

***Mycobacterium pinnipedii* in a captive Southern sea lion (*Otaria flavescens*): a case report**

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ABSTRACT: *Mycobacterium pinnipedii* causes tuberculosis in free-living and captive pinniped species throughout the world. We report on the isolation of this *M. tuberculosis* complex (MTC) member from an imported male Southern sea lion (*Otaria flavescens*) in a zoo in the Czech Republic. Nodular granulomatous lesions were found in the lungs, pleura and mesenteric lymph nodes of this animal and *M. pinnipedii* was isolated from lung, mesenteric and submandibular lymph nodes. Identification of the isolates was confirmed using two independent molecular methods. Direct IS6110 PCR amplification confirmed the presence of an MTC member in these samples. Faecal and oral swabs from three living female sea lions were examined using direct IS6110 PCR and were all found to be negative. Twelve environmental samples were examined using direct microscopy after Ziehl-Neelsen staining and culture methods along with direct IS6110 PCR examination, all yielding negative results. Seven people that came into close contact with the infected animal were examined using a skin tuberculin test and chest x-ray, revealing no evidence of infection by a MTC member.

Keywords: tuberculosis; pinnipeds; epidemiology; zoonosis; water ecology

Tuberculosis in pinnipeds was first described in 1913 (Blair, 1913), but the causative agent remained unknown until 1986, when a *Mycobacterium*, further identified biochemically as *M. bovis*, was isolated from New Zealand fur seals (*Arctocephalus forsteri*) and Australian sea lions (*Neophoca cinerea*) found in a marine park in Western Australia (Forshaw and Phelps, 1991). Later, it was discovered that the isolates from these animals shared biochemical and phenotypic features with *M. bovis*, but differed in their genotypic characteristics and were referred to subsequently as “seal bacillus” (Cousins et al., 1990, 1993). With regard to these differences, the causative agent was then thought to be a separate species among *M. tuberculosis* com-

plex (MTC) members and the name “*M. pinnipedii*” was proposed (Cousins et al., 2003).

Since 1986, tuberculosis caused by *M. pinnipedii* has been found in seven captive and free living pinniped species, in Australia (including Tasmania), Argentina, France, Germany, The Netherlands, New Zealand, Uruguay and Great Britain. According to the literature, the most frequently affected pinniped species has been the Southern sea lion (*Otaria flavescens*), the majority of which has been kept in captivity. The lesions found in the animals comprised mainly of nodular granulomatous lesions with caseation in lung and thoracic lymph nodes, thus indicating the respiratory tract as the most likely route of infection in most of the ani-

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mals (Forshaw and Phelps, 1991; Cousins et al., 1993; Woods et al., 1995; Bernardelli et al., 1996; Thorel et al., 1998; Bastida et al., 1999; Cousins et al., 2003).

The presence of infected pinnipeds in some zoological gardens and marine parks was most probably the source of infection for other animals and humans. During the period between 1992 and 1995, *M. pinnipedii* was diagnosed in two snow leopards (*Panthera uncia*), as well as two Amur leopards (*Panthera pardus orientalis*) and one Southern sea lion in a French zoo. The felid enclosure was localised in the neighbourhood of the basin for sea lions and most probably, formed the source of *M. pinnipedii* infection for leopards from an aerosol formed by a pressure washer used to clean the basin (Moisson et al., 1998; Thorel et al., 1998).

Three different animal species, one Brazilian tapir (*Tapirus terrestris*), one lama (*Lama glama*) and two lowland gorillas (*Gorilla gorilla gorilla*; Cousins et al., 2003; Cousins, 2006) from a zoo in Great Britain most probably became infected from two South American fur seals (*Arctocephalus australis*). An infected Southern sea lion was most probably the source of *M. pinnipedii* infection for one Malayan tapir (*Tapirus indicus*) and a Bactrian camel (*Camelus bactrianus*) in a German zoo (Moser et al., 2008).

