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Use of Multiple Diagnostic Tests to Detect *Mycobacterium pinnipedii* Infections in a Large Group of South American Sea Lions (*Otaria flavescens*)

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Abstract

Since 2000, Heidelberg Zoo has been dealing with tuberculosis caused by *Mycobacterium pinnipedii* within its collection of South American sea lions (*Otaria flavescens*). Recently, more cases became known all across Europe. Various diagnostic methods, including microscopy, PCR, and culture of sputum samples; three serological tests (ElephantTB STAT-PAK® assay, multiantigen print immunoassay [MAPIA], and dual path platform assay [DPP]); and diagnostic imaging, were used to examine 14 animals. *M. pinnipedii* infection was strongly suspected *antemortem* based on the diagnostic results and was confirmed at necropsy in 10 sea lions. ElephantTB STAT-PAK® assay, MAPIA, and DPP test showed the diagnostic potential for rapid detection of this disease in live sea lions. The highest sensitivity was achieved when applying more than one test.

Key Words: diagnosis, *Mycobacterium pinnipedii*, South American sea lions, *Otaria flavescens*, tuberculosis

Introduction

Historically, tuberculosis in pinnipeds has been reported as early as 1965 (Ehlers, 1965). Since then, pinniped tuberculosis (*Mycobacterium pinnipedii*) has occurred in a variety of wild and captive sea lions and fur seals, including Australian sea lions *(Neophoca cinerea*) in Australia; South American sea lions (*Otaria flavescens*) in Argentina, Uruguay, and France; New Zealand sea lions (*Phocarctos hookeri)* in New Zealand; South American fur seals *(Arctocephalus australis*) in Argentina, Uruguay, and the UK; New Zealand fur seals *(A. forsteri*) in Australia and New Zealand; an Australian fur seal (*A. pusillus doriferus*) in Australia; and as a possible common link in the Southern hemisphere in a wild subantarctic fur seal *(A. tropicalis*) in Argentina (Cousins, 2006; Gomis et al., 2008; Lacave et al., 2009). Initially, the seal isolates were determined to be most compatible with *M. bovis* as biochemical testing clearly confirmed the relationship to the *M. tuberculosis* complex, but since the MPB70 protein of *M. bovis* was absent from all isolated strains, they were identified as *M. bovis* "subtype seal" (Cousins et al., 1990, 1993, 2003; Harboe et al., 1990; Forshaw & Phelps, 1991). The restriction fragment length polymorphism (RFLP) technique showed that all pinniped isolates were indeed different from other members of the *M. tuberculosis* complex as well (Cousins et al., 1993).

Although *M. pinnipedii* affects pinnipeds primarily, it has shown pathogenicity in other species, including humans (Thompson et al., 1993; Cousins, 2006). In pinnipeds, clinical signs are usually absent or nonspecific despite extensive tissue involvement (Gomis et al., 2008). In other exotic animals, advanced cases may show anorexia, emaciation, dyspnea, and coughing (Thoen, 1993; Bengis, 1999; Kaneene & Thoen, 2004). Affected animals may shed the bacteria by respiratory aerosols and close contact (Cousins et al., 1993; Thoen & Barletta, 2006).

Gross *postmortem* findings include granulomatous lesions, mostly in thoracic, hepatic,

and gastrointestinal lymph nodes. The organs commonly involved are lungs, pleura, liver, spleen, and peritoneum (Cousins et al., 1993; Cousins, 2006). Pyothorax has also been reported in the literature (Cousins et al., 1993; West, 2006). Clinical diagnosis may be difficult in some animals because lesions may be limited to only a single deep lymph node (Kaneene & Thoen, 2004).

Sputum or tracheal wash samples can be microscopically examined using special staining methods, including acid fast and fluorescent antibody, to help in detecting shedding animals (Kaandorp, 1998). However, it may be difficult to detect acid-fast bacilli (AFB) using this method because at least 1,000 organisms/ml are necessary, yielding a low sensitivity of microscopy (Mikota & Maslow, 1997; Lange et al., 2006). The polymerase chain reaction (PCR) technique can produce results faster, but they are not validated for pinniped tuberculosis. In case of a positive microscopic result of a human sputum sample, the sensitivity is almost 100% (Lange et al., 2006). PCR detects bacterial DNA, even from dead organisms, and it is able to distinguish *M. tuberculosis* complex members from nontuberculous mycobacteria (Mikota & Maslow, 1997; Thomson, 2006). Mycobacterial culture still remains the diagnostic gold standard test; however, it may take up to 8 wks to grow (West, 2006). Tuberculin skin testing has been performed on flipper skin and eyelids of pinnipeds with limited success (Castro Ramos et al., 1998). It relies on a delayed-type hypersensitivity reaction to mycobacterial antigens and does not differentiate between a former infection and an active disease (Kaandorp, 1998; Bengis, 1999).

Serological techniques may be of potential value for detection of mycobacterial infection because they are simple, rapid, and relatively non-invasive and can be accurate as long as appropriate antigens and immunoassay formats are used (Lyashchenko et al., 2000). Multi-antigen print immunoassay (MAPIA) and an ElephantTB STAT-PAK® assay for tuberculosis in elephants demonstrated encouraging performance in various exotic animal species (Miller, 2008).

This paper describes the investigation of multiple cases of pinniped tuberculosis in South American sea lions (*O. flavescens*) and the concurrent use of several diagnostic tests to identify the causative organisms and demonstrate disease-free status in the remaining herd members.

