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Nontuberculous mycobacteria in captive and pet reptiles

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Abstract

The aim of this study was to highlight the importance of nontuberculous mycobacteria species in the pathology of various reptilian pet species as well as their epidemiological significance of infection transmission to humans. Faeces samples from six living reptiles and organs from ten carcasses were submitted to bacteriological testing during the years 2003–2015. Positive colonies from one faeces sample and two organs showed the presence of a gene coding 65kDa antigen common for all mycobacteria. Further identification to the species level revealed that the isolates belong to *Mycobacterium fortuitum* and *Mycobacterium avium* subsp. *hominissuis*, later subjected to drug susceptibility testing which confirmed high resistance levels in both isolates. In conclusion, there is a great significance of the occurrence of nontuberculous mycobacteria in captive and pet reptiles, presenting reptiles as possible hosts representing a serious threat of transmission of high resistance mycobacterial isolates to humans. To our knowledge, this is the first report of *M. avium* subsp. *hominissuis* occurrence in reptiles.

Antimicrobial resistance, MIC, IS901

Mycobacteria are defined as aerobic, non-motile, non-sporulating, acid-alcohol fast, rod-shaped Actinomycetes which belong to the genus *Mycobacterium*, the single genus within the family of *Mycobacteriaceae* (Goodfellow and Wayne 1982; Stackebrandt et al. 1997). The number of species within the genus has rapidly been changing during the last years, now ranging around 175 (LPSN 2016). Most of these species are saprophytes (Garrity et al. 2004). Nontuberculous mycobacteria (NTM), also named as environmental mycobacteria, are isolated from water, soil, dust, and plants. They are divided into two groups: rapidly growing mycobacteria (RGM) where visible colonies appear within seven days, and slowly growing mycobacteria (SGM) which require longer incubation time (Tortoli 2009). The growth rate affects antimicrobial susceptibility as well as clinical signs and pathology of the affected organism (Tortoli 2003; CLSI 2011). One of the characteristics of NTMs is the high level of natural drug resistance as well as inducible and mutational resistance acquired during suboptimal drug exposure and selection (van Ingen et al. 2012).

With regard to reptiles, there are plenty of spontaneous mycobacterial infection cases described in a wide variety of reptiles, including lizards, snakes, crocodiles and turtles (Soldati et al. 2004; Slany et al. 2010). The transmission is not yet well understood, but it is generally believed that skin lesions or ingestion are the most likely way of infection. Affected animals usually develop granulomatous lesions in the form of greyish-white nodules observed in many organs and in the subcutis. Unlike mammalian tubercles, calcification has not been observed in reptiles (Soldati et al. 2004). Many reptilian pet species are hunted from the wild due to the impossibility of breeding in captivity, and are generally imported. Due to these facts such pets are more likely to harbour exotic pathogens (Ebani et al. 2005). With regard to humans, cases of mycobacteria infection being transmitted from reptilian pets to their owners have been described. Although it is considered that immunocompromised patients are at the highest risk of these kinds of

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infections, cases have also been described in healthy people (Hassl et al. 2007; Bouricha et al. 2014).

The aim of the present study was to highlight the importance of NTM species in the pathology of various reptilian pet species as well as their epidemiological significance of infection transmission to humans, especially in terms of high levels of antimicrobial drug resistance of isolates.

Materials and Methods

We conducted a retrospective study using laboratory samples that came for routine examination in the period from 2003 to 2015. The study included 16 animals (six living animals and 10 carcasses): nine snakes, six lizards and one chelonian (Table 1). Referring to living animals, the faeces samples were collected randomly into sterile tubes from cages where each animal was kept individually. Of these, five animals were clinically healthy and one animal (case no. 11) had skin lesions located on the hind leg and above that leg in the form of grey pigmentation changes, irregularly shaped and slightly elevated (Plate VIII, Fig. 1). Basic bacteriological and mycological examination of the specified skin lesions gave negative results. Neither histopathological examination nor mycobacteria testing from skin were performed. With regard to carcasses, delivered organs were all with visible pathological changes in the form of granulomatous inflammation. To our knowledge, these animals were not treated with any antibiotics. The origin of animals (born in captivity or imported from the wild) included in this study is unknown.

Bacterial examination

Specimens of animal tissues and organs were homogenised, concentrated and decontaminated according to the protocol described by Kent and Kubica (1985). The material was inoculated on standard nutrient media: Löwenstein-Jensen slant with pyruvate, Löwenstein-Jensen slant with glycerine, and Stonebrink slant followed by incubation at 37 °C. Each faeces sample was decontaminated using 0.9% hexadecylpyridinium chloride (HPC) (Sigma-Aldrich, USA) for one hour at room temperature (Whittington 2010). For each sample, 200 µl of homogenized suspension was inoculated on standard nutrient media as previously described. Media were checked for growth twice a week for eight weeks. All grown colonies were Ziehl-Neelsen (ZN) stained to confirm that they comprised acid–fact bacilli; positive colonies were subcultivated and identified by molecular methods.

