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

P. Kriz¹, P. Kralik¹, M. Slany¹, I. Slana¹, J. Svobodova², I. Parmova³, V. Barnet⁴, V. Jurek⁴, I. Pavlik¹

¹Veterinary Research Institute, Brno, Czech Republic
²Regional Institute of Public Health, Brno, Czech Republic
³State Veterinary Diagnostic Institute, Prague, Czech Republic
⁴Private veterinary practitioner, Czech Republic

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* complex (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-
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 located 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 (RD1mic and RD2seal), 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 serosal membrane lining the diaphragm (Photo V. Barnet).
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...
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.

*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 also was 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.

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 1. Examination of the male sea lion’s tissues**

<table>
<thead>
<tr>
<th>Origin</th>
<th>Sample</th>
<th>Direct PCR</th>
<th>Culture</th>
<th>Isolate</th>
</tr>
</thead>
<tbody>
<tr>
<td>Respiratory tract</td>
<td>submandibular LN</td>
<td>+</td>
<td>+</td>
<td><em>M. pinnipedii</em></td>
</tr>
<tr>
<td></td>
<td>lungs</td>
<td>+</td>
<td>+</td>
<td><em>M. pinnipedii</em></td>
</tr>
<tr>
<td></td>
<td>laryngeal mass</td>
<td>−</td>
<td>nt</td>
<td></td>
</tr>
<tr>
<td></td>
<td>tracheal mucus</td>
<td>−</td>
<td>nt</td>
<td></td>
</tr>
<tr>
<td>Head</td>
<td>masseter muscle</td>
<td>−</td>
<td>nt</td>
<td></td>
</tr>
<tr>
<td>Gastrointestinal tract</td>
<td>mesenteric LN</td>
<td>+</td>
<td>+</td>
<td><em>M. pinnipedii</em></td>
</tr>
<tr>
<td>Urogenital tract</td>
<td>urinary bladder</td>
<td>−</td>
<td>nt</td>
<td></td>
</tr>
</tbody>
</table>

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)

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

---

Table 2. Examination of environmental samples from the zoo

<table>
<thead>
<tr>
<th>Sampling site (details shown in Figure 3)</th>
<th>Sample</th>
<th>Location</th>
<th>ZN&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Culture&lt;sup&gt;b&lt;/sup&gt; IS&lt;sub&gt;6110&lt;/sub&gt; PCR&lt;sup&gt;c&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>(1) Water source for zoo</td>
<td>sediment</td>
<td>brook bed</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td></td>
<td>sludge</td>
<td>sewer bed</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td></td>
<td>cobwebs</td>
<td>upon entre doors</td>
<td>–</td>
<td>nt&lt;sup&gt;d&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>cobwebs</td>
<td>upon doors to paddock for sea lions</td>
<td>–</td>
<td>nt&lt;sup&gt;d&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>pooled faeces</td>
<td>floor</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>(2) Sea lion enclosure</td>
<td>cobwebs</td>
<td>wall</td>
<td>–</td>
<td>nt&lt;sup&gt;d&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>sludge</td>
<td>sewerage</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>(3) Room for preparation of feed</td>
<td>cobwebs</td>
<td>wall</td>
<td>–</td>
<td>nt&lt;sup&gt;d&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>sludge</td>
<td>sewerage</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>(4) Water ditch in paddock for elephants</td>
<td>sediment</td>
<td>bottom</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>(5) Water outlet from sea lions’ enclosure</td>
<td>fern</td>
<td>in the outlet</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td></td>
<td>biofilm</td>
<td>outlet wall under water surface</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>(6) Lake</td>
<td>sediment</td>
<td>bottom near bank</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td></td>
<td>biofilm</td>
<td>stone in water</td>
<td>–</td>
<td>–</td>
</tr>
</tbody>
</table>

<sup>a</sup>Direct microscopy after the Ziehl-Neelsen staining  
<sup>b</sup>Culture performed using two solid media (Herrold and Stonebrink) and one liquid medium (Sula)  
<sup>c</sup>Direct IS<sub>6110</sub> 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  
<sup>d</sup>Samples not tested because their little amounts were used for IS<sub>6110</sub> PCR examination
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 \textit{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.

**Acknowledgement**

We would like to thank Mariana Blahutkova (Veterinary Research Institute, Brno, Czech Republic) for her help with sample collection and Adil Hussain (University of Birmingham, United Kingdom) for grammatical and language corrections.

**REFERENCES**


cobacterium pinnipedii: transmission from South American sea lion (Otaria byronia) to Bactrian camel (Camelus bactrianus bactrianus) and Malayan tapirs (Tapirus indicus). Veterinary Microbiology 127, 399–406.


Received: 2011–01–25
Accepted after corrections: 2011–06–30

Corresponding Author:
Prof. MVDr. Ivo Pavlik, CSc., Veterinary Research Institute, Department of Food and Feed Safety, Hudcova 70, 621 00 Brno, Czech Republic
Tel. +420 533 331 601, Fax +420 541 211 229, E-mail: pavlik@vri.cz