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Tuberculosis in South American sea lions (*Otaria flavescens*) – diagnostic options and its epidemiologic importance for other mammals within the zoological garden

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To my family and friends, who never stopped supporting me!

CONTENTS

1 INTRODUCTION........ 1

2 REVIEW OF LITERATURE 2

2.1 The South American sea lion (*Otaria flavescens*)........ 2

2.1.1 Taxonomy 2

2.1.2 Distribution and Human Interactions........ 3

2.1.3 Captivity 4

2.2 Pinniped tuberculosis........ 4

2.2.1 Etiology 4

2.2.2 Epidemiology........ 7

2.2.3 Clinical signs 8

2.2.4 Pathology 9

2.2.4.1 Pathogenesis........ 9

2.2.4.2 Lesions........ 10

2.2.5 Diagnostic tests........ 11

2.2.5.1 Diagnostic imaging techniques 11

2.2.5.2 Microscopy 12

2.2.5.3 Polymerase chain reaction (PCR)........ 13

2.2.5.4 Culture........ 13

2.2.5.5 Sequencing........ 14

2.2.5.6 Tuberculin skin testing 16

2.2.5.7 Serological tests 17

2.2.5.7.1 ElephantTB STAT-PAK........ 17

2.2.5.7.2 Multiantigen print immunoassay (MAPIA) 19

2.2.5.7.3 Dual-plate-platform (DPP)........ 20

2.2.5.7.4 ELISA 21

2.2.6 Treatment........ 21

3 PUBLISHED PAPERS........ 23

3.1 Pinniped tuberculosis in Malayan tapirs (*Tapirus indicus*) and its transmission to other terrestrial mammals........ 23

3.2 Use of Multiple Diagnostic Tests to Detect *Mycobacterium pinnipedii* infections in a large group of South American Sea Lions (*Otaria flavescens*)........ 30

3.3 Computed tomographic examination of South American sea lions (*Otaria flavescens*) with suspected *Mycobacterium pinnipedii* infection........ 44

4 DISCUSSION........ 50

5 SUMMARY 54

6 ZUSAMMENFASSUNG 56

7 REFERENCES 58

ABBREVIATIONS

AFB	acid fast bacilli
BTB	blood TB test
CFP-10	culture filtrate protein
cm	centimeter
CT	computed tomography
DNA	desoxyribonucleic acid
DPP	dual plate platform
DR	direct repeat
ELISA	enzyme-linked immunosorbent assay
ESAT-6	early secreted antigenic target 6
FAFLP	fluorescent amplified fragment length polymorphism
Fig	figure
H.E.	hematoxylin eosin
IS	insertion sequence
IU	international unit
kDa	kilodalton
kg	kilogram
kV	kilovolt
m	meter
MAPIA	multiantigen print immunoassay
mAs	milliampere-second
min	minutes
ml	milliliter
mm	milimeter
MTC	mycobacteria of the tuberculosis complex
ND	not done
neg	negative
PCR	polymerase chain reaction
pos	positive
PPD	purified protein derivation
RD	region of difference
RFLP	restriction fragment length polymorphism
RNA	ribonucleic acid

RT	rapid test
SS	seal spoligotype
TB	tuberculosis
TST	tuberculin skin testing
Tx	treatment
wk	week
yr	year
µm	micrometer
°C	degrees Celcius

1 INTRODUCTION

Tuberculosis is a widely spread zoonotic disease caused by acid-fast bacteria of the *Mycobacterium tuberculosis* complex in a variety of mammalian species (LARSEN and SALMAN 2001).

Pinniped tuberculosis has been reported in various captive and wild sea lions and fur seals in South America, Europe, Australia and New Zealand (COUSINS 2006; GOMIS et al. 2008; LACAVE et al. 2009).

The causative agent, *Mycobacterium pinnipedii*, has been distinguished from other mycobacteria of the *M. tuberculosis* complex (BERNARDELLI et al. 1996; COUSINS et al. 1993).

In the past two decades pathogenicity in and possibility of transmission to other mammalian species has been reported (COUSINS 2006; GOMIS et al. 2008).

When a seal trainer developed pulmonary tuberculosis and the culture isolates were identical to the strains obtained from the pinnipeds he had worked with previously, the zoonotic potential was confirmed (THOMPSON et al. 1993).

Since 2000 the Heidelberg zoo has been dealing with tuberculosis in its collection of South American sea lions (*Otaria flavescens*). After a Malayan tapir (*Tapirus indicus*) was transferred to a zoological institution in France it transmitted the disease to the other tapirs that succumbed to tuberculosis. Culturing and spoligotyping confirmed the origin, the sea lions at the Heidelberg zoo.

An investigation of the sea lion group housed at Heidelberg in addition to different species of mammals living in adjacent exhibits revealed multiple cases of pinniped tuberculosis in sea lions as well as in Bactrian camels (*Camelus b. bactrianus*), Crested porcupine (*Hystrix cristata*) and a red panda (*Ailurus fulgens*).

This investigation provided a good opportunity to evaluate ante mortem diagnostic tests, to attempt to understand epidemiology and to describe pathological lesions.

2 REVIEW OF LITERATURE

2.1 The South American sea lion (Otaria flavescens)

2.1.1 Taxonomy

Pinnipeds belong to the order Carnivora and include three families, Otariidae (fur seals and sea lions), Odobenidae (walruses), and Phocidae (true seals).

The 33 living species of pinnipeds are represented by 18 phocid species, 14 otariid species, and the walrus. The otariids have external ear flaps or pinnae, whereas the phocids are also called “earless” seals (BERTA 2009).

The family *Otariidae* consists of two subfamilies, the *Arctocephalinae* (fur seals) and the *Otariinae* (sea lions) (BERTA 2009; CAPPOZZO and PERRIN 2009).

The subfamily of fur seals consists of two genera, the Northern fur seal (*Callorhinus ursinus*) and the Southern fur seals (*Arctocephalus*), which include the following species: the South American fur seal (*A. australis*), the New Zealand fur seal (*A. forsteri*), the Antarctic fur seal (*A. gazella*), the Galapagos fur seal (*A. galapagoensis*), the Juan Fernandez fur seal (*A. philippii*), the Guadalupe fur seal (*A. townsendi*), the Subantarctic fur seal (*A. tropicalis*), and the two subspecies of *Arctocephalus pusillus*: the South African fur seal (*A. p. pusillus*), and the Australian fur seal (*A. pusillus doriferus*) (BERTA 2009).

The sea lions subfamily includes five genus represented by seven living species: The Steller´s or Northern sea lion (*Eumetopias jubata*), the Southern sea lion (*Otaria flavescens*), the Australian sea lion (*Neophoca cinerea*), the California sea lion (*Zalophus californianus californianus*), the Japanese sea lion (*Z. c. japonicus*), the Galapagos sea lion (*Z. c. wollebacki*) and the New Zealand sea lion (*Phocarctos hookeri*) (BERTA 2009; CAPPOZZO and PERRIN 2009).

The Southern sea lion, or preferably the South American sea lion, had next to his common name “Lobo marino de un pelo”, two scientific names for a long time: *Otaria byronia* (DE BLAINVILLE 1820) and *Otaria flavescens* (SHAW 1800), whereas the latter one has been decided on (CAPPOZZO and PERRIN 2009; RODRIGUEZ and BASTIDA 1993).

Otaria flavescens is one of the largest sea lions and has a distinct sexual dimorphism. In the wild, adult males can weigh up to 350 kg and reach a maximum body length of 3 m. They also show a thick neck with hair resembling a mane (CAPPOZZO and PERRIN 2009).

For this reason they are also called “Mähnenrobbe” (maned seals) in German.

Adult females reach a body length of 2 m and a maximum weight of 150 kg (CAPPOZZO and PERRIN 2009).

Figure 2.1 Male South American sea lion

Figure 2.2 Female South American sea lion

2.1.2 Distribution and Human Interactions

The South American sea lion can be found along the Atlantic and Pacific coasts of South America from Brazil to Peru (BERNARDELLI et al.1996; CAPPOZZO and PERRIN 2009).

The total population has been estimated at 110,000 individuals for the southwestern Atlantic coast, 140,000 for the Chilean coast and 100,220 for Peru (CAPPOZZO and PERRIN 2009; OLIVIA et al. 2009)

Next to population decreases due to the thermic effects of El Nino on the whole marine ecosystem, there are also various kinds of human interactions that affect the population size. The South American sea lion was hunted for oil and fur, used as bait for the Southern king crab and was also seen as competitor for the fishing industry. Due to the fact that an important part of their diet includes commercial fish species, predation on fishing catches, damage to fishing gear and aquaculture installations, such as salmon farms, have been reported. (CAPPOZZO and PERRIN 2009; OLIVIA et al. 2009)

2.1.3 Captivity

As of June 30th 2011, there are 307 male and 485 female South American sea lions as well as 41 animals of unknown sex listed in the European regional studbook. 191 males, 208 females and 36 animals of unknown sex were born in captivity (WALTER 2011). These numbers indicate that the breeding programs are efficient but there are still wild born animals living in zoo collections either of an older age or more recent imports from the wild.