Materials and Methods

Animals

The South American sea lions in this study were kept at the Heidelberg Zoo in Heidelberg, Germany. The original group at the Heidelberg Zoo in 2006 consisted of 10 individuals, all housed together. After an outbreak of pinniped tuberculosis at Le Pal Zoo in Le Pal, France, in 2007, three sea lions—two adult females and one subadult female—were moved to the group at the Heidelberg Zoo. One adult female sea lion at Tierpark Hagenbeck was included in the study because it was born in Heidelberg and was supposed to leave Hamburg to return to Heidelberg in 2008. Heidelberg has been keeping *O. flavescens* since 1974. So far, 18 animals have been born, and three of these were stillborn. Twelve animals were wild caught or of unknown origin during this time period. In 2000 and 2001, two animals died of pulmonary disease, later confirmed to be pinniped tuberculosis since *M. pinnipedii* had not yet been identified as such before 2003. After a disease outbreak of *M. pinnipedii* in Malayan tapirs (*Tapirus indicus*) housed in an adjacent exhibit in 2006, a complete investigation among the sea lion group was conducted.

Sputum

Animals were trained to give sputum samples by coughing on command. Parts of sputum and/or saliva were also scraped from the sides of their mouth. Acid-fast staining, PCR, and cultures were performed.

ElephantTB STAT-PAK® Assay

ElephantTB STAT-PAK® assay (Chembio Diagnostic Systems, Inc., Medford, NY, USA), a lateral-flow antibody detection test employing selected *M. tuberculosis* antigens and a blue latex signal detection system, was performed as previously described (Lyashchenko et al., 2006). The test required 30 µl of serum and 3 drops of sample buffer (included in the kit) that were added to the device sequentially. Results were read visually 20 min later. Any visible band in the test area, in addition to the control line, was considered an antibody positive result, whereas no test band was considered a negative result.

Dual Path Platform (DPP) Assay

A new generation point-of-care TB test has been developed using Chembio DPP® technology (Greenwald et al., 2009). The assay has three separate lines—(1) MPB83, (2) CFP10/ESAT-6, and (3) TBF10—and one control line. The DPP assay was performed using 5 µl of serum, 2 drops of buffer added to the sample well, and 4 drops of buffer added to the conjugate well. Results were read visually at 15 min by two independent operators who did not know the true infection status of the animals. Visible reactivity with any of the 3 antigen bands observed at 15 min was considered an antibody positive result. No reactivity with the test antigens was taken as a negative result.

Multi-Antigen Print Immunoassay (MAPIA)
The test was performed as previously described using a panel of 12 proteins of *M. tuberculosis* and horse-radish peroxidase-conjugated protein A (Sigma) along with 3,3',5,5'-tetramethyl benzidine (Kirkegaard & Perry Laboratories, Gaithersburg, MD, USA; Lyashchenko et al., 2006). The following recombinant antigens were immobilized on nitrocellulose membrane: ESAT-6 and CFP10 proteins as well as hybrids CFP10/ESAT-6 and Acr1/MPB83 produced at the Statens Serum Institut (Copenhagen, Denmark); MPB59, MPB64, MPB70, and MPB83 produced at the Veterinary Sciences Division (Stormont, UK); alpha-crystallin (Acr1) and the 38 kDa protein purchased from Standard Diagnostics (Seoul, South Korea); native MPB83 protein supplied by the Veterinary Laboratories Agency (Weybridge, UK); and Mtb8 and Mtb48 proteins and polyepitope fusion TBF10 developed by Corixa Corp. (Seattle, WA, USA). MAPIA results were scored by two independent operators who did not know the true infection status, with a visible band of any intensity being read as a positive reaction.

Computed Tomography (CT) Scan
In Heidelberg, CT scans were carried out at the German Cancer Research Institute on a Toshiba Aquilion (Toshiba Medical Systems, Neuss, Germany). For Hamburg, CT scans were performed on a Philips Tomoscan 7500 at the small animal clinic Magunna, Magunna and Nickel in Norderstedt (Philips Medical Systems, The Netherlands).

To be able to perform the thoracic CT scans, the animals were premedicated with a combination of medetomidine, midazolam, and butorphanol; intubated; and maintained on isoflurane. After the procedures were performed, anesthesia was reversed with flumazenil, atipamezole, and naltrexone.

Radiography
Due to the size of one of the male South American sea lions, CT scanning was impossible; therefore, radiographs were taken by digital radiography. This procedure was performed at a clinic for horses—Walliser in Kirchheim, Germany.

Necropsy and Histopathology
Six of the necropsy examinations were performed within 12 h of the animals' deaths at the Chemisches und Veterinäruntersuchungsamt Heidelberg, known as CVUA Heidelberg, a regional public veterinary and food control service laboratory. Two male sea lions were necropsied at the University of Giessen due to their large size, and one female sea lion was necropsied at a private veterinary pathology practice in Hamburg. Samples of lesions and nonlesional tissues (lymph nodes and lungs) were collected and shipped to the Friedrich-Loeffler-Institute, the Federal Research Institute for Animal Health. Samples of other organs (i.e., spleen, liver, intestine, heart, brain, and urinary bladder) were examined at the institutions that performed the necropsies.

Microbiology
Ziehl Neelsen staining was conducted on tissue impression smears to detect AFB according to standard procedures. For culture testing, tissue samples were homogenized; decontaminated with NALC (0.5%)-NaOH (2%)-Na-citrate (1.45%) for 20 min at room temperature; and neutralized twice with PBS. The sediment was then inoculated onto Stonebrink agar containing pyruvate and Lowenstein-Jensen agar, both containing PACT (polymyxin B, amphotericin B, carbenicillin, trimethoprim), as well as MGIT liquid medium (BD, Heidelberg, Germany), and then cultivated for 12 to 14 wks at 37° C.

