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Isolation and identification of mycobacteria from captive reptiles

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ABSTRACT

The occurrence of *Mycobacterium* species in clinically healthy pet reptiles was studied in Italy during the period 2004–2006. The feces samples of 223 animals were examined bacteriologically. Thirty-seven strains were isolated, in particular from 13/18 (72.2%) ophidians, 13/134 (9.7%) saurians and 11/71 (15.5%) chelonians. The isolates were classified, after HPLC analysis of bromophenacyl esters of cell wall mycolic acids, as *Mycobacterium fortuitum* (14 strains, 37.8%), *Mycobacterium fortuitum*-like (17, 45.9%), *Mycobacterium peregrinum* (4, 10.8%), and *Mycobacterium chelonae* (1, 2.7%). *M. fortuitum* was isolated from seven pythons, five saurians and two turtles; *M. fortuitum*-like from six saurians, six pythons and five turtles; *M. peregrinum* from four turtles; *M. chelonae* from one lizard. One isolate from an *Iguana iguana* could not be identified by HPLC analysis showing a previously unreported profile. Comparative 16S rDNA sequencing showed a low similarity with *Mycobacterium triviale* (97.2%) and *Mycobacterium confluentis* (97.1%). On the basis of such data the unidentified bacterium turned out to belong to a not yet described *Mycobacterium* species.

Genus *Mycobacterium* includes acid fast, aerobic, no-spore-forming, non-motile bacilli with wide variations in host affinity and pathogenic potential. This genus comprises almost 150 species differentiated into two groups: the *Mycobacterium tuberculosis* complex (MTC) and the non-tuberculous mycobacteria (NTM).

The NTM are ubiquitous in environment; they have been frequently isolated from water, soil, dust and plants. Contact with contaminated environments may occasionally be responsible for infection in humans and animals, especially if immunosuppressed. These mycobacteria are divided between rapid growers, which develop visible colonies on solid media within 7 days, and slow growers which require longer incubation time. Rapid and slow growers differ in their antimicrobial susceptibility and pathologies induced in humans (Tortoli, 2009).

Reptiles too may be affected by mycobacteriosis and usually develop granulomatous lesions, in different organs, clinical signs may differ on the basis of the district involved. At necropsy, white–grayish nodules are observed and histopathologic examination reveals typical granulomatous inflammations with multinucleated giant cells. Unlike mammalian tubercles, calcification has not been observed (Soldati et al., 2004). Systemic infections have been reported in lizards (Murray, 1996; Kramer, 2006; Girling and Fraser, 2007), snakes (Kiel, 1977; Quesenberry et al., 1986; Hernandez-Divers and Shearer, 2002), turtles (Greer et al., 2003; Oros et al., 2003; Murray et al., 2009), and crocodiles (Ariel et al., 1997). Mycobacteria reported to be involved in these cases are: *Mycobacterium chelonae*, *M. fortuitum*, *M. intracellulare*, *M. marinum*, *M. phlei*, *M. smegmatis*, *M. ulcerans*, *M. confluentis*, *M. haemophilum*, *M. hiberniae*, *M. neoaurum*, *M. nonchromogenicum* (Soldati et al., 2004).

The transmission of mycobacteria infections to reptiles is not well understood. The prevalent infection root is most likely skin lesions or ingestion. Animals having poor functionality of immune system are most at risk. The risk factors include stress, poor nutrition, and chronic diseases. Many reptiles and amphibians with mycobacterial infections show weight loss despite a good appetite. Captive wild animals are more likely to harbor the bacterial infections.

Reptiles are often household pets, and, if infected, they can be a source of pathogens for the owners. The risk for humans is higher when the infected animals do not show clinical signs, because they are not treated.

The aim of the present study was to assess the role of clinically healthy pet reptiles in the diffusion of mycobacteria in the domestic environment.

From 2004 to 2006, the feces samples of 223 captive reptiles (134 saurians, 71 chelonians and 18 ophidians) were examined bacteriologically to detect *Mycobacterium* spp. by Bacteriology Laboratory of Department of Animal Pathology, Prophylaxis and Food Hygiene, Faculty of Veterinary Medicine in Pisa. All animals were clinically healthy. One hundred and fifty animals lived, when sam-

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ples were collected, in a single pet shop and seventy-three were pets from private owners. Feces were collected into sterile tubes from cages and water pools in which each animal was individually kept.