2.2 Pinniped tuberculosis

2.2.1 Etiology

Mycobacteria are acid-fast aerobic rods, which can be classified based on growth characteristics. Fast-growing mycobacteria produce macroscopically visible colonies within 7 days and include the apathogenic and opportunistic species. “Slow Growers” need more than a week to grow and contain the highly pathogenic species of mycobacteria (LEWIN and SHARBATI-TEHRANI 2005; METCHOCK et al. 1999).

Mycobacterial diseases have caused infections in a variety of mammals, birds, reptiles, amphibians, and fish, in the wild as well as in zoological collections, but tuberculosis is responsible for the infection with species of mycobacteria belonging to the *Mycobacterium tuberculosis* complex (WEST 2006; MILLER 2008).

Next to *M. tuberculosis* and *M. bovis*, the *M. tuberculosis* complex, includes *M. microti*, *M. canetti*, *M. africanum*, *M. pinnipedii* and *M. caprae*, whereas the latter three have evolved from a common side branch of *M. tuberculosis* (THOEN and BARLETTA 2006; COUSINS 2006; COUSINS et al. 2003).

M. tuberculosis is the main pathogen for tuberculosis in humans and elephants, *M. bovis* causes tuberculosis in domestic animals and wild mammals more commonly (KANEENE and THOEN 2004).

M. microti has been isolated from small rodents, hyraxes, llamas, pigs and ferrets, whereas *M. africanum* has been reported to cause tuberculosis in humans, cattle and pigs (MILLER 2008).

Another member of the *M. tuberculosis* complex, *M. pinnipedii*, was initially identified as *M. bovis* due to common biochemical parameters like negative reactions in the nitrate reduction and niacin accumulation tests. Genetic analyses revealed significant differences and *M. pinnipedii* received species status in 2003 (BERNARDELLI et al. 1996; FORSHAW and PHELPS 1991; COUSINS 2006; COUSINS et al. 2003).

M. pinnipedii has been found primarily in seals and its occurrence in several species of pinnipeds indicates that tuberculosis has been endemic in these animals for many thousands of years (COUSINS 2006; COUSINS et al. 1993; THOMSON 2006).

The first reports of tuberculosis in captive pinnipeds have been in hooded seals as early as 1912 in the USA and in a California sea lion in Germany in 1965, but neither mycobacterial identification nor detailed histopathological description was noted (BERNARDELLI et al. 1996; BLAIR 1912; EHLERS 1965).

Until today pinniped tuberculosis has been reported in seven species of sea lions and fur seals listed in table 2.1.

TABLE 2.1 Overview of the pinniped species reportedly infected with pinniped tuberculosis

Species	Country	wild /captive	Year	Reference
A. tropicalis	Argentina	wild	1996	BASTIDA et al. 1999; COUSINS 2006
N. cinerea	Australia	wild & captive	1985-1992	COUSINS et al. 1990, 1993; FORSHAW and PHELPS 1991; THOMPSON et al. 1993
O. flavescens	Argentina	wild & captive	1987-2003	BERNARDELLI et al. 1996
O. flavescens	Uruguay	wild	2001-2004	COUSINS 2006; ZUMARRAGA et al. 1999
P. hookeri	New Zealand	wild	2001-2004	COUSINS 2006
A. australis	Argentina	wild	1989-2000	BERNARDELLI et al. 1996
A. australis	UK	captive	1996-1998	COUSINS 2006; REDROBE 2003
A. australis	Uruguay	wild	1997-2004	COUSINS 2006
A. forsteri	Australia	wild & captive	1986-1995	COUSINS et al. 1990, 1993; THOMPSON et al. 1993
A. forsteri	New Zealand	wild	1997-1998	COUSINS 2006; HUNTER et al. 1998
A. p. doriferus	Australia	wild	1992	COUSINS 2006; COUSINS et al. 1993; WOODS et al. 1995
O. flavescens	France	captive	1992-2009	GOMIS et al. 2008; LACAVE et al. 2009; THOREL et al. 1998
O. flavescens	The Netherlands	captive	2008	JURCZYNSKI, et al. 2007; KIERS et al. 2008
O. flavescens	Germany	captive	2008	JURCZYNSKI et al. 2007

Although pinnipeds appear to be the natural host, *M. pinnipedii* is pathogenic to other animals as well (COUSINS et al. 2003).
Experimental infection of guinea pigs and rabbits was possible as well as natural infection of cattle in New Zealand (COUSINS 2006).
In two zoological collections in Europe *M. pinnipedii* proved that it has a wide host range by causing infection in Brazilian tapir (*Tapirus terrestris*), llama (*Lama glama*), Chilean pudu (*Pudu puda*), Western Lowland gorilla (*Gorilla g. gorilla*), snow leopard (*Panthera uncia*) and amur leopard (*Panthera pardus*) (COUSINS 2006; COUSINS et al. 2003).
This wide host range does not exclude humans. In 1989 a seal trainer developed active pulmonary tuberculosis, three years after three seals died of tuberculosis at the park he had worked at. An identical strain of *M. pinnipedii* was identified, but still referred to as *M. bovis* at that time (COUSINS 2006; MASLOW 1997; THOMPSON et al. 1993).

2.2.2 Epidemiology

Various ways of transmission have been identified, whereas aerosols are the most likely route of spreading mycobacteria (COUSINS et al. 2003). These can be aerosols created by animals with active disease via direct contact to other animals and humans or even aerosols created during necropsy or during handling of infected animals (MASLOW 1997).
In addition to an actively infected and shedding animal there are more factors to increase the likelihood of transmission. A sufficient amount of bacilli is as important as the total contact time to the infected animal, as well as the droplet size carrying the bacilli (MASLOW 1997).
Another possible way of transmission is the oral route. To accomplish this, body excretions containing a sufficient number of infected live organisms have to be consumed (KANEENE and THOEN 2004; ALEXANDER et al. 2002). A transmission through biting has been identified in black-footed ferrets and domestic cats (DE LISLE 1990, 1993).
Vertical transmission is possible, but in many cases infection may be a result of transmission of infectious organisms via close contact during grooming of offspring or consumption of contaminated milk (KANEENE and THOEN 2004; PALMER 2002).

2.2.3 Clinical signs

In most cases in many species clinical signs are usually seen in advanced stages of the disease only and the diagnosis is considered only when caseous granulomas are recovered at necropsy (BENGIS 1999; GREENWALD et al. 2009; MASLOW 1997; MILLER 2008; THOEN 2006; WEST 2006).
The general signs are highly non-specific and include anorexia, weakness, depression, dyspnoea, lowgrade fluctuating fever and progressive emaciation despite good appetite. In cases where the disease has affected the respiratory system coughing and reduced exercise tolerance may be noticeable (ALEXANDER et al. 2002; BENGIS 1999; DE LISLE et al. 2002; FROST 2006; KANEENE and THOEN 2004; MASLOW 1997; MICHEL et al. 2003; MILLER 2008; STETTER et al. 1995; THOEN 2006; THOEN and HIMES 1980; WEST 2006).
The majority of even the most susceptible species does not show clinical signs at all (DE LISLE et al. 2002; GREENWALD et al. 2009).
Camelids may only show chronic emaciation (WEST 2006).
Due to the slow progression of the disease it is often difficult to recognize affected animals and interfere early in the course of infection (THOEN and HIMES 1980). Many infected animals are already shedding bacilli at that time and pose a risk of infection to other animals or humans (KANEENE and THOEN 2004).
In pinnipeds clinical signs are similar to terrestrial mammals, as dyspnoea, anorexia, weakness, depression and weight loss has been described in most cases (BASTIDA et al. 1999; BERNARDELLI et al. 1996; FORSHAW and PHELPS 1991; KIERS et al. 2008; MOISSON et al. 1998; STEIGER et al. 2003). Coughing has not been a prominent feature despite massive pulmonary tissue involvement (FORSHAW and PHELPS 1991; GOMIS et al. 2008; KIERS et al. 2008).

Figure 2.3 Emaciated male South American sea lion

2.2.4 Pathology

2.2.4.1 Pathogenesis

The general path of transmission is by aerosol infection between individuals in close proximity (BENGIS 1999; COUSINS et al. 1990; THOEN and BARLETTA 2006). The pathogens enter via inhalation, pass the mucociliary layer of the upper respiratory tract and, due to their small size of 1-4 µm, are able to advance as far as the terminal bronchioles where the bacteria are ingested by phagocytes. The phagocytes enter the circulation and are transported to the regional lymph nodes or other organs. The phagocytes fail to kill virulent tubercle bacilli and, in addition, serve as a protection from bactericidal components in the serum. After multiplication of the bacilli, phagocytes are destroyed and the tubercle bacilli are ingested by other phagocytes. Clustering cells form a granuloma at the start and further accumulation of phagocytes progress to the formation of macroscopic lesions referred to as tubercles. Within the next two weeks cell-mediated immunity responses recruit macrophages with an increased capacity to destroy the intracellular bacilli. Cell death and tissue destruction produce caseous necrosis. In some cases enzymatic action leads to liquefaction and cavity formation. When these cavities rupture, the bacilli are spread via blood and/or lymphatic circulation and may gain access to the airways, facilitating

transmission to other animals or humans. Encapsulation does not terminate the disease process but may contain viable bacilli that can be reactivated. Lymph nodes trap mycobacteria in the meshwork of trabeculae and are therefore common organs to get infected and develop granulomatous lesions (BENGIS 1999; MASLOW 1997; THOEN and BARLETTA 2006).