Polymerase Chain Reaction (PCR)
PCR was used to identify mycobacterial growth. DNA was extracted in parallel from tissue using the DNeasy Blood and Tissue Kit (Qiagen, Hilden, Germany), according to the manufacturer's instructions, except that proteinase K treatment was extended overnight followed by heating at 95° C for 15 min to inactivate the pathogens. Mycobacterial DNA in tissue extracts was detected using an in-house nested PCR (Moser et al., 2008). DNA from culture was prepared by ultrasonication and boiling of heat-inactivated mycobacteria for 10 min each. PCR was performed according to Rodriguez et al. (1999) using primer sequences modified according to the GenBank database (Moser et al., 2008). The following sequences were used: 5'- GAA CCC GCT GAT GCA AGT GC-3' as forward and 5'- ACG CCG CTG ACC TCA AGA AG-3' as reversed primer. PCR was run using the following cycling conditions: a denaturation step at 96° C for 60 s, followed by 30 cycles of 96° C for 15 s, 63° C for 60 s, 72° C for 60 s, and a final extension at 72° C for 300 s. An amplicon of 499 bp was generated (Moser et al., 2008). The nested PCR based on the modified PCR of Rodriguez at al. (1999) was performed using 5'-GCA AGT GCC ACA ATG CTG-3' as forward and 5'-CGA ACG CTT CGA CCA GCT CG-3' as reversed primer. A 435 bp DNA stretch was amplified. The following cycling conditions were used: a denaturation step at 96° C for 60 s, followed by 30 cycles of 96° C for 15 s, 67° C for 60 s, 72° C for 60 s, and a final extension at 72° C for 300 s (Moser et al., 2008).

Spoligotyping
Spoligotyping (Isogen Bioscience, The Netherlands) was performed using DNA extracted from pure cultures targeting the spacers 1 through 43 (Kamerbeek et al., 1997).

MIRU/VNTR-Typing
MIRU/VNTR-typing was performed targeting 26 chromosomal loci (Anonymous, 2006; Supply et al., 2006). The MIRU copy numbers were determined by gel electrophoresis.

Treatment
Animals that were treated received a combination of rifampicin (7.5 mg/kg) and isoniazide (5 mg/kg) orally once per day. One animal (Case 10) was treated with a triple combination, adding myambutol at a dosage rate of 15 mg/kg. This animal had seroconverted within the last 6 mo and was actively shedding mycobacteria at the time of treatment initiation. All of the animals were treated for 14 mo or until euthanasia was performed after calcified lesions were detected in CT scans.

Results

Clinical and diagnostic findings are summarized in Table 1. Based on the results of PCR and culture, as the gold standard methods for diagnosing animal tuberculosis, 10 sea lions were determined to be infected with *M. pinnipedii*. Six out of 10 animals were culture positive for *M. pinnipedii*. In the remaining four, histopathology was consistent with tuberculosis and, in addition to that, PCR produced positive results for *M. tuberculosis*-complex. All of the animals were in good nutritional condition.

Case 1
A 14-y-old wild-born female South American sea lion showed anorexia and respiratory distress in June 2006. Radiographic examination revealed a diffuse pneumonia. On the following day, the animal was found dead. Necropsy findings included an opaque, tan fluid thoracic effusion, bronchi filled with creamy purulent debris, a right lung lobe containing white miliary foci, and severe hyperplasia of the pulmonary lymph nodes.

Histopathological findings included a severe purulent to necrotizing pneumonia with calcified foci and a severe suppurative lymphadenitis. AFB were detected in the lesions, and *M. pinnipedii* was isolated 10 wks later.

Case 2
A 16-y-old wild-born female was noticed to cough occasionally. Microscopy examination of a sputum sample revealed AFB, and PCR identified the organism as a member of the *M. tuberculosis* complex.

The animal was anaesthetized, and a blood sample was taken from the right caudal gluteal vein. ElephantTB STAT-PAK® assay performed on serum showed a positive result within a few minutes. The decision was made to euthanize the animal.

Significant necropsy findings included atrophy of the left lung lobe, which also contained white miliary foci. The right lung lobe contained small nodules 1 to 2 cm in size. Furthermore, a severe ulcerative bronchitis and tracheitis were detected. Histopathological findings included a severe ulcerative tracheitis, a severe purulent lymphadenitis, and a severe granulomatous and suppurative pneumonia. No AFB was detected. *Streptococcus canis* was isolated from the lesions. Additional samples of lung tissue were sent in for culture and sequencing. Three months later, *M. pinnipedii* was isolated.

Serological techniques, performed *postmortem*, showed serum antibodies against ESAT-6, CFP-10, and MPB83 proteins.

Case 3
ElephantTB STAT-PAK® assay and MAPIA were conducted in a wild-born 17-y-old male in August 2006 and produced antibody negative results. During follow-up testing in February 2007, the ElephantTB STAT-PAK® assay remained negative, but the MAPIA classified the animal as a suspect for tuberculosis infection. A sputum sample taken 3 mo later showed the presence of AFB, while PCR confirmed the *M. tuberculosis* complex followed by identification of *M. pinnipedii* by culture. At that time, the ElephantTB STAT-PAK® assay and MAPIA were both positive. A decision was made to initiate a treatment for the whole group of sea lions. Due to the animal's advanced age, Case 3 was excluded from treatment, and the animal was euthanized in June 2007.

Necropsy findings included a severe granulomatous pneumonia and enlarged pulmonary lymph nodes with white nodules 1 to 2 cm in diameter. Histopathological findings indicated a chronic pyogranulomatous pneumonia and chronic pyogranulomatous lymphadenitis. No AFB was detected at necropsy, but *M. pinnipedii* was isolated 10 wks later.

