

OCURRENCE AND EXPERIMENTAL INFECTION OF TOADS
(*BUFO MARINUS* AND *B. GRANULOSUS*) WITH
MYCOBACTERIUM CHELONEI SUBSP. *ABSCCESSUS*

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SUMMARY. In a survey of 234 Amazonian toads and frogs, six strains of *Mycobacterium chelonae* subsp. *abscessus* were isolated from the liver or spleen of four of 66 *Bufo marinus* (6·1%) and from the kidney or peritoneal fluid of two of 86 *B. granulosis* (2·3%). There were no histopathological lesions in the viscera of the infected animals. Experimental infection of 29 captive *B. marinus* and *B. granulosis*, by the intraperitoneal route, with a pooled inoculum of *M. chelonae* subsp. *abscessus* caused five deaths near the end of a 2-month observation period. *M. chelonae* subsp. *abscessus* was isolated from the liver, spleen, kidney, gonad, heart and lung of toads killed at various intervals after inoculation, and intracellular acid-fast bacilli were seen in these organs. Histological evidence of invasion of tissues by mycobacteria became apparent from the 45th day after infection. The susceptibility to infection of *B. marinus* and *B. granulosis* suggests that these toads may serve as a fortuitous animal host for *M. chelonae* subsp. *abscessus*.

INTRODUCTION

Mycobacterial infection in amphibians was first described in frogs by Küster, Weber and Taüte (1905, cited by Reichenbach-Klinke and Elkan, 1965) and later by Lichenstein (1921, cited by Reichenbach-Klinke and Elkan, 1965). A case of tuberculosis of the liver, lungs and intestines caused by acid-fast bacilli designated "*Mycobacterium giae*", and later identified as *M. fortuitum*, was reported in a frog of the species *Leptodactylus pentadactylus* by Darzins (1952). Schwabacher (1959) identified the causative agent of skin lesions in a toad of the species *Xenopus laevis* as *M. xenopei*.

In amphibians, mycobacterial disease of the skin, respiratory tract and intestines is generally a secondary infection of captive, debilitated animals. The source of infection is often attributed to environmental contamination. In nature, neither overt disease nor infection has been observed among amphibians. The possible role of herpetofauna as a potential reservoir of acid-fast bacilli has been examined only by Marcus, Stottmeier and Morrow (1976). However, the results of experimental infection of anole lizards, *Anole carolinensis*, with *M. ulcerans* were equivocal.

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We report here the isolation of *M. chelonae* subsp. *abscessus* from the viscera of marine toads, *Bufo marinus* and *B. granulosis*, and experiments to investigate the susceptibility of these toads to *M. chelonae* infection.

MATERIALS AND METHODS

Isolation of mycobacteria. The recovery of *M. chelonae* subsp. *abscessus* from the internal organs of amphibians was a fortuitous finding in a study of fungi harboured by tropical anurans (see Mok, in press). Toads and frogs were collected every 2 weeks from a forested area 10 km from Manaus. The collection sites were sparsely inhabited clearings amidst primary forests, and creeks and ponds along the forest edge. All animals were killed with chloroform and kept at 4°C until necropsy.

Two portions of liver, spleen, kidney and lung were removed from each animal, homogenised and inoculated on to Mycosel agar (Merck), a selective medium for pathogenic fungi (peptone from soy meal, 10 g; D(+)-glucose, 10 g; cycloheximide, 0.4 g; chloramphenicol, 0.05 g; agar agar, 12.5 g/L). Occasionally, peritoneal fluid and gonads were also sampled and cultured. The culture tubes were kept at room temperature (18°C) for 6 weeks and examined weekly. Presumptive mycobacterial colonies were transferred on to Lowenstein-Jensen (L-J) medium and identified by morphology and by biochemical tests (Runyon *et al.*, 1980). A third portion of the liver, spleen, kidney and lung was removed and kept in 10% buffered formalin. Sections of tissues from which *Mycobacterium* spp. had been isolated were stained by haematoxylin and eosin (H & E) and Wade's acid fast stain (Luna, 1968).

Experimental infection in toads. Young adult and older adult *B. marinus* and *B. granulosis* caught in the same area were used.