In pinnipeds the most probable route of transmission is inhalation, because most of the lesions involve pulmonary tissue and thoracic lymph nodes (COUSINS et al. 1993; FORSHAW and PHELPS 1991). Alimentary may be possible due to the fact that liver and hepatic lymph node lesions have been reported, as well as lesions in the liver as the exclusively affected organ (COUSINS 2006; FORSHAW and PHELPS 1991).

In a microscopic view a tuberculous granuloma, encapsulated by fibrous connective tissue, shows a caseous necrosis in a central area bordered by a zone of epithelioid cells, lymphocytes, and granulocytes. Some epitheloid cells may form multi-nucleated giant cells of the Langerhans type close to the necrotic area, whereas mineralization may be present within the necrotic centers. Both are not prominent features of this disease in pinnipeds (BASTIDA et al. 1999; BERNARDELLI et al. 1996; CASTRO RAMOS 1998; FORSHAW and PHELPS 1991; FROST 2006; THOEN and BARLETTA 2006; WEST 2006).

2.2.4.2 Lesions

In many animals, as well as in human beings, tuberculous lesions consist of tumor-like masses in various organs such as lungs, mediastinum and abdominal cavity. Tuberculous granulomas may not be grossly visible lesions and may pass unrecognized in routine post-mortem examination (THOMPSON et al. 1993). Miliary patterns may be seen in disseminated disease as well as calcification or caseo-calcification may occur in lesions (LACAVE et al. 2009; THOMPSON et al. 1993; WEST 2006).

Common lesions in exotic species include enlarged lymph nodes, solitary granulomas or miliary scattered abscesses in axillary and mediastinal lymph nodes, lung, liver, pancreas spleen, kidney, granulomatous pneumonia (ALEXANDER et al. 2002; DE LISLE et al. 2002; STETTER et al. 1995; THOEN and BARLETTA 2006).

In pinnipeds extensive granulomatous pneumonias affecting major parts of the lung tissue, granulomatous pleuritis and marked generalized lymphadenopathy have been seen commonly as well as lesions in liver, spleen and peritoneum (BERNARDELLI et al. 1996; COUSINS 2006; COUSINS et al. 1993, 2003; HUNTER et al. 1998; KIERS et al. 2008; STEIGER et al. 2003; THOMPSON et al. 1993). Granulomatous lesions, caseous or calcified, in lungs and thoracic lymph nodes were primary findings during pinniped post mortem examination (BASTIDA et al. 1999; BERNARDELLI et al. 1996; FORSHAW and

PHELPS 1991; KIERS et al. 2008). In addition, large amounts (up to 8 liter) of yellowish liquid exudate in the thoracic cavity exhibiting a marked pleural reaction have been reported in sea lions and fur seals (BASTIDA et al. 1999; COUSINS et al. 1993; FORSHAW and PHELPS 1991). Bilateral pyothorax has been described in some animals (WOODS et al. 1995). Cases in pinnipeds can be limited to single organs like lung or lymph node or disseminated throughout multiple organs (COUSINS 2006; HUNTER et al. 1998).

Macroscopically some lesions caused by parasites, various bacterial genera (*Rhodococcus, Actinomyces, Actinobacillus, Cryptococcus, Blastomyces, Histoplasma*), and neoplasia can resemble tuberculosis. Histology is appropriate for differentiation (DE LISLE et al. 2002).

2.2.5 Diagnostic tests

To diagnose tuberculosis in live captive wild animals is still very challenging because next to the occurrence of subclinical infections, available diagnostic tests are not validated in exotic species (DE LISLE et al. 2002; MIKOTA and MASLOW 2002; MILLER 2008; WEST 2006).

Direct methods to identify mycobacteria from biological samples include acid-fast and fluorescent smears, culture and polymerase chain reaction (PCR) are the most definitive ways to confirm infection. Indirect methods detect antigen or antibody, or measure cellular reactivity against mycobacterial antigen (KAANDORP 1998; MIKOTA and MASLOW 197; MILLER 2008).

2.2.5.1 Diagnostic imaging techniques

In human beings, non-human primates and small animals thoracic radiography can complement other diagnostic tests for tuberculosis (FROST 2006; KAANDORP 1998). In human beings and animals, computed tomography is considered to be superior to thoracic radiology, especially in identifying the initial stages of tuberculosis (BOSCH-MARCET et al. 2004; LANGE et al. 2006; SONNTAG and MIHALJEVIC 2009). DELACOURT et al. (1993) found that in 60 per cent of children with tuberculosis, no abnormalities were detected on thoracic radiography, yet enlarged lymph nodes were identified with CT.

The use of radiographs in pinnipeds is very limited and case reports show that thoracic and abdominal radiographs failed to detect lesions that were demonstrated during necropsy afterwards (FORSHAW and PHELPS 1991). Although various species of pinnipeds have been kept in zoological collections for decades, there are no documented normal or pathological CT images of the thorax published in the literature.

2.2.5.2 Microscopy

Microscopic examination requires material from lesions or liquid samples of the respiratory system or the gastrointestinal tract, such as sputum, contents of broncheolavage or gastric fluid. To retrieve mycobacterial organisms from a sputum sample is not very efficient, because it has a low sensitivity (22-78% sensitivity) due to the required amount of bacilli of 1000 organisms/ml sputum (KAANDORP 1998; LANGE et al. 2006; MIKOTA and MASLOW 1997).

The sensitivity can be increased by examining tissue sections or concentrated homogenised tissues (DE LISLE et al. 2002).

After a special staining method, such as fluorchrome, Ziehl-Neelsen or Kinyon stain, acid-alcohol-fast, non spore-forming, and nonmotile rods may be observed microscopically (BENGIS 1999; BERNARDELLI et al. 1996; COUSINS 2006; COUSINS et al. 2003; FROST 2006; KANEENE and THOEN 2004; MILLER 2008; ROMEIS 1989; STEIGER et al. 2003; THOEN and HIMES 1980).

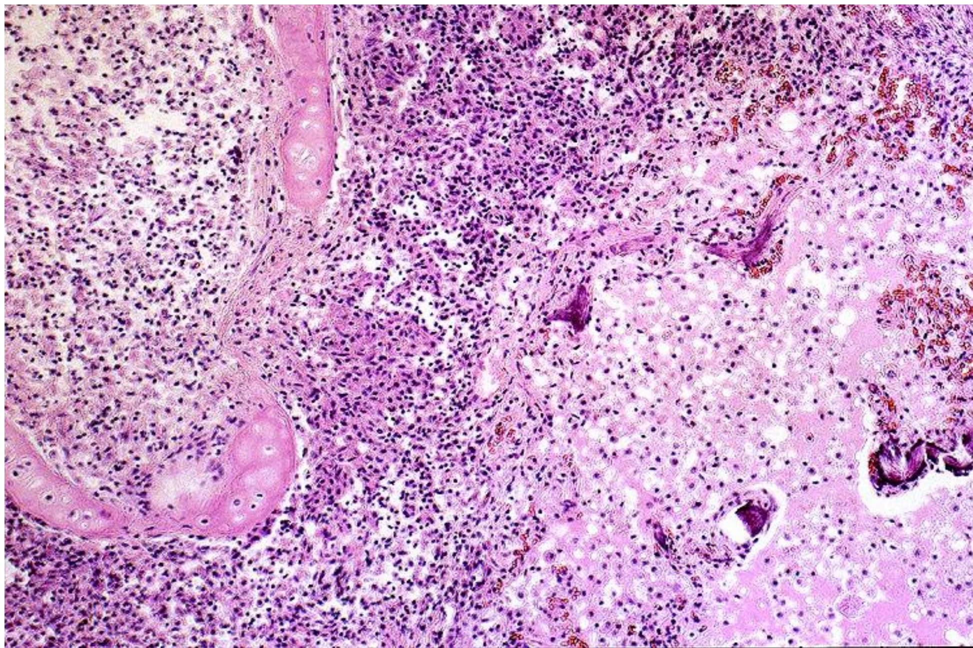

Figure 2.4 Hematoxylin eosin stain of a sea lion lung, acid-fast bacteria in red

Due to the low sensitivity, microscopy is not a reliable method of detection and although the animal is infected with tuberculosis acid-fast organisms may not be seen within lesions (COUSINS 2008; DE LISLE et al. 2002; FORSHAW and PHELPS 1991; KIERS et al. 2008).