Deoxyribonucleic acid (DNA) isolation

The isolation of DNA was performed by resolving a loop full of culture containing one to three CFU in 100 µl of distilled water (AccuGENE®, Lonza, Belgium) followed by incubation at 95 °C for 20 min with shaking at 350 rpm (Thermomixer comfort, Eppendorf) and then centrifugation at 14 000 *g* for one minute (SL8, Thermo Scientific, Germany). After cooling to room temperature, the supernatant was used in polymerase chain reactions (PCR) as a DNA template.

Molecular identification of cultivated mycobacteria

Amplification of DNA sequence containing the gene coding 65kDa antigen common for all mycobacteria was used in order to identify the colonies as members of the genus *Mycobacterium*. The primers TB1 (5'- GAG-ATC-GAG-CTG-GAG-GAT-CC-3') and TB2 (5'- AGC-TGC-AGC-CCA-AAG-GTG-TT-3') were used to amplify the product size of 383 base pairs (Hance et al. 1989).

For further identification, isolated mycobacteria were tested with Geno Type® Mycobacterium CM (Hain Lifescience, Germany), commercial DNA strip assay used for the detection and identification of mycobacteria to the species level.

The isolates confirmed to be *M. avium* subspecies by Geno Type® Mycobacterium CM were subjected to PCR amplification of integrated insertion sequence IS*901* using primers P1 FR300 (5'- CAG-CCA-GCC-GAA-TGT-CAT-CC-3') and P2 FR300 (5'- CAA-CTC-GCG-ACA-CGT-TCA-CC-3'). The amplification product size depends on the presence or absence of the insertion sequence IS*901*, so for *M. avium* subsp. *hominissuis* the amplification product of Flanking Region gives 300 base pairs (no incorporated IS*901*) and for *M. avium* subsp. *avium* 1700 base pairs (Kunze et al. 1992).

Drug susceptibility testing

Drug susceptibility testing of selected isolates was performed by the microdilution technique using VersaTREK kit (TREK Diagnostic System, Cleveland, Ohio, USA) commercial microplates divided for RGM and SGM. The process of preparing a bacterial suspension and its application to the microplate was performed according to manufacturer's instructions. Incubation for RGM lasts for 3 days at 30 °C (up to 2 days more if the growth is poor) and 7 days for SGM at 35 °C (up to 14 days if the growth is poor). Interpretation of results (susceptible, intermediate susceptible, and resistant) was carried out according to the Clinical and Laboratory Standards Institute (CLSI 2011) and according to European Committee on Antimicrobial Susceptibility Testing (EUCAST 2006). Results are expressed as minimum inhibitory concentration (MIC) – the lowest concentration of antimicrobial

substance that inhibits > 99% of mycobacterial growth. *Staphylococcus aureus* ATCC 29213 strain was used as a positive control for both RGM and SGM.

Results

Bacteriological investigation

Three isolates were obtained from three lizards (*Iguana iguana*). Colony growth was observed on Löwenstein-Jensen slant with glycerine for all three samples (Table 1). The culture growth dynamic in case no. 10 was: the first tiny dust-like colonies became visible after 72 h of incubation at 37 °C and reached a diameter of 1–2 mm after 120 h, which indicates that these mycobacteria are RGM. In case no. 4, the first colonies appeared on 44th day of incubation at 37 °C and in case no. 11 on the 23rd day, which indicates SGM.

Ziehl-Neelsen staining showed the presence of acid fast bacilli in all three cases.

Table 1. Review of searched reptile material and mycobacteria detection.

Case no.	Year of investigation	Animal species	Owner	Tested samples	Culture results
1	2003	lizard (*Iguana iguana)*	ZOO	liver	negative
2	2003	snake (unknown species)	private	skin	negative
3	2006	snake *(Morelia spilota)*	ZOO	bowel	negative
4	2006	lizard (*Iguana iguana)*	ZOO	liver	*M. avium* subsp. *hominissuis*
5	2007	snake (unknown species)	ZOO	liver, skin	negative
6	2011	snake *(Chondropython viridis)*	ZOO	liver	negative
7	2012	chelonian (unknown species)	private	lung	negative
8	2013	lizard *(Chamaeleo calyptratus)*	private	liver	negative
9	2015	lizard *(Iguana iguana)*	private	liver, skin	negative
10	2015	lizard *(Iguana iguana)*	ZOO	liver, skin	*M. fortuitum*
11	2015	lizard *(Iguana iguana)*	private	faeces	*M. avium* subsp. *hominissuis*
12	2015	snake *(Python regius)*	private	faeces	negative
13	2015	snake *(Python regius)*	private	faeces	negative
14	2015	snake *(Python regius)*	private	faeces	negative
15	2015	snake *(Python regius)*	private	faeces	negative
16	2015	snake *(Python regius)*	private	faeces	negative