The inoculum was prepared from pooled 14-day-old cultures of the six isolates of *M. chelonae* subsp. *abscessus* in 0.01% aqueous Tween 80. It contained 10⁸ cfu/ml. Twenty-one *B. marinus* and eight *B. granulosis* were given inocula by the intraperitoneal route. *B. marinus* weighing 33–191 g were each given 1 ml of inoculum/25 g body weight. *B. granulosis* weighing 8 to 16 g were each given 1 ml of inoculum/10 g body weight. A group of 20 *B. marinus* and 12 *B. granulosis* were given inocula containing 0.01% aqueous Tween 80 only as controls. The infected and control toads were caged separately. They were fed thrice weekly with crickets and flies.

The toads were observed for 2 months and were killed in groups of 3 or 4 on the 5th, 10th, 17th, 20th, 30th, 45th and 50th days after inoculation. The viscera were examined at necropsy for lesions and cultures were made of the liver, spleen, gonad, kidney, heart and lung on L-J medium and Mycosel agar. Impression smears were made of the same organs and examined with the Ziehl-Neelsen stain. Sections of the above organs were processed and stained by H & E and Wade's acid fast stains.

Experimental infection in mice. Inocula at concentrations of 3 × 10⁶, 3 × 10⁷ and 3 × 10⁸ cfu/ml were prepared from 14-day-old cultures of the *M. chelonae* subsp. *abscessus* isolates in 0.01% Tween 80. Three groups of 42 Swiss white mice aged 8 weeks were used. Mice in each group received 1 ml of the inoculum at different concentrations by intraperitoneal injection. Six mice from each group were killed on the 5th, 12th, 18th, 25th, 30th, 45th and 60th days after inoculation. Cultures were made of the liver, spleen, kidney and lung. Impression smears of the same organs were stained for acid-fast bacilli. All organs from which mycobacteria were isolated and some from which no isolations were made were examined histologically.

RESULTS

Isolation of M. chelonae. From April to December 1982, 234 toads and frogs belonging to seven genera and 16 species were examined (see table). Two-thirds of the animals were toads belonging to the Bufonidae. None of the animals examined by necroscopy showed external or internal signs of disease.

Samples from six animals produced smooth and rough white colonies after incubation for 14–21 days (fig. 1). Microscopic examination with lactophenol cotton

TABLE
Species of toads and frogs examined

Species	Number examined	Number infected*
Bufonidae		
<i>Bufo granulosis</i>	86	2
<i>Bufo marinus</i>	66	4
<i>Dendrophryniscus</i> sp.	6	0
Hylidae		
<i>Hyla boans</i>	12	0
<i>Hyla calcarata</i>	7	0
<i>Hyla geographica</i>	4	0
<i>Hyla lanciformes</i>	14	0
<i>Hyla raniceps</i>	2	0
<i>Hyla</i> sp.	1	0
<i>Ololygon rubra</i>	3	0
Leptodactylidae		
<i>Adenomera hylaedactyla</i>	5	0
<i>Adenomera</i> sp.	1	0
<i>Eleutherodactylus fenestratus</i>	6	0
<i>Leptodactylus fuscus</i>	10	0
<i>Leptodactylus ocellatus</i>	5	0
<i>Leptodactylus pentadactylus</i>	6	0
Total	234	6

* With *M. chelonei* subsp. *abscessus*.

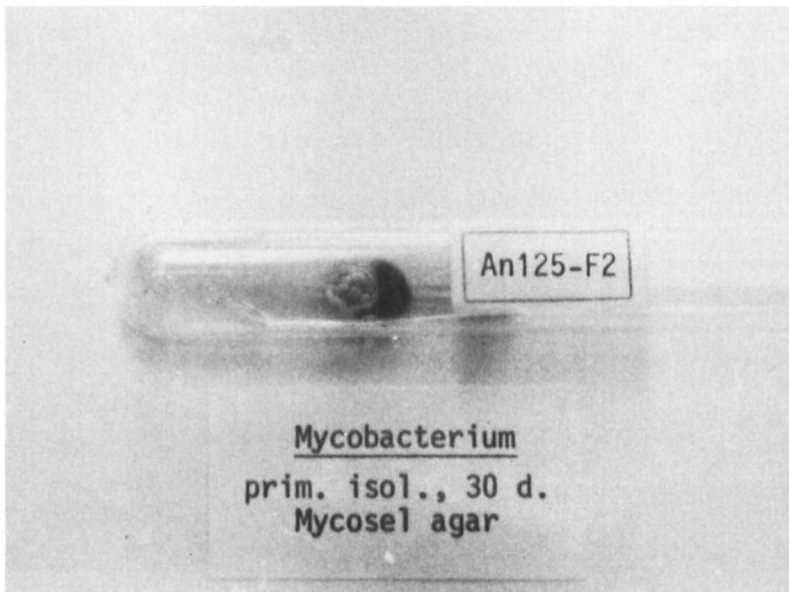


FIG. 1.—Primary isolation of *M. chelonei* subsp. *abscessus* from the liver of *B. granulosis*.

blue revealed aggregated hyaline microorganisms intermediate in appearance between yeasts and bacteria. Smears stained by Ziehl-Neelsen's method showed acid-fast bacilli (AFB). The isolates were transferred on to L-J medium and incubated at 25°C.