2.2.5.3 Polymerase chain reaction (PCR)

The polymerase chain reaction (PCR) is a nucleic acid amplification technique that is carried out on biological samples such as culture, sputum and histopathological specimens collected during necropsy. Specific sequences of desoxyribonucleic acid (DNA) or ribonucleic acid (RNA) are replicated to be able to detect a very small quantity of a particular bacterial target sequence, using highly specific primers and other reagents. Primers are oligonucleotide sequences that bind complementary DNA such as a fragment of the insertion sequence 6110 (IS 6110), which has been used for the identification of members of the M. tuberculosis complex. With this technique as few as 20-50 mycobacteria / ml can be identified and a high sensitivity and specificity has been reported. The sensitivity may be as high as 99% in acid fast positive, culture positive samples, but only 60-80% sensitivity at detecting acid fast negative, culture positive samples. PCR can rapidly identify if the mycobacteria are within the M. tuberculosis complex but does not discriminate the members from each other. More DNA-specific probes have been developed for further speciation (BERNARDELLI et al. 1996; COUSINS et al. 1993; DE LISLE et al. 2002; FROST 2006; KAANDORP 1998; LANGE et al. 2006; LARSEN and SALMAN 2001; MICHEL et al. 2003; MIKOTA and MASLOW 1997; MILLER 2008; THOMSON 2006).
An advantage of PCR testing is that fresh as well as formalin-fixed, paraffin-embedded tissue blocks can be used, which allows retrospective investigations of archived samples. The disadvantage of detecting dead or dying organisms is that it cannot be used to monitor treatment (DE LISLE et al. 2002; MIKOTA and MASLOW 1997; MILLER et al. 1997; MILLER 2008; THOMSON 2006).
In pinnipeds PCR has been used successfully to identify *M. tuberculosis* complex bacteria in various biological samples (COUSINS et al. 1993; HUNTER et al. 1998; KIERS et al. 2008; WOODS et al. 1995).
A negative result does not assure that the animal is not infected with tuberculosis, it simply means that the test did not detect the infectious agent in that specific sample. False negative results can occur due to prolonged formalin fixation, which will increase DNA degradation (THOMSON 2006).

2.2.5.4 Culture

Culture, still considered the „gold standard“, can be performed on fresh tissue samples or other material such as sputum and faeces and can detect approximately 100 organisms/ml (DE LISLE et al. 2002; KANEENE and THOEN 2004; LARSEN and SALMAN 2001; MIKOTA and MASLOW 1997; THOMSON 2006; WEST 2006). Preferred specimens have included lung, lymph nodes, and abscesses (LARSEN and SALMAN 2001).

Although tracheal wash is the currently recommended sample for living non-domestic hoofstock, it will like trunk washes in elephants, only detect animals with extensive shedding of mycobacteria (LYASHCHENKO et al. 2006; NATIONAL TUBERCULOSIS WORKING GROUP FOR ZOO AND WILDLIFE SPECIES 2001). Media for culturing mycobacteria causing tuberculosis can be solid or liquid. A liquid media may be more sensitive requiring as few as 10-100 organisms/ml to reveal a positive culture result (LANGE et al. 2006). The tuberculous strains grow slowly on egg-based media like Stonebrink or Löwenstein Jensen at 37°C (BASTIDA et al. 1999; BERNARDELLI et al. 1996; DE LISLE et al. 2002; MILLER 2008). This growth is generally enhanced by sodium pyruvate and within 3-6 weeks of incubation dysgonic, rough, flat, and nonphotochromogenic colonies emerge (COUSINS 2006; COUSINS et al. 2003). A significant disadvantage, in comparison to PCR, is that 6-8 weeks are needed to complete the testing (BERNARDELLI et al. 1996; KANEENE and THOEN 2004; LANGE et al. 2006; MILLER 2008; THOEN 1993; THOMSON 2006). Further speciation may take another 4-6 weeks (FROST 2006). False negative culture results are possible due to inadequate numbers of bacteria, improper collection, or contamination causing overgrowth with other bacteria (LARSEN et al. 2001).

2.2.5.5 Sequencing

After PCR identified the isolate of being member of the *M. tuberculosis* complex, sequencing leads to determination of the species involved (LANGE et al. 2006).

The insertion sequence IS6110 is a transposable element found only in members of the *M. tuberculosis* complex. The total number of IS6110 copies found in the genome differs between the members of the complex. *M. bovis* isolates often harbour only a single copy of IS6110, while isolates of *M. pinnipedii* and *M. tuberculosis* contain several copies of this element. Restriction fragment length polymorphism (RFLP) analysis visualizes the variations (DE LISLE et al. 2002; HARRIS 2006; NEEDHAM and PHELPS 1990). RFLP revealed that all seal-related isolates analysed by COUSINS et al. (1993) appeared identical and were clearly different from other members of the *M. tuberculosis* complex. In sea lions and fur seals from the south-western Atlantic coast IS6110-RFLP showed that three bands (1.70, 1.90 and 4.00 kilobase) were shared by all four isolated strains, two bands (1.95 and 5.55 kilobase) were shared by three strains and six bands were observed in one strain. The differences in the position and number of the copies of the IS6110 element, the so-called IS6110 fingerprint patterns, indicate diversity between these strains and suggests that this disease may be endemic in wild seals (BERNARDELLI et al. 1996; ROMANO et al. 1995).

Techniques to genotype isolates help to trace the origin of the disease and discover the epidemiological relatedness in a disease outbreak. This strain differentiation is carried out

through the spacer oligotyping (spoligotyping) (KAMERBEEK et al. 1997). This focuses on a region of the *M. tuberculosis* complex chromosome called the direct repeat (DR) locus, composed of a series of 43 identical DR sequences. Each DR is interspersed by spacer sequences. In spoligotyping PCR products are amplified using primers specific for the DR sequences. Visualization in a specialized dot-blot apparatus enables the detection of the specific spoligotype patterns. Deletions of spacer sequence regions are characteristic for the different members of the *M. tuberculosis* complex (HARRIS 2006; ZUMARRAGA et al. 1999).

All of the pinniped isolates from Australia and all but one of the Argentinian isolates had a unique but identical pattern, designated seal spoligotype 1 (SS-1). The remaining Argentinian isolate was designated SS-2. The three isolates from Great Britain had identical spoligotypes (SS-3) that differed by one spacer from the others. The isolates from New Zealand and the isolate from a New Zealand bovine had identical spoligotypes (SS-4) that lacked six spacers that were present in all other pinniped isolates. When compared to reference strains of *M. tuberculosis*, *M. africanum*, *M. microti*, *M. bovis*, *M. canetti* and *M. caprae* the seal isolates formed a distinct cluster within the *M. tuberculosis* complex. Spoligotyping confirmed that the seal isolates from Australia, Argentina, Uruguay and Great Britain were closely related (COUSINS et al. 2003).

ZUMARRAGA et al. (1999) described that the spoligotypes isolated from South American seals lack spacers 39-43, as does *M. bovis*, but the MPB70 antigen was not detected in these mycobacteria. This has been reported from Australian seal isolates as well (COUSINS 2006; WOODS et al. 1995). The MPB70 antigen is highly expressed in *M. bovis* (COUSINS et al. 1993; ZUMARRAGA et al. 1999).

A more recently developed technique to characterize the pinniped strains is the fluorescent amplified fragment length polymorphism (FAFLP) (AHMED et al. 2003; COUSINS et al. 2003). This technique highlights base substitutions across the genome that can be linked to different stress factors. FAFLP profiling seems to be more promising for taxonomic studies than searching for bands in methods as IS6110 RFLP. Many of the Australian seal isolates were identical to the Argentinean ones with 100% similar genotypes. Phylogenetic analyses of *M. pinnipedii* revealed different insertion deletion polymorphisms and therefore this method suggests that *M. pinnipedii* is evolutionarily closer to *M. bovis* than to any other member of the *M. tuberculosis* complex (AHMED et al. 2003). Previous studies based on more routine analyses came to a different conclusion (COUSINS et al. 1993, 2003; ZUMARRAGA et al. 1999). In 2005 BIGI et al. conducted microarray-based comparative genomics and identified two novel genomic deletions (PiD1 and PiD2) being exclusive to *M. pinnipedii*.

2.2.5.6 Tuberculin skin testing

The delayed tissue hypersensitivity reaction to mycobacterial antigens is performed with an intradermal tuberculin test. The corresponding resonse is primarily cell-mediated and depends on the previous exposure to the antigens (HAAGSMA and EGER 1990; KAANDORP 1998; LARSEN and SALMAN 2001; MILLER 2008; WEST 2006). To perfom this test purified protein derivative (PPD) tuberculin is prepared from the culture filtrate of *M. bovis* by precipitation with ammonium sulfate or trichloracetic acid (THOEN 1993; THOEN and EBEL 2006). Bovine PPD tuberculin contains MPB70 antigen and formation of antibody to this was highly specific for infection with *M. bovis* (HARBOE et al. 1990). A simultaneous, comparative skin test using PPD tuberculin made of *M. avium* may assist in the interpretation of a reaction (BENGIS 1999; MILLER 2008). A small aliquot of both tuberculins is injected intradermally into a skin fold near the base of the tail or into the skin of the cervical region, the thorax, abdomen, ear or eyelid (MIKOTA and MASLOW 1997; MILLER 2008; THOEN 1993). The injection site is visually observed and palpated or measured at 72 hours following injections, and any inflammatory reaction is considered suspicious (MILLER 2008; THOEN and EBEL 2006). The local reaction of the skin is characterized by inflammation and swelling in infected animals and results from a local influx of lymphocytes (KANEENE and THOEN 2004; MIKOTA and MASLOW 1997).