M. pinnipedii infection has also been described in humans. People who were in close contact with the infected animals (mainly zookeepers) either through direct contact with the infected pinnipeds or exposure to an environment contaminated with *M. pinnipedii*, e.g., during the cleaning of pinniped enclosures, were found to be infected using a number of different methods (Thompson et al., 1993; Thorel et al., 1998; Kiers et al., 2008; Moser et al., 2008).

Differentiation between MTC members requires the use of molecular methods because there is very little possibility of distinguishing between them using only their phenotypic features, biochemical features, or drug sensitivity. The added possibility of conducting various epidemiological studies is an additional merit of molecular methods (Haddad et al., 2004). In the last few years several molecular studies on the differentiation between the MTC members were performed. Djelouadji et al. (2008) have developed a single-step method based upon sequencing the Exact Tandem Repeat D sequence in the genome of MTC members, distinguishing seven out of eight MTC members. However, it was not possible to distinguish *M. pinnipedii* from

M. microti. Bigi et al. (2005) identified two specific genomic deletions (PiD1 and PiD2) in *M. pinnipedii*, which can be used to determine its presence. A further method for identifying *M. pinnipedii*, based on the detection of differing genomic regions (RD1^{mic} and RD2^{seal}), was described one year later (Warren et al., 2006).

The aims of this paper were (i) to describe the first ever case of *M. pinnipedii* infection in a captive Southern sea lion (*Otaria flavescens*) kept in a zoo in the Czech Republic, confirmed by the two molecular methods mentioned above; (ii) to investigate the remaining sea lions exposed to the infected individual; and (iii) to investigate the environment of the zoo for *M. pinnipedii* contamination, with regard to the health risk for zoo staff and other animals.

Case description

The colony of Southern sea lions in the zoo numbered four animals. The seven year-old male sea lion, imported from a zoo in Germany in 2005, died during gastric surgery performed for the removal of a foreign body in November 2009. The animal did not have a healthy physical appearance, but neither did it show any signs of pulmonary disease before surgery. During the subsequent autopsy, nodular granulomatous lesions of various sizes were found on the left lung, as well as on the

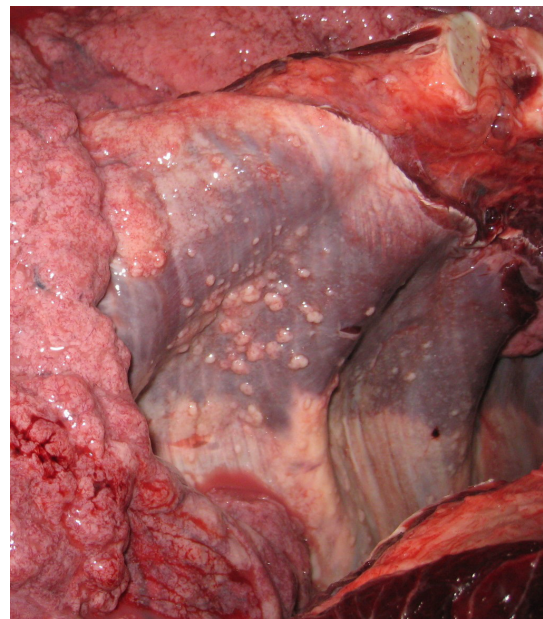


Figure 1. Nodular lesions on the serosal membrane lining the diaphragm (Photo V. Barnett)

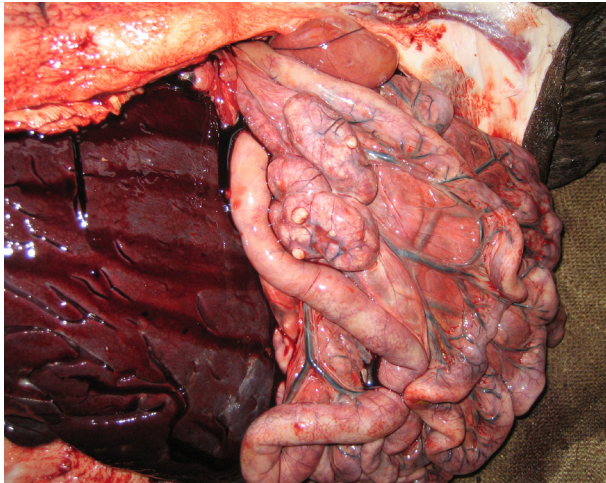


Figure 2. Enlarged mesenteric lymph nodes with nodular lesions (Photo V. Barnett)