Case 4
A wild-born 11-y-old female tested MAPIA-negative in October 2006 but started showing weak antibody response by this assay in February 2007. The ElephantTB STAT-PAK® assay was nonreactive at that time. In June 2007, examination of a sputum sample produced no AFB. PCR was inconclusive (due to nonspecific inhibitors), but the culture revealed *M. pinnipedii*.

By that time, the MAPIA showed progressive seroconversion, while the ElephantTB STAT-PAK®

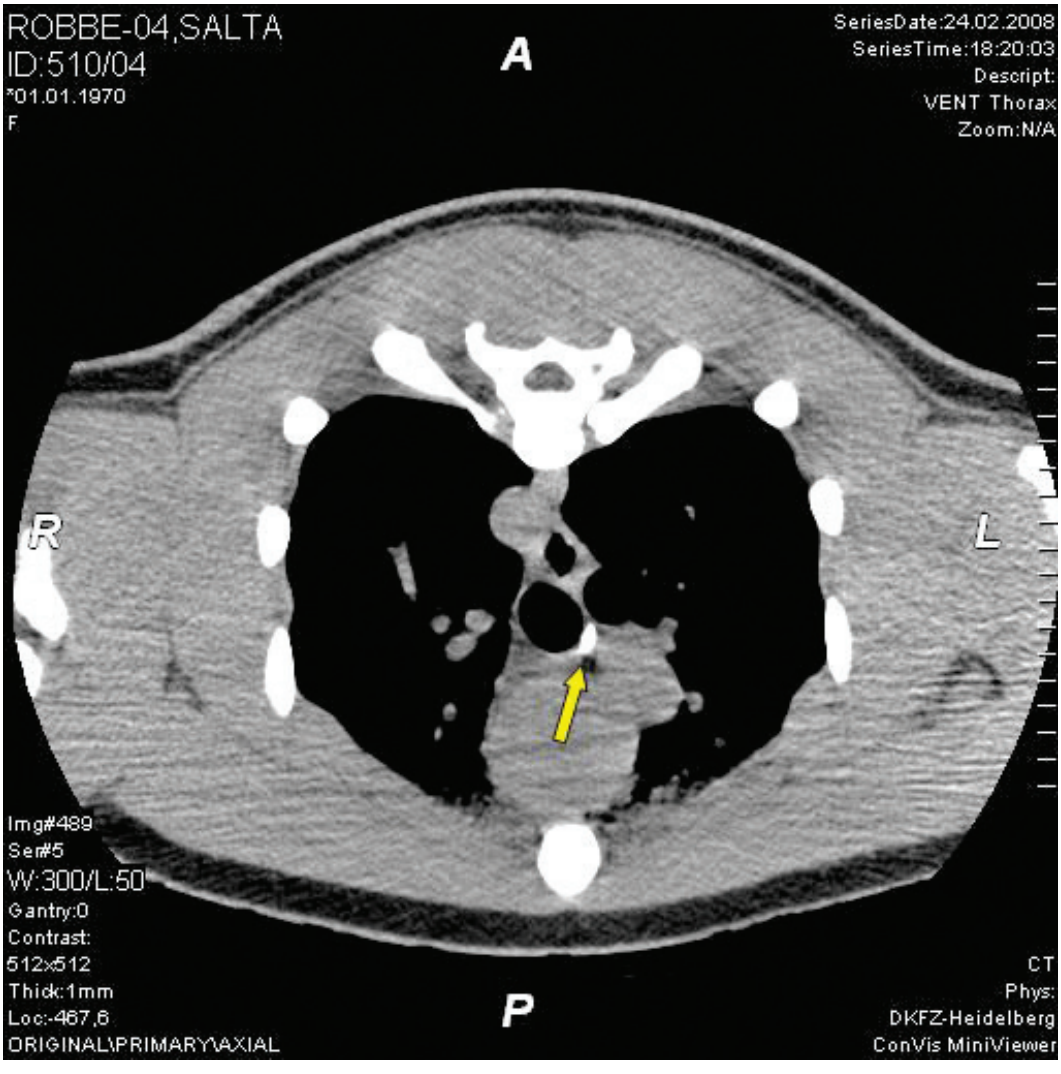

Figure 1. CT scan of female sea lion; yellow arrow is pointing to calcified mediastinal lymph node.

assay remained negative. Treatment was initiated. In July 2007, the animal gave birth, but the female offspring died 2 wks later for unknown reasons. The placenta was found positive for mycobacteria-like bacteria by culture. Using PCR, these were identified as *Gordonia* sp. After 9 mo of treatment, a thorough examination was performed. No AFB was found in a sputum sample. PCR and culture remained negative. The ElephantTB STAT-PAK® assay and MAPIA were reactive. A DPP test showed antibodies to MPB 83, E6/P10, and F10. A CT scan detected foci of calcifications in the mediastinal lymph nodes. Euthanasia was performed.

Significant necropsy findings included moderately enlarged cranial mediastinal lymph nodes with dry yellow masses within the parenchyma. Histopathology revealed a granulomatous lymphadenitis. Microscopy showed AFB, but direct PCR and culture results were negative.

Case 5

A 1-y-old female, born in Heidelberg, tested negative by ElephantTB STAT-PAK® assay, MAPIA, and DPP in June 2007. Sputum examination by microscopy was initiated, and PCR and culture produced negative results. Nevertheless, treatment was initiated, and the animal was re-examined 11 mo later. ElephantTB STAT-PAK® assay and MAPIA were still negative, but a DPP test showed antibodies against MPB83, E6/P10, and F10. A CT scan was performed and showed calcified areas in the mediastinal lymph nodes only. The animal was euthanized.