Skin testing detects only infection and not active disease and can therefore not differentiate between earlier exposure and active infection (KAANDORP 1998; MASLOW 1997).

Intradermal tests are typically the primary tests for detecting *M. bovis* infections in zoo animals. However, many difficulties occur. These tests have rarely been validated in non-domestic animals. There may be not only variation between species but a reaction may also depend on the injection site, antigen strength or interpretation of the result (MIKOTA and MASLOW 1997).

Several reasons for false-positive or false negative readings are possible. Low sensitivity may be due to encountering the infection recently after exposure, too early for a cell-mediated response in competent animals, as well as in advanced cases, where anergy may develop in severely infected animals and test results are negative (BENGIS 1999; FROST 2006; KAANDORP 1998; LANGE et al. 2006; LARSEN and SALMAN 2001; MILLER 2008; THOEN and BARLETTA 2006; THOEN and EBEL 2006). This has also been reported in animals with healed lesions, dormant infections or animals with lymphokine induced immunosuppression, which challenges the surveillance of these animals (FROST 2006; THOEN and BARLETTA 2006). Low specificity is encountered in animals that, due to their environment, are exposed to large numbers of atypical mycobacteria, resulting in cross-sensitization to standard tuberculin antigens (BENGIS 1999; DE LISLE 2002; LANGE et al.

2006; LARSEN and SALMAN 2001; MILLER 2008). In many species the specificity is not known and false-positive results may lead to unneccesary euthanasisa of genetically important, endangered species, whereas false-negatives may result in further spread of the disease. Nonspecific reactions have been observed in species like non-human primates, camelids, tapirs, bongo antelope, reindeer, and orangutans aggravating interpretation of the test result (KAANDORP 1998; MILLER 2008; WEST 2006). Nonspecific reactions may have been due to trauma as a result of administration or as a reaction to the adjuvant (FROST 2006).

Another disadvantage is that animals have to be handled, or even immobilized, twice for initial injection and again for injection site measuring (BENGIS 1999; LARSEN and SALMAN 2001; THOEN and EBEL 2006).

In pinnipeds tuberculin skin tests are typically carried out by intradermal injection of tuberculin in flipper skin or eyelids (CASTRO RAMOS 1998). An increase in thickness of the skin of 3-4 mm or greater at 72 hours past inoculation was considered a positive reaction (CASTRO RAMOS 1998; NEEDHAM and PHELPS 1990). Due to a low number of animals that have tested reportedly, this test has not been validated in pinnipeds (FORSHAW and PHELPS 1991). False-negative results have occured (REDROBE 2003).

2.2.5.7 Serological tests

Serological tests are rapid, relatively non-invasive, and do not require isolation of the bacteria (LYASHCHENKO et al. 2000). The tests applied here, use antigens of the region of difference (RD)-1 in the genome of *Mycobacterium tuberculosis*. These antigens are the „early secreted antigenic target 6kDa“ (ESAT-6) and the „culture filtrate protein 10kDa“ (CFP-10) (LANGE et al. 2006). In elephants, serum immunoglobulin G antibodies to ESAT-6 were detected up to 3,5 years prior to culture of *M. tuberculosis* from trunk washes (LYASHCHENKO et al. 2006).

2.2.5.7.1 ElephantTB STAT-PAK

This is a rapid test (RT), initially developed for elephants, which uses lateral-flow technology and selected recombinant antigens. The antibody detection test employs a cocktail of several selected *M. tuberculosis* and/or *M. bovis* antigens and a blue latex bead-based signal detection system. The portable ready-to-use test requires 30 µl of serum sample (plasma or whole blood) and 3 drops of sample diluent buffer (included in the kit) that are added sequentially to the sample pad (Figures 2.5, 2.6). As the diluted test sample migrates to the conjugate pad, the latex particles conjugated to antigen bind antibody, if present in the

sample, thus creating a coloured immune complex. Driven by capillary forces, the complex flows laterally across the nitrocellulose membrane impregnated with specific antigen and binds to the immobilized antigen, thus producing a blue band in the test area. The liquid continues to migrate along the membrane, producing a similar blue band in the control area (Figure 2.7). This demonstrates that the tests reagents are working properly. Results are read 20 minutes after addition of the sample and diluent buffer (Figure 2.8). Any visible band in the test area, in addition to the control line, was considered an antibody-positive result, whereas no band was considered a negative result (Figure 2.9) (LYASHCHENKO 2006).
This lateral flow chromatographic assay uses three *M. tuberculosis* complex specific antigens: MPB83, ESAT-6 and CFP-10 (DEAN et al. 2009).
The rapid test has shown promise in diagnosing naturally infected Old World and New World camels (DEAN et al. 2009; MILLER 2008). In llamas the sensitivity increases after a skin test (DEAN et al. 2009).

Figure 2.5 Addition of serum to the RT

Figure 2.6 Addition of buffer

Figure 2.7 20 minutes to test result

Figure 2.8 Negative test result after 20 minutes

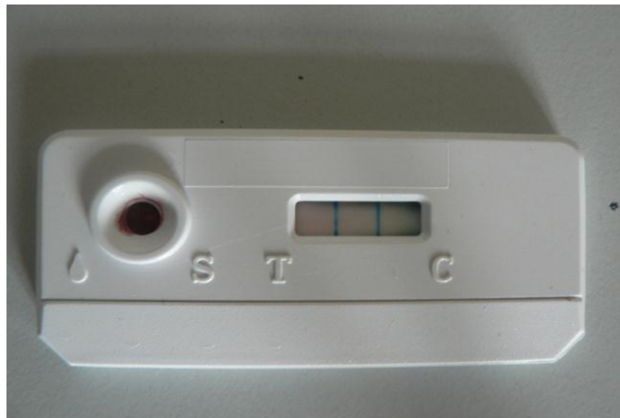

Figure 2.9 Positive test result after using whole blood

2.2.5.7.2 Multiantigen print immunoassay (MAPIA)

In the multiantigen print immunoassay (MAPIA) a panel of defined antigens of *M. tuberculosis* and *M. bovis* are applied to nitrocellulose membranes by semi-automated microspraying, followed by incubation with test sera and detection using standard chromogenic immunodevelopment (LYASHCHENKO et al. 2000, 2006). The used antigens include ESAT-6, CFP-10, MPB-64, MPB-59, MPB-70, MPB-83, Acr-1, 38kDa, CFP-10/ESAT-6, Acr-1/MPB-83, PPD-B (DEAN et al. 2009).

This assay originally developed for the identification of seroreactive antigens in human TB, was successfully used in cattle, white-tailed deer, reindeer, elephants and European badgers infected with *M. bovis* (LYASHCHENKO et al. 2000, 2006). In reindeer, MAPIA has been proven very sensitive and detected antibodies against *M. bovis* earliest after infection at 4 weeks (MILLER 2008).

2.2.5.7.3 Dual-plate-platform (DPP)

A new generation point-of-care TB test has been developed using Chembio DPP® technology. The assay has three separate lines (MPB83, CFP10/ESAT-6, and 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 at 15 minutes visually by two independent operators who did not know the true infection status of the animals. Visible reactivity with any of the three antigen bands observed at 15 minutes was considered an antibody positive result (Figure 2.10). No reactivity with the test antigens was taken as a negative result. Reader device-generated data demonstrated clear-cut discrimination between TB-infected and non-infected elephants using the DPP VetTB assay (GREENWALD et al. 2009).

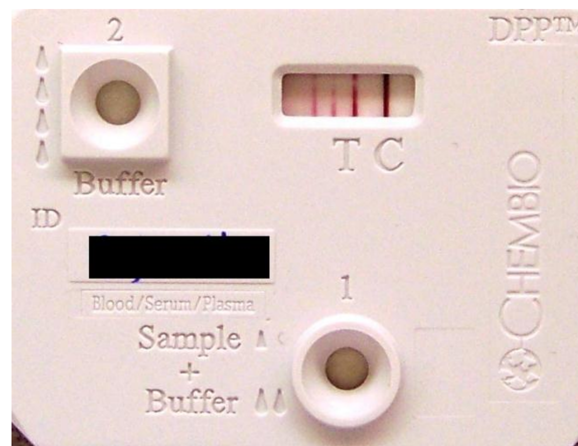

Figure 2.10 Positive DPP test

2.2.5.7.4 ELISA

The enzyme-linked immunosorbent assay (ELISA) has been described for detection of antimycobacterial antibodies in the sera of certain exotic species exposed to clinically significant mycobacteria (THOEN 1993).