serosal membrane lining the diaphragm (Figure 1). Nodular granulomatous lesions with mineralization were found in the mesenteric lymph nodes (Figure 2). Samples taken from the lesioned lung lobe, mesenteric and submandibular lymph nodes underwent both culture and direct IS6110 PCR examinations. Additional samples of laryngeal mass, tracheal mucus, masseter and urinary bladder were examined using direct IS6110 PCR (Table 1).

The three remaining female sea lions were in a good state of health and appearance and did not display any clinical symptoms suggestive of pulmonary disease. The two adult females were 17 years old and have lived in the zoo since 1996, when they were bought from a merchant in Great Britain (both were captured in Uruguay). The juvenile female

(the offspring of the male sea lion and one of the two adult females) was born in the Czech zoo, one year before the male died. Oral swabs and faeces were collected from the three females for direct IS6110 PCR examination.

The Southern sea lions were kept in an enclosure with an outdoor pool. Water for the pool was drawn from a forest lake around the vicinity of the zoo. It was then cleaned by passing it through a water treatment device (for the removal of scum) and subsequently used for various cleaning purposes, e.g., for elephant or panda paddocks. In parallel to this, the water was also run off through an outlet into a lake with water birds. A total of 12 environmental samples were collected from different places in the zoo (Table 2, Figure 3).

The samples obtained from the lymph nodes and lung of the male sea lion were smeared and stained using fluorochromes (Auramine O and Rhodamine B) and examined by fluorescence microscopy. Acid-fast bacilli (AFB) were detected in lung tissue and in the submandibular lymph node.

Culture examination of the samples was performed in two independent laboratories. In the first laboratory, the samples were decontaminated using a modified Petroff's method. Briefly, 4 ml of 2M NaOH was added to each sample. The suspension was shaken for 15 min and then centrifuged (20 min, 4000 × g). Fifteen millilitres of 0.1M HCl was added to the sediment. After centrifugation, the sediment was resuspended in 0.5 ml of sterile distilled water and then inoculated onto two solid culture media (Loewenstein-Jensen and Ogawa) and one liquid culture system (Bactec MGIT 960

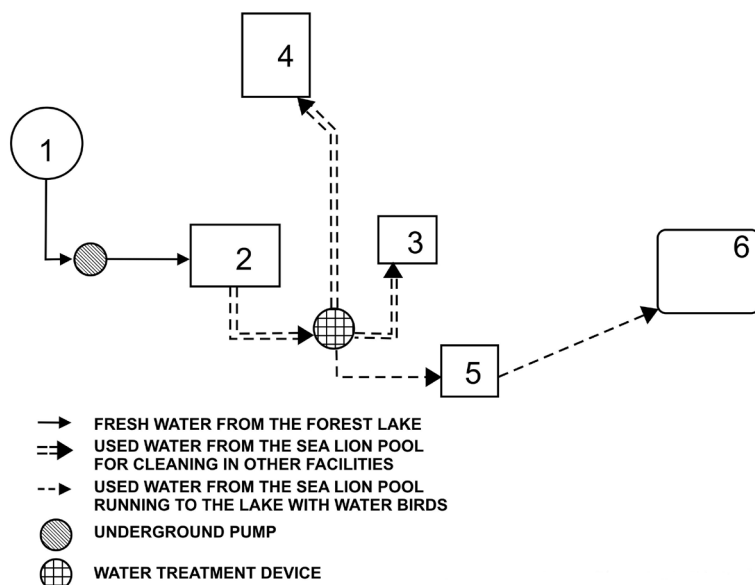


Figure 3. Sampling sites in the zoo (numbers 1 to 6 correspond with marking of sampling sites in Table 2)

