *M. fortuitum* (8.2%). *M. arupense* was isolated from five environmental samples (Table 2). Three isolates originated from one Zoo, namely from two neighbouring enclosures, one with bongo antelopes and the other one with three okapi. One isolate was discovered in dog feed and another one in the soil collected from an eagle's nest near a reservoir with freshwater fish infected with NTM (Table 2).

DISCUSSION

Differentiation of mycobacteria to the species level by evaluation of phenotypic and biochemi-

cal tests is time-consuming because of the slow growth rate of mycobacteria. Phenotypic and biochemical test results may vary depending on the growth conditions, sometimes leading to inaccurate results. With respect to these limitations, 16S rDNA sequencing analysis was used to improve this approach. We were unable to identify a group of 40 mycobacterial isolates to the species level, because the sequence data were not in the range of the stated conditions used for species identification.

A broad-range of bacterial 16S rDNA PCR followed by direct sequencing provides valuable information, especially in culture-negative cases (Woo et al., 2008). The advantage of using ribosomal sequences for genotypic identification of mycobacteria is that it allows the possibility of discovering previously undescribed species (Springer et al., 1996).

There are several limitations to the sequencing-based approach, such as the impossibility of detecting bacteria in polymicrobial samples without committing to a time-consuming and laborious cloning strategy, the risk of contamination, or the insufficient quality of public sequence databases due to faulty/poorly characterized sequence entries. The major difficulties and controversies

in the interpretation of sequence data concern the assignment of bacterial species according to similarity search results, as no threshold values are available (Woo et al., 2008). Janda and Abbott (2007), in their recommended guidelines, suggested that a minimum of > 99%, and ideally > 99.5%, sequence similarity be used as the criteria for species identification, and that other properties, e.g., phenotype, should be considered in final species identification.

Another disadvantage of using 16S rDNA sequencing analysis is the inability to distinguish between some closely related mycobacterial species. Further differentiation can be accomplished by analysis of other gene targets (Telenti et al., 1993).

A review of the literature revealed four articles reporting five cases of *M. arupense* isolation from humans (Cloud et al., 2006; Masaki et al., 2006; Tsai et al., 2008; Neonakis et al., 2010). No diagnostic criteria for classification of *M. arupense* as a causative pathogen currently exists, because it is difficult to interpret clinical, radiological and above all bacteriological findings, especially in those patients with co-existing chronic lung diseases (Jogi and Tyring, 2004). Generally, *M. arupense* could grow in human sputum cultures because it is present either as a casual bacterium with no clinical relevance or as a mycobacterial infection with observed symptoms and self-limiting disease. When we consider all the data published in the literature, *M. arupense* can likely be found in human clinical samples because the human organism can be contaminated with bacteria through the respiratory tract, the gastrointestinal tract or soft tissues (Scannapieco, 1999). Especially in patients with immunodeficiency or HIV/AIDS, this opportunist pathogen could be the cause of rare infections.

The mycobacterial isolate found in the first case originated from a patient with pulmonary complications, and results indicate that *M. arupense* was the only causative pathogen. The patient's clinical symptoms as well as radiographic lung opacities were stabilized without antituberculous therapy. Results were conclusive when the patient was checked 12 months later. The reported patient worked in a bakery for a long period of her life. Similarly to the findings of *M. arupense* in mouse organs (Table 2), we propose that the possible mode of transmission of *M. arupense* into the respiratory tract was the inhalation of contaminated dust in the bakery environment.

In the case of the second patient the presence of *M. arupense* cannot be designated as either clinically significant or insignificant. The presence of *M. arupense* may have been accidental and the reported symptoms caused by other factors. Also, the culture-negative results may have been down to the augmentin treatment, while *M. arupense* was still present. The third case report can be explained in two ways: the presence of *M. arupense* may have represented an infection, but since the patient was otherwise healthy, the infection was self-limited, or, initial contact with *M. arupense* resulted in the colonization of human micro-flora without any influence on health.

We found *M. arupense* in the liver tissue of wet field and urban mice *Microtus arvalis* before its discovery and characterisation (Table 2). Our ascertainment is in accordance with identification of *M. arupense* isolates obtained from small terrestrial mammals: from the lung of *Mastomys natalensis* and from the liver of *Criteromys gambianus* (Durnez et al., 2008).

It should be noted that surface waters can be sources of *M. arupense* for spread to the environment (Lee et al., 2008). Recently, published work revealed bioaerosols as a possible source of *M. arupense* spread to humans in duck houses, as *M. arupense* was present at high concentrations up to 10⁴ CFU per cubic meter (Martin et al., 2010). Our data demonstrate that soil or dust could be an environmental source of *M. arupense*. The isolate obtained from the environment of the okapi and the bongos demonstrates the presence of *M. arupense* in soil and urine. Leftover refrigerated organic dog food contaminated with *M. arupense* could become a source of human infection. The diagnosis of open pulmonary tuberculosis in this case was made precisely (*M. tuberculosis* confirmed by commercial assay GenoType MTBC; Hain LifeSciences, Germany). Therefore, this type of contamination most likely originates from dust in the environment or, maybe, *M. arupense* is part of the normal respiratory flora as suggested earlier and the owner contaminated the dog food by coughing. *M. arupense* present in the sea eagle nest could originate from contaminated water, soil or small terrestrial mammals, as described in previous publications (Durnez et al., 2008; Lee et al., 2008).

It can be concluded that *M. arupense*, a relatively member of the NTM, is an environmentally-derived mycobacterium, occasionally detected in human sputum.

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