ELISA measures antibody formation against specific antigens. The test is performed by adding serum to a well of a microtiter plate that contains the test antigen. Antibody against the antigen will bind to the antigen (protein, lipid, glykolipid, etc.) and remain after washing. The adherent antibody is then detected by a fluorescent tagged antibody against the animal´s antibody (MIKOTA and MASLOW 1997).

Two technical factors limit the application of ELISA. First, unless antibodies against the animals being tested are available, detection is severely limited since cross-reactivity between species is limited (FROST 2006; MIKOTA and MASLOW 1997). Secondly, the antigen against which antibodies are measured must be chosen carefully. The BTB (Blood-TB-test) ELISA test measures antibodies against MPB70, a protein characteristic for *M. bovis* (COUSINS et al. 1993, 2003; HARBOE et al. 1990; MIKOTA and MASLOW 1997). This is not a reliable method to differentiate *M. bovis* from *M. tuberculosis* because this protein is also present in *M. tuberculosis* (ZUMARRAGA 1999). In wild and captive pinnipeds as well as an infected seal trainer no MPB70 antigen was detected in isolates (COUSINS 2006; COUSINS et al. 1990, 2003; THOMPSON et al. 1993; WOODS 1995).

An additional limitation is the contact with environmental mycobacteria, which may lead to the development of low levels of immune sensitization. The application of a post-skin test antibody response test may be useful, because it has been shown that exposure to mycobacterial antigens during tuberculin testing increases humoral antibody levels in animals infected with tuberculosis (BENGIS 1999; DE LISLE et al. 2002).

2.2.6 Treatment

There are different options to address this problem. Refusal to control the disease is irresponsible and does not consider the tremendous impact the disease may have. An intensive monitoring of potentially diseased animals without any control efforts is the absolute minimum of action. Another extreme measurement is the complete culling of infected herds. For small populations of endangered animals this as well as the inability to control tuberculosis will lead to a dramatic further reduction of rare species. In this case to test and cull infected animals is more appropriate unless a sensitive and specific antemortem test is available (BENGIS 1999; THOEN and HIMES 1980).

In human beings treatment consists of a combination of different drugs such as rifampicin, isoniacide and ethambutol and it is carried out for the duration of at least six months (SCHABERG et al. 2001).

In animals treatment of tuberculosis is controversial and of unknown effectiveness. Experimental treatment has been carried out on various animals (HAAGSMA and EGER 1990; THOEN 1993; WEST 2006). Toxicity and poor acceptance of the drugs can affect the success of the treatment. In some animals, like elephants, it was possible to measure serum drug levels and monitor that adequate levels were maintained (WEST 2006). In other species this results in multiple anesthesias to perform the blood collection. In every species culture is necessary for identification and antibiotic sensitivity testing (MILLER 2008). Long-term monitoring of affected individuals is essential for determining the success of TB treatment (MIKOTA and MASLOW 2002).

M. pinnipedii isolates are susceptible to isoniacide, rifampicin, streptomycin, ethambutol and pyrazinamide (COUSINS et al. 2003; KIERS et al. 2008).

There are no reports of treatment of tuberculosis in pinnipeds other than culling.

3 PUBLISHED PAPERS

3.1 Pinniped tuberculosis in Malayan tapirs (*Tapirus indicus*) and its transmission to other terrestrial mammals

Kerstin Jurczynski, Konstantin P. Lyashchenko, David Gomis, Irmgard Moser, Rena Greenwald, and Pierre Moisson.

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PINNIPED TUBERCULOSIS IN MALAYAN TAPIRS (*TAPIRUS INDICUS*) AND ITS TRANSMISSION TO OTHER TERRESTRIAL MAMMALS

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Abstract: In the last 7 yr, three different species of terrestrial mammals were diagnosed with *Mycobacterium pinnipedii* either within one collection or through the introduction of an infected animal from another zoo. The affected species included the Malayan tapir (*Tapirus indicus*), Bactrian camel (*Camelus bactrianus bactrianus*), and crested porcupine (*Hystrix cristata)*. In the first zoo, all of these were living in exhibits adjacent to a group of South American sea lions (*Otaria flavescens*) and were cared for by the same keeper. One infected tapir was transferred to a different zoo and transmitted *M. pinnipedii* infection to three other Malayan tapirs. The tapirs were tested with various diagnostic methods, including comparative intradermal tuberculin test, PCR and culture of sputum samples, Rapid Test (RT), and multiantigen print immunoassay (MAPIA). The *M. pinnipedii* infection was confirmed at postmortem examination in all animals. RT and MAPIA showed the diagnostic potential for rapid antemortem detection of this important zoonotic disease.
Key words: *Mycobacterium pinnipedii*, Malayan tapirs, MAPIA, tuberculosis, zoonosis.

INTRODUCTION

Tuberculosis is a widespread zoonotic disease caused by acid fast bacteria of the *Mycobacterium tuberculosis* complex in a variety of mammalian species.[11] Pinniped tuberculosis has been reported in captive Australian sea lions (*Neophoca cinerea*) in Australia, captive New Zealand fur seals (*Arctocephalus forsteri*) in Australia and the United Kingdom,[4,6] captive South American sea lions (*Otaria flavescens*) in Uruguay and France,[6,7,17,24] wild South American sea lions and wild New Zealand fur seal (*Arctocephalus australis*) in Argentina,[2] wild Australian sea lions (*N. cinerea*) and wild New Zealand fur seals (*A. forsteri*),[5] a wild Australian fur seal (*Arctocephalus pusillus doriferus*) in Tasmania,[27] and a wild Subantarctic fur seal (*Arctocephalus tropicalis*) from Argentina.[6]

The causative agent, *Mycobacterium pinnipedii*, has been distinguished from *Mycobacterium bovis* and *M. tuberculosis*.[2,5] In 2003, Cousins et al.[4] recommended elevation of the "seal bacillus," formerly called M. tuberculosis–type seal, to species level within the *M. tuberculosis* complex. In the past two decades, pathogenicity of *M. pinnipedii* has been reported in experimentally infected guinea pigs and rabbits, a Brazilian tapir (*Tapirus terrestris*), llama (*Lama glama*), western lowland gorilla (*Gorilla gorilla gorilla*), and two felid species (*Panthera uncia, Panthera pardus orientalis*).[3,7,17,24] This highlights the risk of infection for highly endangered species in zoo collections as well as for humans.

The zoonotic potential of *M. pinnipedii* was demonstrated when a seal trainer developed pulmonary tuberculosis 2 yr after an outbreak of pinniped tuberculosis occurred in the marine park where he was working.[6] Culture isolates obtained from this patient and infected animals revealed identical strains of *M. pinnipedii*.[23] In tapirs, tuberculosis is more commonly caused by *M. tuberculosis* or *M. bovis.* Pulmonary disease has been reported for Brazilian tapirs (*T. terrestris*) and Malayan tapirs (*Tapirus indicus*).[9,15,19,20] Antemortem diagnostic testing is always a challenge; therefore, multiple methods should be applied.

The present study documents a multihost species outbreak of *M. pinnipedii* infection involving Malayan tapirs (*T. indicus*), Bactrian camel (*Camelus bactrianus bactrianus*), and Indian crested porcupine (*Hystrix cristata*). The infection presumably originated from a group of South American sea lions (*O. flavescens*) housed in an adjacent exhibit (to the tapir index case) at the Heidelberg Zoo, Germany, and subsequently spread through animal exchange. New antemortem diagnostics were evaluated on some of the infected animals.

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MATERIALS AND METHODS

Animals

Case 1 was a 5-yr-old female Bactrian camel (*C. b. bactrianus*), born in Eastern Europe in 1997 and living at the Heidelberg Zoo since 1998. In 2002, the animal was humanely euthanized because of deteriorating health.

Case 2 was a male adult Malayan tapir (*T. indicus*, "tapir 1"). Tapir 1 was born in South Africa in 1992 and came to the Heidelberg Zoo in 2000. It was transferred to the Mulhouse Zoo in France in May 2004. Before the transfer, the animal had been tested for tuberculosis (TB) by comparative tuberculin skin testing (TST) for *M. bovis* and *M. avium* with no visible reaction. Six months after its arrival, the animal started to lose weight and demonstrated respiratory signs in November 2004. After further investigations, the animal had to be euthanized in January 2005.

Cases 3 and 4 were female Malayan tapirs ("tapir 2" and "tapir 3") living at the Mulhouse Zoo when tapir 1 arrived. Neither animal displayed clinical signs. However, because of positive TB tests on each, these animals were euthanized in August 2005.

Case 5 was a male adult Malayan tapir ("tapir 4"), which had been sent to Mulhouse Zoo from another zoo in May 2005. Having been negative on the Rapid Test and comparative skin test before its arrival, it had progressively increased its seroconversion from January to May 2006. After an induced antibody boost with TST, tapir 4 was finally euthanized in June 2006.

Case 6 was a male crested porcupine (*H. cristata*), which died in 2002 at the Heidelberg Zoo; tuberculosis was confirmed at postmortem examination.

At the Heidelberg Zoo, the exhibits of South American sea lions, Malayan tapirs, Bactrian camels, and crested porcupines were located next to each other, and the animals were cared for by the same keepers (Fig. 1). At the Mulhouse Zoo, tapirs 1, 2, and 3 were in direct contact, whereas tapir 4 only shared their outdoor exhibit.