Table 1. Examination of the male sea lion's tissues

Origin	Sample	Direct PCR ^a	Culture	Isolate ^b
Respiratory tract	submandibular LN	+	+	<i>M. pinnipedii</i>
	lungs	+	+	<i>M. pinnipedii</i>
	laryngeal mass	–	nt	
	tracheal mucus	–	nt	
Head	masseter muscle	–	nt	
Gastrointestinal tract	mesenteric LN	+	+	<i>M. pinnipedii</i>
Urogenital tract	urinary bladder	–	nt	

LN = lymph node; nt = not tested

^aPCR detection of IS6110 (EliGene MTB RT, Elisabeth Pharmacon, Czech Republic)

^bisolate identification performed using the method described by Warren et al. (2006) and confirmed by the method described by Bigi et al. (2005)

Mycobacterial Detection System; Becton Dickinson, USA). In the second laboratory, tissue samples (approx. 2 cm³) were homogenized and then decontaminated with 7 ml of 1M HCl. The suspension was poured into a test tube through a section of sterile gauze. After 15 min 2–3 droplets of Bromothymol blue were added to each sample and the suspension was neutralized with 1M NaOH. Upon centrifugation (20 min, 3000 × g), the supernatant was discarded and the sediment was resuspended in 3 ml of sterile physiological solution and inoculated onto culture media (one Stonebrink with crystal violet without glycerol; two Loewenstein-Jensen media, one liquid Sula medium and one liquid Sula medium with neotetrazolium chloride). Incubation was performed for two months and mycobacterial colony growth was recorded every two weeks.

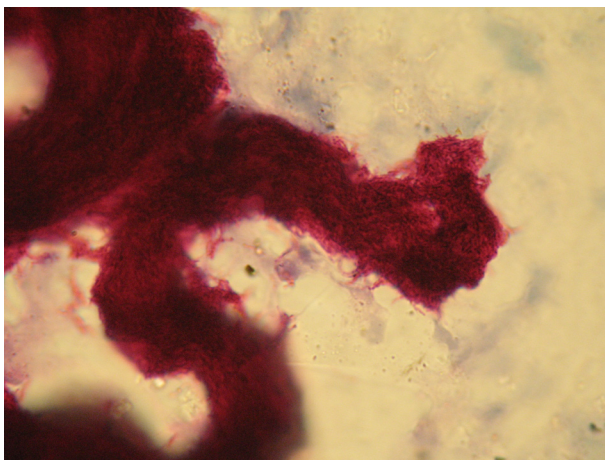


Figure 4. "Cords" of *M. pinnipedii* grown in the Bactec MGIT medium (ZN; 1000× magnification; Photo P. Kriz)

M. pinnipedii was isolated from lung tissue, submandibular and mesenteric lymph nodes in both laboratories and identified in parallel using two independent molecular methods (Bigi et al., 2005; Warren et al., 2006). The presence of an MTC member in all three tissues was confirmed by direct IS6110 PCR (Table 1). Culture isolation was successful in the automated system Bactec MGIT 960 and on solid culture media in both laboratories (Loewenstein-Jensen, Ogawa and Stonebrink with crystal violet without glycerol). Microscopic examination of the isolate grown in the Bactec MGIT medium revealed cords of mycobacterial cells (Figure 4). The isolate was also susceptible to the major anti-tuberculosis drugs (isoniazid, rifampicin, streptomycin, ethambutol and pyrazinamide). Direct IS6110 PCR testing yielded negative results in the remaining tissue samples from the male sea lion and oral swabs and faeces from the three females.

The smears made from environmental samples were stained according to the Ziehl-Neelsen (ZN) method for acid-fast bacilli (AFB) detection and observed under oil immersion with 1000 × magnification. For each sample, at least 100 fields of view were evaluated. AFB were not found in any environmental sample.