Molecular identification

Members of the genus *Mycobacterium* were identified by amplification of 65 kDA antigen specific DNA sequence using conventional PCR. The hybridisation procedure (Geno Type® Mycobacterium CM) revealed that isolate no. 10 is *M. fortuitum* and isolates nos. 4 and 11 belong to the *M. avium* complex (MAC). Further examination used for species determination within MAC showed the amplification product sized 300 base pairs (Flanking Region; FR) which was found in both isolates no. 4 and no. 11, identifying isolated mycobacteria as *M. avium* subsp. *hominissuis*.

Drug susceptibility testing

Drug susceptibility testing was performed on two isolates from two different NTM species isolated from two lizards (*Iguana iguana*), *M. fortuitum* in case no. 10 and *M. avium* subsp. *hominissuis* in case no. 11. In case no. 4, no drug susceptibility testing was performed due to poor isolate recovery.

Table 2. Breakpoints used for rapidly growing mycobacteria drug susceptibility testing and minimum inhibitory concentration (MIC) values for *M. fortuitum* (case no. 10, *Iguana iguana*) of all drugs included in the panel.

Antimicrobial agent	MIC (μg/ml) for category			MIC *M. fortuitum*	Results
	S	I	R		
Trimethoprim/sulphamethoxazole[1]	≤2/38	-	≥4/76	≤0.25/4.78	S
Ciprofloxacin[1]	≤1	2	≥4	0.5	S
Moxifloxacin[1]	≤1	2	≥4	≤0.25	S
Cefoxitin[1]	≤16	32-64	≥128	≤4	S
Amikacin[1]	≤16	32	≥64	≤1	S
Doxycycline[1]	≤1	2-4	≥8	16	R
Tigecycline[3]	≤0.25	-	≥0.5	0.12	S
Clarithromycin[1]	≤2	4	≥8	2	S
Linezolid[1]	≤8	16	≥32	8	S
Imipenem[1]	≤4	8-16	≥32	32	R
Cefepime[2]	≤8	16	≥32	>32	R
Amoxicillin/clavulanic acid 2:1 ratio[2]	≤8/4	16/8	≥32/16	32/16	R
Ceftriaxone[2]	≤8	16-32	≥64	>64	R
Minocycline[2]	≤1	2-4	≥8	>8	R
Tobramycin[1]	≤2	4	≥8	4	I

[1]These breakpoints are recommended by the Clinical and Laboratory Standards Institute (CLSI 2011) for rapidly growing mycobacteria
[2]These breakpoints are recommended by CLSI (2011) for Nocardia and other Actinomycetes
[3]These breakpoints are recommended by the European Committee on Antimicrobial Susceptibility Testing (EUCAST 2006)
S – susceptible; I – intermediate susceptible; R – resistant
MIC – minimum inhibitory concentration

Table 3. Breakpoints used for *M. avium* complex drug susceptibility testing and minimum inhibitory concentration (MIC) values for *M. avium* subsp. *hominissuis* (case no. 11, *Iguana iguana*) of all drugs included in the panel.

Antimicrobial agent	MIC (μg/ml) for category			MIC *M. avium* subsp. *hominissuis*	Results
	S	I	R		
Clarithromycin[1]	≤8	16	≥32	4	S
Linezolid[1]	≤8	16	≥32	64	R
Moxifloxacin[1]	≤1	2	≥4	8	R
Amikacin	No interpretations available			16	/
Ethambutol	No interpretations available			8	/
Rifabutin	No interpretations available			<0.25	/
Rifampin	No interpretations available			4	/
Streptomycin	No interpretations available			64	/
Isoniazid	No interpretations available			>8	/
Trimethoprim/sulfamethoxazole	No interpretations available			4/76	/
Ciprofloxacin	No interpretations available			16	/
Doxycycline	No interpretations available			>16	/
Ethionamide	No interpretations available			5	/

[1]These breakpoints are recommended by the Clinical and Laboratory Standards Institute (CLSI 2011) for *M. avium* complex
S – susceptible; I – intermediate susceptible; R – resistant
MIC – minimum inhibitory concentration

With regard to the *M. fortuitum* isolate, bacterial growth was observed after five days of incubation. According to the CLSI (2011) standard, RGM resistance was determined for the following antimicrobial compounds: imipenem and doxycycline. The isolate was susceptible to trimethoprim sulphamethoxazole, linezolid, ciprofloxacin, moxifloxacin, cefoxitin, amikacin, and clarithromycin. Intermediate susceptibility was determined to tobramycin (Table 2). In case of RGM, other tested antimicrobial breakpoints have not yet been established by CLSI so values were interpreted by CLSI for *Nocardia* and other *Actinomycetes* according to a recent study (Broda et al. 2013). Therefore, the isolate showed resistance to cefepime, amoxicillin/clavulanic acid at a 2:1 ratio, ceftriaxone, and minocycline. Breakpoints for tigecycline have not been determined, so we presented our results according to EUCAST (2006). The isolate was susceptible to tigecycline (Table 2).