Tuberculin tests

Comparative TST was performed by intradermal injections of 0.1 ml of bovine purified protein derivative (PPD; 2,000 IU, Bovituber®, Synbiotics Europe, 69367 Lyon Cedex 07, France) and 0.1 ml of avian PPD (2,500 IU, Avituber®, Synbiotics Europe, 69367 Lyon Cedex 07, France).

Figure 1. Exhibit structure at the Heidelberg Zoo showing the close proximity.

Rapid Test (RT): Available fresh and frozen serum samples were evaluated by RT on the basis of lateral flow technology (ElephantTB STAT-PAK) recently developed by Chembio Diagnostic Systems Inc. (Medford, New York 11763, USA). This test was performed as described previously.[13,18]

Multiantigen print immunoassay (MAPIA): A panel of 12 defined antigens was used in MAPIA to identify serologic correlates of active disease. The following recombinant antigens of *M. bovis* were purified to >95% purity as polyhistidine-tagged proteins: ESAT-6 and CFP-10 produced at the Statens Serum Institut (2300 Copenhagen, Denmark) and MPB59, MPB64, MPB70, and MPB83 produced at Veterinary Sciences Division (BT4 3SD Stormont, Belfast, United Kingdom). Alpha-crystallin (Acr1) and the 38-kDa protein of *M. tuberculosis* were purchased from Standard Diagnostics (156–68, Seoul, South Korea). Polyprotein fusions CFP-10/ESAT-6 and Acr1/MPB83 were constructed at the Statens Serum Institut by overlapping polymerase chain reaction (PCR) with the use of gene-specific oligonucleotides to amplify the genes from *M. tuberculosis* H37Rv chromosomal DNA. The fused polygene PCR products were cloned into the pMCT6 *Escherichia coli* expression vector using *Sma*I and *Bam*HI restriction enzymes. The polyproteins were purified to >95% purity by exploiting the polyhistidine tag encoded by the vector. *Mycobacterium bovis* culture filtrate (MBCF) was obtained from a field strain of *M. bovis* (T/91/1378;

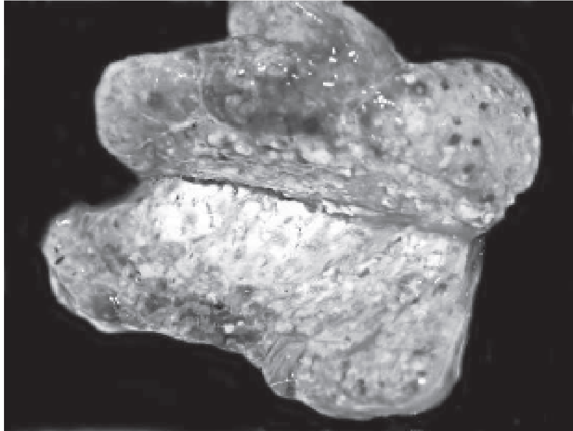

Figure 2. Granulomatous lesion in the mesenteric lymph node of a tapir.

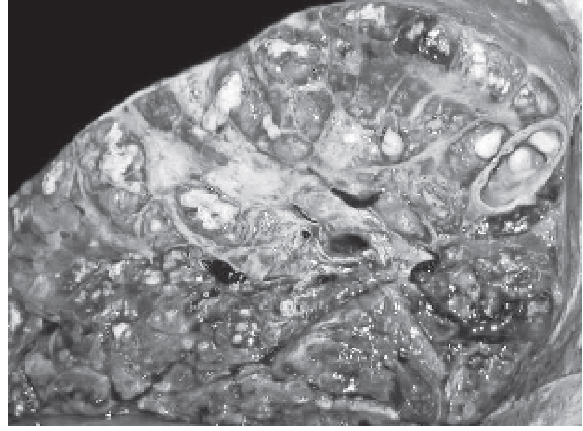

Figure 3. Tapir 1 lung whole section shows granulomatous lesions.

Veterinary Sciences Division) cultured in synthetic Sauton's medium.

MAPIA was performed as described previously.[13,14] Briefly, antigens were immobilized on nitrocellulose membrane (Schleicher & Schuell, Keene, New Hampshire 03431, USA) at a protein concentration of 0.05 mg/ml using a semiautomated airbrush printing device (Linomat IV, Camag Scientific Inc., Wilmington, Delaware 28401, USA). The membrane was cut perpendicular to the antigen bands into 4-mm-wide strips. Strips were blocked for 1 hr with 1% nonfat skim milk in phosphate-buffered saline with 0.05% Tween 20 (PBST; Sigma-Aldrich, St. Louis, Missouri 63178, USA) and then incubated for 1 hr with serum samples diluted 1:50 in blocking solution.

After washing, strips were incubated overnight with peroxidase-conjugated Protein A (Sigma-Aldrich) diluted 1:1,000, followed by another wash step. Immunoglobulin G antibodies bound to printed antigens were visualized with 3,3',5,5'-tetramethyl benzidine (Kirkegaard and Perry Laboratories Inc., Gaithersburg, Maryland 20878, USA).

Postmortem examination

Postmortem examination was performed on all euthanized or deceased animals. The examinations were conducted by certified pathologists. Samples of lungs, liver, kidney, spleen, intestines, heart, trachea, and varius lymph nodes (axillary, mediastinal, intestinal) were taken and examined for histology, bacteriology (culture), and virology. Ziehl–Neelsen stains were performed on lungs and lymph nodes.

RESULTS

Case 1

Postmortem examination of the Bactrian camel revealed numerous caseous nodules up to 30 mm in diameter in the lungs and in the enlarged thoracic lymph nodes. Histologically, these granulomas contained a central zone of caseous necrosis and mineralization. Acid-fast bacilli were detected in the lungs and lymph nodes. PCR and culture for tuberculous bacteria were positive as well. A spoligotyping carried out by the Friedrich Loeffler Institute in Jena (Germany) identified *M. pinnipedii*. Unfortunately, no serum was available for RT and MAPIA testing.

Case 2

A sputum sample of tapir 1 submitted for microbiology stained positive for acid-fast bacilli. The animal was euthanized. A postmortem serum sample was RT-positive, and MAPIA confirmed antibody reactivity. The postmortem examination revealed macroscopically calcified lesions in the mesenteric lymph nodes and cicatrical, cavernous lesions in the lungs (Figs. 2, 3). Histology revealed severe pneumonic lesions characteristic of tuberculosis, and spoligotyping identified the causative agent as *M. pinnipedii*.

Cases 3 and 4

The female tapirs 2 and 3 both tested positive by RT in 2005. The animals were euthanized. Additional tests (Ziehl–Neelsen staining, RT, MAPIA) were conducted postmortem, with positive results for *Mycobacterium*. Several granulomatous lesions in the lungs and mediastinal

lymph nodes were noted at postmortem examination. Culture and spoligotyping confirmed *M. pinnipedii*.

Case 5

The last Malayan tapir (tapir 4) at Mulhouse was negative by TST and RT before its arrival from the Copenhagen Zoo (Denmark) in May 2005. Although tapir 4 never had direct contact to either female at the Mulhouse Zoo, they shared the outdoor exhibit.

Chronologic RT testing of tapir 4 demonstrated seroconversion starting with the first positive RT in January 2006, followed by a gradual increase of antibody levels (intensity of test band in RT) in the following months. MAPIA results supported the RT findings. In May 2006, an antibody boost was provoked with TST. It was expected to induce an antibody boost that could be followed with MAPIA bands shortly after tuberculin injection. Both RT and MAPIA revealed significantly elevated antibody responses 2 wk after PPD injection (Fig. 4). The animal was euthanized in June 2006. Postmortem examination findings included granulomatous tracheobronchial lymph nodes. Culture and subsequent poligotyping confirmed *M. pinnipedii*.

Case 6

Postmortem examination of the crested porcupine revealed caseous lesions in the lobes of the right lung and throughout both kidneys. Acid-fast bacteria were isolated, and the kidneys were submitted for mycobacterial culture. PCR was positive for the *M. tuberculosis* complex. The spoligotyping at that time was unable to distinguish between *M. tuberculosis* and *M. africanum*. The pattern of the crested porcupine was compared with the pattern of *M. pinnipedii* 7 yr later. The reference laboratory declared the two patterns identical and confirmed the infection of the porcupine as caused by *M. pinnipedii*.