Environmental samples were decontaminated and cultured as described previously (Matlova et al., 2003). No mycobacterial growth was observed after culture examination of any of the environmental samples and direct IS6110 PCR also gave negative results for MTC members in all but one sample, in which inhibition of the PCR reaction occurred (Table 2).

Table 2. Examination of environmental samples from the zoo

Sampling site (details shown in Figure 3)	Sample	Location	ZN ^a	Culture ^b	IS6110 PCR ^c
(1) Water source for zoo	sediment	brook bed	–	–	inh.
	sludge	sewerage	–	–	–
(2) Sea lion enclosure	cobwebs	upon entre doors	–	nt ^d	–
	cobwebs	upon doors to paddock for sea lions	–	nt ^d	–
	pooled faeces	floor	–	–	–
(3) Room for preparation of feed	cobwebs	wall	–	nt ^d	–
	sludge	sewerage	–	–	–
(4) Water ditch in paddock for elephants	sediment	bottom	–	–	–
(5) Water outlet from sea lions' enclosure	fern	in the outlet	–	–	–
	biofilm	outlet wall under water surface	–	–	–
(6) Lake	sediment	bottom near bank	–	–	–
	biofilm	stone in water	–	–	–

^adirect microscopy after the Ziehl-Neelsen staining

^bculture performed using two solid media (Herrold and Stonebrink) and one liquid medium (Sula)

^cdirect IS6110 PCR method (EliGene MTB RT, Elisabeth Pharmacon, Czech Republic); results are given as “+” for presence or “–” for absence of any member of *Mycobacterium tuberculosis* complex

^dsamples not tested because their little amounts were used for IS6110 PCR examination

Seven people came into close contact with the male sea lion: three veterinary practitioners and four zookeepers. They were all subjected to a tuberculin skin test Mantoux II and chest x-ray examination. No evidence of tuberculous infection was detected by any of the diagnostic methods used on them.

DISCUSSION AND CONCLUSIONS

This is the first ever report of *M. pinnipedii* infection in pinnipeds found in the Czech Republic. The infected male of the Southern sea lion species was originally imported from a German zoo four years before the diagnosis. As both parents of the male died from tuberculosis in Germany, it is very likely that they were the source of infection for their offspring (respective zookeeper, personal communication). Both adult females were captured on Uruguay's coastal waters as juveniles in 1992 and were imported into the Czech Republic in 1996. Southern sea lions concentrate along rookeries on the eastern coast of South America, including Uruguay, where they can also come into contact with other pinniped species (Bernardelli et al.,

1996). It is highly probable that if they were exposed to or infected by any MTC member as juveniles from other free-living pinnipeds, the infection would present as clinical disease sooner or later, after the animal underwent certain stress conditions, such as those created by import or delivery. Infection with *M. pinnipedii* was not demonstrated in them, although they lived for four years in close contact with the infected male. However, repeated examinations of faeces and oral swabs were recommended to be undertaken trimonthly.

The infected male did not display any clinical symptoms suggestive of tuberculosis and thus the prolonged latency of the infection may be of greater concern when considering the possibility of shedding *M. pinnipedii* into the environment. *M. pinnipedii* was found in the lungs, which corresponds to a respiratory route of infection, similar to many other cases of infected pinnipeds around the world (Bernardelli et al., 1996; Moser et al., 2008). Notably, it was not found in the sample of tracheal mucus, but the presence of *M. pinnipedii* in the submandibular and mesenteric lymph nodes suggests that it could have been, shed by sputum and then swallowed.

To evaluate the potential risk of infection to sea lions and other animals, as well as to humans, we decided to screen the zoo environment for the presence of *M. pinnipedii*. According to the recommendations of previous studies we mainly collected samples of cobwebs, biofilms and sediments, where mycobacteria are said to be most prevalent within the environment (Kaevska et al., 2011). We obtained negative results for both ZN microscopy and culture in all samples. Direct IS6110 PCR examination confirmed the negative results. Thus, it seems likely that the health risk for zoo staff and other animals in the vicinity of the sea lion enclosure as well as in paddocks cleaned using water from the sea lion pool was low.

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