Each sample (about 1–3 g) was decontaminated in 10 ml of a 1.5% hexadecylpyridinium chloride (HPC, Sigma, St. Louis, MO, USA) for 1 h at room temperature. For each sample, 1 ml of the homogenized suspension was transferred onto one tube of Dubos solid medium (Difco, Becton, Dickinson and Company, Sparks, MD, USA) which was incubated at 37 °C in a slanting position with partially unscrewed cap. After 4–5 days, after the evaporation of the liquid phase, the caps were tightly screwed and the tubes were incubated at 37 °C for two months. The cultures were observed daily in the first week, then weekly thereafter. All grown colonies were microscopically examined using the Ziehl–Neelsen staining. Acid fast colonies were subcultured onto new Dubos tubes and kept in collection until typing.

All the isolates were identified at the Regional Reference Center for Mycobacteria of Careggi University Hospital in Florence.

The identification of mycobacteria was carried out by means of HPLC analysis of bromophenacyl esters of cell wall mycolic acids according to the standard procedure (Butler et al., 1992; Tortoli and Bartoloni, 1996). A C_{18} Ultrasphere XL cartridge column (Beckman) on a System Gold Beckman instrument was used. Low and high molecular mass internal standards (Ribi, ImmunoChem, USA) were added for identification of peaks.

One mycobacterial isolate ambiguously identified by means of HPLC was further investigated by genetic sequencing. Extraction of DNA and amplification of the first 500 bp of 16S rRNA gene were carried out as described previously (Kirschner et al., 1993). The 16S rDNA sequence was established by overlapping direct sequencing of both strands of the amplicons using fluorescence-labeled dideoxy dye terminators on an automated DNA sequencer (ABI 373A; Perkin-Elmer Biosystems).

The mycobacterial cultures grew 37 strains identified as belonging to *Mycobacterium* genus on the basis of the acid fastness of the colonies. The isolates were obtained from 13/18 (72.2%) ophidians, 13/134 (9.7%) saurians and 11/71 (15.5%) chelonians. Twenty-five (16.6%) strains were isolated from animals of the pet shop, and 12 (16.4%) from the animals of different private owners.

The strains were classified, after HPLC analysis, as *M. fortuitum* (14, 37.8%), *M. fortuitum*-like (17, 45.9%), *Mycobacterium peregrinum* (4, 10.8%), and *M. chelonae* (1, 2.7%).

M. fortuitum was isolated from seven pythons, five saurians and two turtles; *M. fortuitum*-like from six saurians, six pythons and five turtles; *M. peregrinum* from four turtles; *M. chelonae* from one lizard (Table 1).

One isolate obtained from the fecal sample of an *Iguana iguana* could not be identified conclusively by HPLC analysis as it showed a previously unreported profile. No overlapping 16S rDNA sequence was detected in GenBank database; the most closely related species were *Mycobacterium triviale* and *Mycobacterium confluentis*, both were characterized by low similarity (97.2% and 97.1% respectively). On the basis of such data the unidentified organism turned out to belong to a not yet described *Mycobacterium* species. The GenBank accession number for the 16S rDNA sequence of this strain is JN592446.

Reptiles can carry a wide variety of pathogens responsible for infections in humans. Herpetologists, zoo personnel, veterinarians and owners are at risk through the frequent exposure to cold-blooded animals.

The results obtained in the present research show a relevant prevalence (16.59%) of mycobacteria among captive cold-blooded animals, even when they are in good health.

All the isolates belonged to the rapidly growing species *M. chelonae*, *M. peregrinum* and *M. fortuitum*. A wide variety of infections have been associated with these mycobacteria in humans, involving lungs, bone, central nervous system, prosthetic heart valves (Silcox et al., 1981; Schlossberg and Aaron, 1991; Wallace et al., 1992; Lessing and Walker, 1993; Nagao et al., 2009; Amorim et al., 2010). These mycobacteria can also be the cause of disseminated disease (Woods and Washington, 1987; Drabick et al., 1990; Rodriquez-Barradas et al., 1992). Skin infections, often evolving into draining subcutaneous abscesses, are particularly common (Hanson et al., 1987).