Necropsy findings included an enlarged caudal mediastinal lymph node with multifocal miliary calcified areas. A mild subacute purulent bronchopneumonia was also detected. Histopathological examination revealed a severe chronic granulomatous lymphadenitis with central dystrophic calcification. A mild purulent bronchopneumonia was detected as well. Although no AFB was found by microscopy and the culture remained negative, a PCR of lymph nodes revealed the *M. tuberculosis* complex.

Case 6

Another 1-y-old captive-born female came from Le Pal Zoo, France, to Heidelberg. Sputum examination by microscopy, PCR, and culture, which were performed while the sea lion was still in France, produced negative results. The sea lion had been treated with the same combinations of drugs in France as chosen in Heidelberg, and treatment was continued. In April 2008, ElephantTB STAT-PAK®

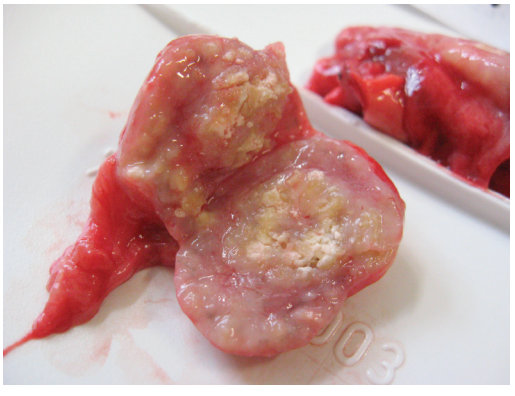

Figure 2. Mediastinal lymph node showing miliary calcifications

assay and MAPIA showed negative results, and DPP found antibodies to F10 antigen only. A CT scan revealed calcified foci in the mediastinal lymph nodes only. The animal was euthanized.

Necropsy findings included enlarged cranial and caudal mediastinal lymph nodes with multifocal areas of calcification and central necrosis. Histopathology confirmed this to be a severe chronic granulomatous lymphadenitis with central dystrophic calcification. As with the previous case, the Friedrich-Loeffler-Institute detected PCR signals specific for *M. tuberculosis* complex, but the culture remained negative.

Case 7

A wild-born 12-y-old female was examined in June 2007. A sputum sample was negative by microscopy and culture. Serological tests (ElephantTB STAT-PAK® assay, MAPIA, and DPP) gave negative results. Prophylactic treatment was initiated. In February 2008, sputum microscopy, PCR, and culture remained negative. ElephantTB STAT-PAK® assay, MAPIA, and DPP performed 2 mo later were nonreactive. A CT scan showed small calcified areas in the mediastinal lymph nodes. The animal was euthanized.

Necropsy findings included single calcifications in the caudal mediastinal lymph nodes and a male fetus in good developmental condition. Histopathology of the female showed a granulomatous lymphadenitis. PCR signals specific for *M. tuberculosis* complex were detected, but the culture remained negative.

Case 8

A 7-y-old female, born in Heidelberg, had lived at Tierpark Hagenbeck in Hamburg since 2003 and

Figure 3. Seroconversion of Case 10 shown in a series of ElephantTB STAT-PAK® assays

was supposed to move back to Heidelberg in 2008. As a pre-shipment test, a CT scan was carried out in November 2008. Calcifications of mediastinal lymph nodes were detected during this examination. The animal was euthanized.

Pathological findings included enlarged axillary lymph nodes and enlarged cranial mediastinal lymph nodes with a central calcification. Histopathology revealed a chronic granulomatous lymphadenitis with central calcifications of the cranial mediastinal lymph node. A few AFB were detected in macrophages by Ziehl-Neelsen-staining but not in the cytology of bronchial mucus. *M. pinnipedii* was isolated from the lymph nodes. By spoligotyping and VNTR-typing, it was confirmed that the strain was identical to that found in Heidelberg.

Case 9

A 10-y-old male born in Heidelberg died unexpectedly in July 2009. In February 2007, he was serologically negative by ElephantTB STAT-PAK® assay and MAPIA. Four months later, a sputum sample was AFB-negative but yielded positive PCR results for the *M. tuberculosis* complex. Treatment was initiated. Three months, 5 mo, 10 mo, and 21 mo after the start of treatment, sputum remained negative by microscopy, PCR, and culture. Due to his enormous size and weight, a CT scan was not possible. The animal was examined by digital x-rays in a horse clinic in April 2008. No abnormalities were seen.

Serological testing performed at the same time yielded a negative result with ElephantTB STAT-PAK® assay and MAPIA, while DPP carried out with the same serum retrospectively found antibody against F10.

In July 2009, the animal showed listlessness and anorexia for a few days before he died. The most significant necropsy finding was a large amount (30 to 40 l) of serosanguinous fluid in the thoracic cavity. In addition, the superficial cervical and the axillary lymph nodes were enlarged and showed abscess formation. The cranial mediastinal lymph node was severely calcified. Histopathology showed a granulomatous lymphadenitis and a bronchopneumonia. *S. equi* spp. *zooepidemicus* was cultured from the lesions.

Case 10

An 8-y-old female showed seroconversion by ElephantTB STAT-PAK® assay, MAPIA, and DPP. In addition, the animal started shedding mycobacteria in sputum. AFB were identified by microscopy and detected with sputum PCR. Sputum culture confirmed *M. pinnipedii*. After treatment was initiated, the animal stopped shedding within 2 wks. Post-treatment monitoring performed regularly showed no evidence of active disease in this sea lion.