All six AFB isolates grew rapidly and did not produce yellow pigment; they were arylsulphatase positive, nitrate reductase negative and did not utilise iron. The cultures grew on MacConkey agar without crystal violet and produced both smooth and rough colonies, without aerial hyphae, on corn-meal glycerol agar. They were classified as *M. chelonei* and identified as *M. chelonei* subsp. *abscessus* by the Mycobacteriology Branch of the Centers for Disease Control, Atlanta, USA.

Three isolates were from the spleen and one from the liver of *B. marinus* (four out of 66 animals, 6.1%); one was from the kidney and one from the peritoneal fluid of *B. granulosis* (two out of 86 animals, 2.3%). Neither histopathological lesions nor any AFB were detected in the viscera of these six naturally infected toads.

Experimental infection in toads. The infected toads appeared normal during the first month after inoculation but a few became emaciated during the second half of the experiment. Four *B. marinus* died on the 48th, 56th, 57th and 58th days, and one *B. granulosis* died on the 58th day.

At necropsy, no macroscopic lesions were observed in the viscera of the infected toads. Pulmonary nematode or trematode infestation was visibly greater in the infected toads than in the controls. From the fifth day after inoculation, aggregates of intracellular AFB were detected in the liver, spleen, gonad, kidney, heart and lung (fig. 2). The spleen and liver showed the most AFB and gonads were the least infected. The numbers of AFB increased with the infection period.

M. chelonei subsp. *abscessus* was recovered from all organs examined during the study. Cultures from the gonads yielded the fewest colonies. Growth of the

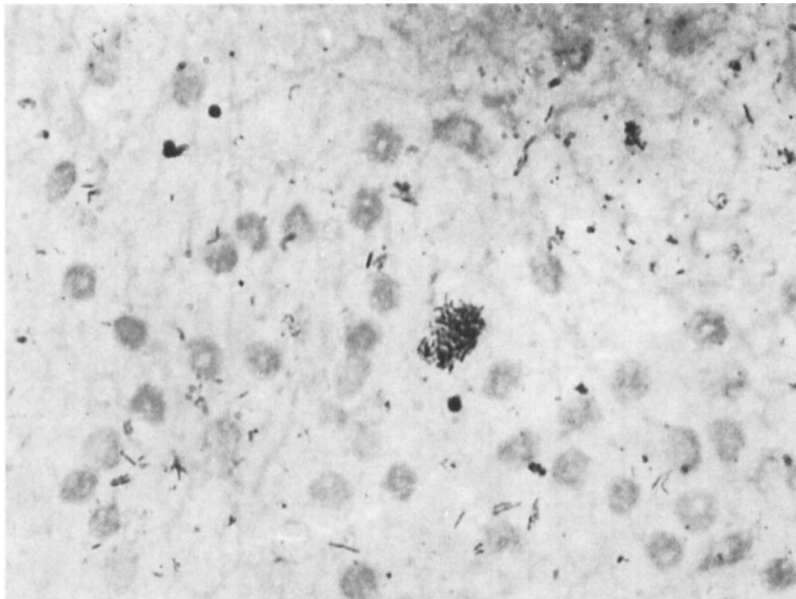


FIG. 2.—Presence of AFB in an impression smear from spleen of experimentally infected *B. marinus* 20 days after inoculation (Ziehl-Neelsen stain; $\times 973$).



FIG. 3.—Aggregate of AFB (arrow) in liver of *B. marinus* 45 days after inoculation (H & E stain; $\times 973$).

mycobacterium appeared after 4–6 days on both L-J medium and Mycosel agar and was heavier in the latter medium.

Histological evidence of mycobacterial infection appeared on the 45th day after inoculation. Clusters of AFB were seen in the liver, spleen, gonad, kidney, heart and lung (fig. 3). The inflammatory response was minimal. A few areas of non-caseous necrotic tissue without AFB were present in the liver, spleen and heart.