During the present research, *M. chelonae* was isolated only from a chinese water dragon (*Physignathus cocincinus*). Previously, *M. chelonae* sepsis and disseminated intravascular coagulation has been observed in an eastern spiny soft-shell turtle (*Apalone spinifera spinifera*), which presented pale mucous membranes, hemorrhagic nasal discharge, petechiae on all limbs. The turtle was euthanized and showed hemorrhagic coelomic effusion, bilaterally hemorrhagic lungs and petechiae on the serosal surfaces of the intestinal tract (Murray et al., 2009). Ulcerative stomatitis and subcutaneous granulomas in a *Boa constrictor*; osteoarthritis in a Kemp's Ridley sea turtle (*Lepidochelys kempii*); multiple cutaneous and hepato-splenic granulomas have also been associated to *M.*

Table 1
Mycobacterium species isolated from feces samples of reptiles.

Mycobacterial species	No. of isolates	Host	Source
Mycobacterium chelonae	1	Chinese water dragon (*Physignathus cocincinus*)	Pet shop (*n*=1)
Mycobacterium fortuitum	14	River cooter turtles (*Chrysemys concinna*)	Private owner (*n*=1); Pet shop (*n*=1)
		Chinese water dragon (*Physignathus cocincinus*)	Private owner (*n*=1)
		Giant spiny chameleons (*Chamaeleo verrucosus*)	Pet shop (*n*=2)
		Argentine black and white tegu (*Tupinambis merianae*)	Pet shop (*n*=1)
		Bearded dragon (*Pogona vitticeps*)	Pet shop (n=1)
		Ball pythons (*Python regius*)	Private owners (*n*=2); pet shop (*n*=5)
Mycobacterium fortuitum-like	17	Ball pythons (*Python regius*)	Private owner (*n*=1); Pet shop (*n*=5)
		Giant spiny chameleons (*Chamaeleo verrucosus*)	Pet shop (*n*=2)
		Green iguanas (*Iguana iguana*)	Pet shop (*n*=2)
		Bearded dragon (*Pogona vitticeps*)	Pet shop (*n*=1)
		Argentine black and white tegu (*Tupinambis merianae*)	Pet shop (*n*=1)
		River cooter turtles (*Chrysemys concinna*)	Pet shop (*n*=1); Private owners (*n*=3)
		Painted turtle (*Chrysemys picta*)	Private owner (*n*=1)
Mycobacterium peregrinum	4	River cooter turtles (*Chrysemys concinna*)	Pet shop (*n*=1); Private owners (*n*=2)
		Painted turtle (*Chrysemys picta*)	Private owner (*n*=1)
Mycobacterium spp.	1	Green iguana (*Iguana iguana*)	Pet shop (*n*=1)

n represents the number of isolates per source.

chelonae (Quesenberry et al., 1986; Greer et al., 2003; Rhodin and Anver, 1977).

We isolated *M. fortuitum* from saurians, turtles and pythons, whereas *M. peregrinum* from turtles only. *M. fortuitum* (including *M. fortuitum*-like), and *M. peregrinum* were isolated with high prevalence degrees. Clinical cases of reptiles associated with these mycobacteria are not reported in literature. In contrast, these species have been often isolated from fishes with or without macroscopic lesions. This high prevalence is probably related to the common presence of *M. fortuitum* and *M. peregrinum* in the aquatic environment where their presence is strongly related to management factors such as the quality and quantity of nutrients, water supply and temperature (Zanoni et al., 2008).

Members of *Mycobacterium tuberculosis* complex have never been isolated, suggesting that poikilothermic animals are not suited to harbor this species and thus they do not have a role in the transmission of human or animal tuberculosis.

During the present research a strain, belonging to a not yet described *Mycobacterium* species, has been isolated from a green iguana. The animal did not show clinical signs. However, it is not excluded that this mycobacterium is able to induce pathologies in reptiles, other animal species, and humans.

The results of this investigation confirm that pet reptiles can act as possible reservoirs of pathogens and represent a serious health risk to humans, in particular immune compromised patients, and other domestic animals. Moreover, pet reptiles are generally introduced from foreign countries and may harbor exotic pathogens not present in the country of importation (Ebani et al., 2005).

Treatment or euthanasia of reptiles with manifested pathologies is recommended. However, a strict attention to cage hygiene and regular sanitation and personal hygiene after handling the animals is always indispensable to minimize the exposure to zoonotic pathogens.

Conflict of interest statement

None of the authors of this paper has a financial or personal relationship with other people or organisations that could inappropriately influence or bias the content of the paper.

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