M. avium subsp. *hominissuis* bacterial growth was observed after eight days of incubation. According to CLSI (2011), the isolate was susceptible to clarithromycin while being resistant to moxifloxacin and linezolid. Referring to other tested antimicrobials, there are no available breakpoint data so it was not possible to interpret these results (Table 3).

Discussion

To our knowledge, this is the first study showing NTM species, more precisely *M. fortuitum* and *M. avium* subsp. *hominissuis*, being isolated from reptilian samples in Croatia.

This is also one of the few studies on determination of antimicrobial susceptibility of NTMs isolated from reptiles and generally from animals.

According to recent research, the presence of *M. fortuitum* was proven in saurians, turtles and pythons that had no visible clinical signs (Ebani et al. 2012). In the present case, the isolate was cultured from granulomatous lesions of the liver and skin of a carcass. Drug susceptibility results confirmed high resistance levels in the present *M. fortuitum* isolate. Cefoxitin showed antimicrobial activity whereas other tested cephalosporins (cefepime and ceftriaxone) did not (Table 2). The isolate was resistant to amoxicillin-clavulanic acid which was also observed in a previous study (Swenson et al. 1982). With regard to tetracycline, the present study showed tigecycline antimicrobial activity, while minocycline and doxycycline did not, as previously reported by Wallace et al. (2002). The isolate was resistant to imipenem which is in accordance with a previous study (Set et al. 2010). Among aminoglycosides, amikacin was active, whereas tobramycin showed intermediate activity. Drugs within fluoroquinolones, moxifloxacin and ciprofloxacin, were active, which was also the case in a recent study (Pang et al. 2015). The remaining tested drugs clarithromycin and linezolid were also active against the *M. fortuitum* isolate in the present study. The isolate was susceptible to trimethoprim sulphamethoxazole which is in agreement with a previous study where it was one of the most active drug against *M. fortuitum* (Tang et al. 2015). *Mycobacterium fortuitum* is medically one of the most important RGMs among humans and is associated with traumatic and surgical wound infections, skin and soft tissue infections, and less often with the pulmonary disease (Rastogi et al. 2001). A case of transmission of *M. fortuitum* infection between a reptilian pet and a human has been described, where it was identified in both the reptilian pet and the owner who was in good general condition and without symptoms except for enlarged lymph nodes (Hassl et al 2007).

Referring to *M. avium* subsp. *hominissuis*, the excretion of *M. avium* subsp. *hominissuis* in faeces could also be explained by isolation of the same most commonly found mycobacterial species in the environment, so it could be the result of a passage through the gastrointestinal tract (Pavlik et al. 2000). Moreover, NTM species are a common cause of granulomatous lesions in the subcutis of reptiles, so we can assume that it was also

a cause in the present case. To our knowledge, this is the first description of *M. avium* subsp. *hominissuis* occurrence in reptiles. Previously, there was a report on isolation of mycobacteria belonging to MAC in the Komodo dragon (Skoric et al. 2012). In the present study, the tested MAC isolate (case no. 11) showed susceptibility to clarithromycin (Table 3) which is in accordance with other surveys (Wallace et al. 1996). The present isolate showed resistance to linezolid and moxifloxacin (Table 3) which is also in accordance with previous study (Griffith et al. 2007). Species within MAC are the most frequently isolated and the most common cause of the human pulmonary NTM disease (Simons et al. 2011).

As there are limited data on effective drug treatments against animal mycobacterial isolates, we compared our results with different human isolates. In conclusion, this study shows great similarity in drug resistance between reptilian and human *M. fortuitum* and *M. avium* subsp. *hominissuis* isolates, which points to the interconnectedness and possible mutual transmission of the pathogen. It is important to note that these animals were not treated with any antibiotics. These results highlight the epidemiological significance of the occurrence of environmental mycobacteria in captive and pet reptiles, presenting reptiles as possible hosts and a serious threat of transmission of highly resistant mycobacterial isolates to humans.

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Fig. 1. Skin lesions located on the hind leg and above that leg in the form of grey pigmentation changes irregularly shaped and slightly elevated (*Iguana iguana*, case no. 11)