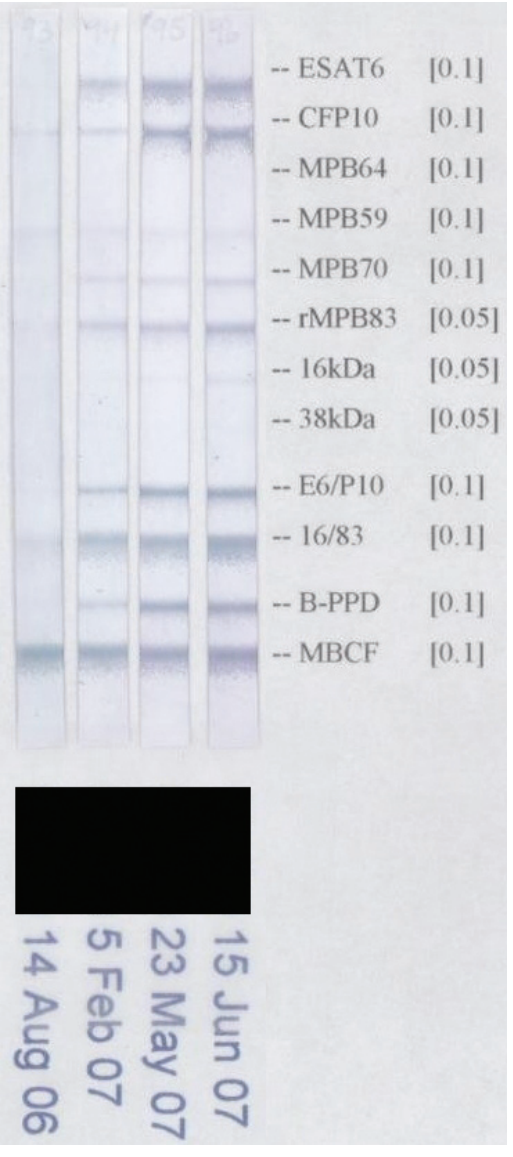

Figure 4. Seroconversion of Case 10 shown in a series of MAPIAs

Discussion

During the investigation of pinniped tuberculosis, several diagnostic methods were applied. It is commonly believed that the definitive diagnosis of animal tuberculosis can only be made by isolating tuberculous bacteria. However, only a few animals that were extensively shedding in the present

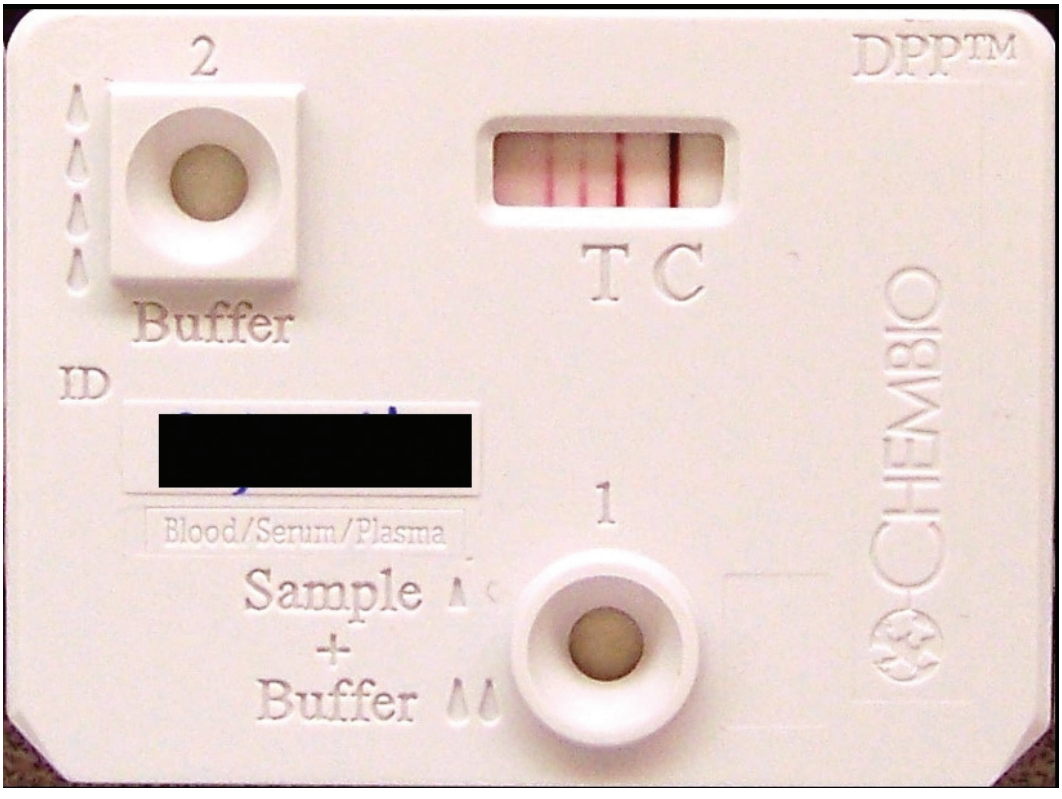

Figure 5. A positive DPP test, showing antibodies against MPB83, E6/P10, and F10

study could be identified *antemortem* using this method.

In contrast, the serological results helped identify most of the infected sea lions. ElephantTB STAT-PAK® assay, MAPIA, and DPP were useful point-of-care tools to detect seroconversions and identify seroreactive antigens. Furthermore, the DPP assay showed the highest sensitivity compared to that of ElephantTB STAT-PAK® assay and MAPIA, suggesting its greater agreement with the necropsy findings.

Spoligotyping was used to determine the *M. pinnipedii* strains involved. When the spoligotyping patterns of Le Pal and Hamburg were compared to that of the strain identified at the Heidelberg Zoo, it was shown that the strain in Le Pal was different, but the animal from Hamburg, born in Heidelberg, had an identical pattern. These findings were confirmed by MIRU/VNTR patterns. This was considered to be a strong indication that the animal in Hamburg harboured the infectious agent since its arrival from Heidelberg years earlier.