Results of similar necroscopy examinations on the control toads were negative.

Experimental infection in mice. On the fifth day after inoculation, AFB were seen and recovered from the liver, spleen, kidney and lung. On the 12th day, they were still seen and recovered from the liver and spleen, and were also isolated from the kidney. By the 18th day, the liver, spleen, kidney and lung were negative for AFB on direct examination but the spleen cultures from all six mice remained positive for mycobacteria. Cultural examinations thereafter were negative. Throughout the 60-day observation period, no AFB were seen in histological sections of the mouse viscera.

DISCUSSION

M. chelonae and *M. fortuitum* are the only rapidly growing, non-photochromogenic mycobacteria that are opportunist pathogens for man and animals (Thoen and Himes, 1977; Foz *et al.*, 1978; Harris, 1980; Weiszfeiler and Karasseva, 1981). The growing recognition of the clinical importance of these mycobacteria has accompanied the establishment of distinct taxonomic status for each subspecies and biovariant (Silcox, Good and Floyd, 1981; Tsukamura, 1981). The two subspecies of *M. chelonae* are subsp. *chelonae* and subsp. *abscessus* (Stanford *et al.*, 1972). *M. chelonae* and *M. fortuitum* have been found as saprophytic microorganisms in aquaria, water tanks, river, soil and dust (Chapman, 1971; Paull, 1973; Romanelli *et al.*, 1980). However,

information about animal infections with these mycobacteria is scarce. *M. chelonae* was originally isolated from diseased turtles by Friedmann (1903). Garcia-Rodriguez *et al.* (1975) isolated *M. chelonae* from the ganglion of an apparently healthy domestic hog in Spain. Kantor (1978) reported the isolation of *M. chelonae* and "*M. fortuitum-chelonae*" from the cervical, axillary, inguinal and bronchial lymph nodes of nine wild "peludo" armadillos, *Chaetophractus villosus*, caught in the pampas region of Argentina. To date, the domestic pig and "peludo" armadillo are the only reported animal hosts for *M. chelonae*.

The toads *B. marinus* and *B. granulosis* are the most common members of the Bufonidae in tropical America. Their natural habitats are open areas often associated with human dwellings. Our attention was drawn to the possible isolation of atypical mycobacteria on mycological culture medium from the unintentional isolation of "*M. fortuitum-chelonae*" from human tissues on Sabhi agar (Restrepo *et al.*, 1981). The occurrence of *M. chelonae* subsp. *abscessus* in apparently normal internal organs of the commonest tropical American Bufonidae is a new finding. The toads might have acquired the mycobacterial infection from their environment because *M. chelonae* can be found in soil and water. They may thus have been a fortuitous animal hosts for the mycobacterium. We are currently examining soil and water from the areas in which the toads were caught for the presence of *M. chelonae*.

The susceptibility of *B. marinus* and *B. granulosis* to *M. chelonae* was confirmed by the successful establishment of experimental infections. Frogs and toads are highly resistant to contagious cutaneous or systemic tuberculosis infection (Reichenbach-Klinke and Elkan, 1965). It was not surprising, therefore, that large inocula and prolonged observation periods were required for the manifestation of histopathological signs in captive toads. The liver and spleen were the most susceptible organs and gonads the most resistant. As amphibians do not possess a diaphragm, mechanical bathing of the viscera by the peritoneal fluid, and haematogenous and circulatory spread might all be involved in the systemic dissemination of the mycobacteria.

M. chelonae subsp. *abscessus* was isolated from the spleen, liver, kidney and peritoneal fluid of the wild toads. If there is an environmental source of infection for this mycobacterium, the microorganism may be absorbed through the richly vascularised skin and then circulate throughout the internal body cavity of the toads. Alternatively, because *B. marinus* and *B. granulosis* are more terrestrial than aquatic in their habits, mycobacteria may be ingested and then disseminated via the circulatory system. Experimental studies with metamorphosing tadpoles are presently being undertaken to attempt to elucidate the mode of mycobacterial entry into the organs of amphibia.

The absence of detectable histopathological lesions and the low frequency of naturally infected toads suggest that the host-parasite relationship between the toad and *M. chelonae* subsp. *abscessus* is benign and facultative. The relationship also appears to be host-specific because no mycobacterial infection was detected in frogs belonging to the Hylidae or Leptodactylidae frogs examined and *M. chelonae* subsp. *abscessus* was unable to persist in mouse tissues after experimental infection.

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