CT has proven to be a good diagnostic imaging method for the detection of calcified lymph nodes in animals up to 150 kg. X-rays are usually more problematic due to the size of the animals and the blubber layer, and therefore may generally offer no sufficient penetration.

Treatment has been carried out after antibiotic sensitivity testing. One animal (Case 10) that seroconverted within a few months of infection, and in which *M. pinnipedii* has been identified by sputum culture ante-treatment, has been negative in multiple CT scans and sputum cultures since then. It needs to be considered that treatment of exposed animals, if initiated early enough, may prevent manifestation of infection. Post-treatment euthanized animals revealed detection of mycobacteria in the calcified lymph nodes but only by PCR. Culture remained negative in all these animals, whereas *M. pinnipedii* was cultured out of the lesions from the nontreated animals. Therefore, at least up to now, there is a strong indication that treatment has been successful in eliminating the bacteria.

Clinical signs were observed only in three out of 10 infected animals, and these were highly unspecific (e.g., anorexia, respiratory distress). In four out of nine necropsied animals, *M. pinnipedii* was isolated from the mediastinal lymph nodes only. During *postmortem* examinations, these lymph nodes were hard to find as they are usually 1 to 2 cm in size and embedded deeply along

the bifurcation of the trachea. These lymph nodes can be overlooked easily in a regular *postmortem* protocol. Necropsy of Case 9 showed lesions in the mediastinal lymph nodes with a strong similarity to those seen in the confirmed cases; however, *M. pinnipedii* was not isolated from these lesions. It remains unknown if the 14 mo of treatment are responsible for this as live mycobacteria were not recovered from any of the post-treatment euthanized animals.

In *postmortem* examinations, lesions associated with tuberculosis have been found exclusively in lungs and the surrounding lymph nodes. This led to the assumption that the disease was transmitted by aerosol. Among the sea lion group, close physical contact is the most probable source of infection. The sea lion pool at the Heidelberg Zoo is routinely subjected to weekly high-pressure cleaning, and this might have been the source of infection for Malayan tapirs, the Bactrian camel (*Camelus bactrianus*), and the Indian crested porcupine (*Hystrix cristata*). These three species were housed in enclosures adjacent to the sea lion pool and were cared for by the same keepers (Jurczynski et al., 2011).

In the four animals that did not produce culture positive results to confirm *M. pinnipedii*, the involvement of *M. pinnipedii* was more than likely under the pressure of known exposure. Histopathology results and positive PCR results strongly support this.

Determining the sensitivity and specificity for the testing modalities described requires some assumptions, namely that an animal is considered a true positive if it had either a positive PCR, culture, or the presence of acid-fast bacterium (AFB) in sputum samples. This is an assumption that has limitations, and readers are advised to consider that positive PCR and the presence of AFB in sputum samples do not indicate active clinical tuberculosis. In addition, the cohort is limited in number and, taking the assumption above, there were no negative animals within this study group, leading to an inability to determine hypothetical specificities for the tests (i.e., there were no true negative or false positive cases). Another consideration is the limitation of the tests in that they are not specifically designed for the species in question and the fact that the time of infection and duration of infection were unknown; therefore, to compare the tests and the ability to determine sensitivities required knowledge of the time infection occurred. To determine this was impossible; and for sensitivities, all known animals were considered to be positive cases (i.e., active infection) when at least one of the tests was positive. This is a major limitation in predicting the sensitivities of each of the tests as it is possible that infection occurred earlier

Table 1. Clinical and diagnostic data obtained for South American sea lions included in the study

Case #	Gender	Age (y)	Clinical signs	X-ray/CT scan	AFB	PCR	Culture	Histo	STAT-PAK®	MAPIA	DPP	Tx
1	Female	14	Yes	Pos	Pos	Pos	Pos	Pos	ND	ND	ND	No
2	Female	16	Yes	ND	Pos	Pos	Pos	Pos	Pos	Pos	Pos	No
3	Male	17	No	ND	Pos	Pos	Pos	Pos	Pos	Pos	Pos	No
4	Female	11	No	Pos	Pos	Neg	Pos	Pos	Pos	Pos	Pos	Yes
5	Female	1	No	Pos	Neg	Pos	Neg	Pos	Neg	Neg	Pos	Yes
6	Female	1	No	Pos	Neg	Pos	Neg	Pos	Neg	Neg	Pos	Yes
7	Female	12	No	Pos	Neg	Pos	Neg	Pos	Neg	Neg	Neg	Yes
8	Female	7	No	Pos	Pos	Pos	Pos	Pos	ND	ND	ND	No
9	Male	10	Yes	Neg	ND	Pos	Neg	Pos	Neg	Neg	Pos	Yes
10	Female	8	No	Neg	Pos	Pos	Pos	ND	Pos	Pos	Pos	Yes

y = year, AFB = acid-fast bacteria, Tx = treatment, ND = not done

Table 2. Timeframe and outcome of the various tests performed in the group of South American sea lions

Case #	June 06	July 06	Aug 06	Oct 06	Feb 07	May 07	June 07	Treatment initiation (case 4-7 & 9-10)	Sep 07	Feb 08	April 08	May 08	Nov 08	July 09	Oct 09	Aug 10
1	Onset of clinical signs **Death**															
2	Occasionally coughing	RT: pos MAPIA: pos DPP: pos **Euthanasia**														
3			RT: neg MAPIA: neg		RT: neg MAPIA: suspect	RT: pos MAPIA: pos Sputum: pos	**Euthanasia**									
4				RT: neg MAPIA: neg	RT: neg MAPIA: suspect	Sputum: pos RT: neg MAPIA: pos				Sputum: neg CT: pos	RT: pos MAPIA: pos DPP: pos **Euthanasia**					
5							Sputum: neg RT: neg MAPIA: neg DPP: neg				RT: neg MAPIA: neg DPP: pos	CT: pos **Euthanasia**				
6									Sputum: neg		RT: neg MAPIA: neg DPP: pos	CT: pos **Euthanasia**				
7							Sputum: neg RT: neg MAPIA: neg DPP: neg			Sputum: neg CT: pos	RT: neg MAPIA: neg DPP: neg **Euthanasia**					
8													CT: pos **Euthanasia**			
9			RT: neg	RT: neg MAPIA: neg	RT: neg MAPIA: neg		Sputum: pos		Sputum: neg		Tracheolavage: neg Digital X-rays: neg RT: neg MAPIA: neg DPP: pos			Onset clinical signs **Death**		
10			RT: neg MAPIA: neg		RT: pos MAPIA: pos DPP: pos	Sputum: pos	Sputum: neg RT: pos MAPIA: pos DPP: pos		Sputum: neg	Sputum: neg CT: neg					Sputum: neg CT: neg RT: pos	CT: neg RT: pos

and that the tests failed to pick up the infection or presence of *M. pinnipedii*. However, it is also possible that the animals simply had not been infected at that time, and this is the assumption made here. As it can be seen, there are several unknown factors here that are impossible to determine which could be overcome when using experimental infection modelling which is not ethically or morally acceptable in this or many zoo and wildlife species. This is one of the major challenges facing emerging infectious disease testing in the clinical setting. However, based on this data, and accepting the assumptions above, the following can be said of the different testing modalities utilised in this study.

Sputum, as in other species, is poorly sensitive in diagnosing mycobacterial infection (Lecu & Ball, 2011). In this case, the sensitivity was 28.6%. This is to be expected as animals with active tuberculosis are thought to shed bacteria for limited periods of time (Lecu & Ball, 2011). The serological tests ranged in their sensitivities from 58% (ElephantTB STAT-PAK®), to 60% (MAPIA), to 87.5% (DPP). In combination, the results showed a poorer sensitivity (80%) than for the DPP alone. The CT testing for detection of *M. pinnipedii* infection was 71.4%; however, this is not the purpose of the test. The CT is used for the detection of calcified lymph nodes; for the animals that underwent *postmortem* examination, the sensitivity was 100%. This should not be confused with the ability to detect mycobacterial infection, but it is a staging of the clinical progression of the disease. Currently, it is not known how long calcification of the lymph nodes takes to develop and whether it occurs in all sea lions with active tuberculosis infection. However, its presence is a strong indicator of infection when used in combination with other testing modalities in the face of a known history of exposure. CT also has the potential to allow evidence-based decisions on whether to treat or not, and to monitor the efficacy of treatment. It is hypothesised that the presence of calcified lymph nodes is unlikely to be treatable with anti-tuberculosis drugs.

Combining the testing modalities with a minimum of two positives of the three tests (sputum, serology, and CT) produced a sensitivity of 71.4%. However, if one of the two tests being positive was CT, then the sensitivity increased to 85.7%. If only one of the three testing modalities had to be positive, then the sensitivity increased further to 100%. Utilising the later testing regime, the interpretation of one out of three as a positive is useful in a screen and cull program for which the risk of a positive animal to a collection is considered high. There is still the risk that animals that have been exposed to but are not currently showing signs of active disease may be euthanized, and these animals may well have been candidates for successful treatment. Utilising the former two out of three tests as a positive allows a more ethical approach to managing the animals in a collection; however, it comes with the consideration that positive animals may well be missed or considered negative and go on to maintain the infection within the population. As with tuberculosis in other species, testing and its interpretation requires assessment of the history, knowledge of exposure to mycobacteria, and management decisions for a particular collection in combination with disease risk analysis for the individual situation.

Conclusions

There is no single, 100% reliable *antemortem* test for detecting tuberculosis in captive wildlife animals. Most tests developed for domestic species have not been validated for exotic animals and, therefore, may have sub-optimal sensitivity and/or specificity. However, depending on interpretation, and in combination, the tests presented here can be utilised to provide strong evidence of infection of *M. pinnipedii* related disease in South American sea lions. This small study highlights the potential usefulness and efficacy of sputum, serological, and computer tomographical diagnostics used in combination for the diagnosis of tuberculosis in this species. Evaluation and validation of this testing regime will require the addition of other animals into the cohort. This in itself is a challenge due to the limited population sizes in captivity compounded by the known difficulties in determining the epidemiology of *Mycobacterium* sp. in sea lions. Until that time, or until alternative tests become available, the interpretation using the testing regime described will have to be based on individual institutions and their own biosecurity and disease management plans. The serological assays evaluated in this study showed particularly encouraging results and should be considered as very important and, up to now, very reliable tools to detect, *antemortem*, sea lions infected with *M. pinnipedii*. These *antemortem* findings are of extreme importance for subsequent decisions in regard to particular populations. The spoligotyping and MIRU/VNTR-typing indicated that the origin of infection might have been identical in all these cases except for the animal from Le Pal. These methods remain important tools for tracing back transmission routes of zoonotic infectious diseases. Animal exchanges and imports of wild animals of unknown disease status are considered a strong factor that may compromise biosecurity and facilitate further spread of this important zoonotic disease. Preshipment screenings with improved diagnostic tests for tuberculosis can help with disease eradication in zoological gardens. The best strategy for high

sensitivity of tuberculosis detection in sea lions and other animals is the use of multiple diagnostic methods rather than a single test.

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