DISCUSSION

Several diagnostic methods were applied during the tuberculosis outbreak described in the present report. The "gold standard" of confirming an infection with the *M. tuberculosis* complex organisms is the isolation of the bacteria. This method, however, can only identify extensive shedders.[13] PCR is a more sensitive method of detection and can be used on feces, sputum, or tracheal lavage samples.[11] It can identify organisms belonging to

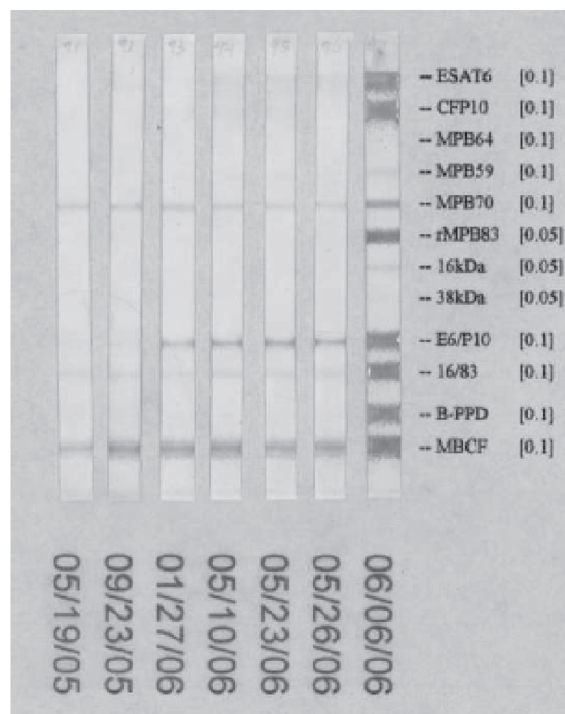

Figure 4. MAPIA of tapir 4 shows a significantly elevated antibody response 2 wk after TST.

the *M. tuberculosis* complex, but it cannot distinguish between *M. bovis* and *M. tuberculosis*.[5]

The TST used for domestic ruminants has been similarly applied in many exotic species. This test relies on a delayed-type hypersensitivity reaction.[1] PPD tuberculins prepared from *M. bovis* and *M. avium* are widely used.[21,22] In tapirs, PPD should be injected in the inguinal region near the nipples, although the skin around the perineum may also be used.[9,16] Injection site is visually observed and palpated at 72 hr after injection, and any inflammatory response is classified as suspect.[22]

However, the TST raises several issues. First, it requires double injections for execution and reading of the test. Second, it detects only infection, not necessarily active disease.[10] Third, it has a low sensitivity. Animals with advanced disease can give anergic responses to tuberculin.[1,16,22] Finally, the low specificity of this test can cause false-negative test results in animals other than domestic hoofstock and nonhuman primates.[1,10,11] In some instances, even false-positive reactions can occur. Tapirs are known to have an increased likelihood of nonspecific reactions.[16]

In the present study, male Malayan tapir 1 had a negative response to the TST when it left the Heidelberg Zoo, although it was suspected that it was already infected with *M. pinnipedii* because

the camel and porcupine living near its enclosure were confirmed to have the same *Mycobacterium* strain. The TST proved useful in the post–skin test antibody response test that was performed on tapir 4 before it was euthanized. In this test, a blood sample was taken before performing a TST. The results were compared with a second one taken 2–3 wk later. It had been reported that exposure to tuberculin antigens during skin testing increases humoral antibody levels in tuberculous animals.[2] The MAPIA results clearly demonstrate the expected antibody boost. This approach applied in TB-negative Malayan tapirs resulted in no reaction,[7] suggesting high diagnostic specificity.

The RT developed by Chembio was carried out on all tapirs. The first tapir tested positive postmortem in January 2005. The two females were tested after its death and were also confirmed positive. After a post–skin test antibody response test in tapir 4, the seroconversion was first demonstrated in January 2006. In this animal, the RT was positive at a very early stage of the infection—weak at the beginning and getting gradually stronger—long before clinical signs were observed. Antibody-positive results were confirmed by MAPIA. MAPIA revealed a gradual change in the band pattern as time progressed, indicating the differential emergence of mycobacterial antigens in the course of infection (early antigens, late antigens) leading to the production of antibodies of different specificities. Both test results were consistent with postmortem examination findings. The RT and MAPIA have been used successfully in elephants, cattle, white-tailed deer, reindeer, and Eurasian badgers infected with *M. bovis*.[8,12–14,25,26]

Spoligotyping conducted on the isolates helped determine the suspected origin of the *M. pinnipedii* strain. When the spoligotyping patterns of all four tapir isolates were compared with those from the Bactrian camel and South American sea lion identified at the Heidelberg Zoo, the strains appeared identical and characteristic for *M. pinnipedii*.[18] This finding confirmed that tapir 1 likely brought the infectious agent from Heidelberg to Mulhouse, where tapirs 2, 3, and 4 became infected by direct contact or sharing of the exhibit and then subsequently developed clinical disease. The spoligotyping pattern of the porcupine revealed *M. pinnipedii*, but the exact strain was never identified because the animal died 7 yr before.

In the infected tapirs, camel, and sea lions, lesions have been found in the lungs and surrounding lymph nodes predominantly. This supports the assumption that the disease was transmitted by aerosol, as it has been suspected in previous reports.[7] The sea lion pool at the Heidelberg Zoo is routinely subjected to weekly high-pressure cleaning, and this could be the source of infection for the tapir, camel, and porcupine in the adjacent enclosures. Additionally, the same keepers take care of all these species, and fomites could be another source of infection.

CONCLUSIONS

No antemortem test is 100% reliable for detecting tuberculous infections in captive wildlife animals. Most tests are not validated and have low sensitivity, low specificity, or both in nondomestic host species. In this study, RT and MAPIA showed promising results in identifying tuberculosis due to *M. pinnipedii* in tapirs. The spoligotyping confirmed the origin of infection. This method remains an important tool for epidemiology of zoonotic infectious diseases. The reported findings further demonstrate the ability of *M. pinnipedii* to cause fatal disease in multiple species of zoo animals other than pinnipeds, as well as the importance of preshipment testing for tuberculosis.

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LITERATURE CITED

1. Bengis, R. G. 1999. Tuberculosis in free-ranging mammals. *In:* Fowler, M. E., and R. E. Miller (eds.). Zoo & Wild Animal Medicine, 4th ed. W. B. Saunders Co., Philadelphia, Pennsylvania. Pp. 101–114.

2. Bernadelli, A., R. Bastida, J. Loureiro, H. Michelis, M. I. Romano, A. Cataldi, and E. Costa. 1996. Tuberculosis in sea lions and fur seals from the southwestern Atlantic coast. Rev. Sci. Tech. Off. Int. Epizoot. 15: 985–1005.

3. Cousins, D. 2006. Tuberculosis in fur seals and sea lions caused by *Mycobacterium pinnipedii*. *In:* Thoen, C. O., J. H. Steele, and M. J. Gilsdorf (eds.). *Mycobacterium bovis* Infection in Animals and Humans. Blackwell Publishers, Boston, Massachusetts. Pp. 258–270.

4. Cousins, D. V., R. Bastida, A. Cataldi, V. Quse, S. Redrobe, S. Dow, P. Duignan, A. Murray, C. Dupont, N. Ahmed, D. M. Collins, W. R. Butler, D. Dawson, D. Rodriguez, J. Loureiro, M. I. Romano, A. Alito, M. Zumarraga, and A. Bernardelli. 2003. Tuberculosis in

seals caused by a novel member of the Mycobacterium tuberculosis complex: *Mycobacterium pinnipedii* sp.nov. Int. J. Syst. Evol. Microbiol. 53: 1305–1314.

5. Cousins, D. V., S. N. Williams, R. Reuter, D. Forshaw, B. Chadwick, D. Coughran, P. Collins, and N. Gales. 1993. Tuberculosis in wild seals and characterisation of the seal bacillus. Aust. Vet. J. 70: 92–97.

6. Dunn, J. L., J. D. Buck, and T. R. Robeck. 2001. Bacterial diseases of cetaceans and pinnipeds. *In:* Dierauf, L. A., and F. M. D. Gulland (eds.). CRC Handbook of Marine Mammal Medicine, 2nd ed. CRC Press, Boca Raton, Florida. Pp. 309–335.

7. Gomis, D., P. Moisson, K. P. Lyashchenko, I. Moser, D. Bakker, M. C. Gutiérrez, M. L. Boschiroli, and A. Gravet. 2008. Tuberculosis outbreaks in Mulhouse Zoo, 1992–96 and 2005–06: diagnostic perspectives. Proc. Eur. Assoc. Zoo Wildl. Vet. Conf. 2008: 157–171.

8. Greenwald, R., J. Esfandiari, S. Lesellier, R. Houghton, J. Pollock, C. Aagaard, P. Andersen, R. G. Hewinson, M. Chambers, and K. Lyashchenko. 2003. Improved serodetection of *Mycobacterium bovis* infection in badgers (*Meles meles*) using multiantigen test formats. Diagn. Microbiol. Infect. Dis. 46: 197–203.

9. Janssen, D. L., B. A. Rideout, and M. E. Edwards. Medical management of captive tapirs (Tapirus spp.). Proc. Am. Assoc. Vet. 1996: 1–11.

10. Kaandorp, J. Diagnosis in mycobacteriosis. Proc. Eur. Assoc. Zoo Wildl. Vet. Conf. 1998: 85–91.

11. Larsen, R. S., and M. D. Salman. 2001. Diagnostic testing for *M. bovis* in nondomestic hoofstock. Proc. Am. Assoc. Zoo Vet. 2001: 203–208.

12. Lyashchenko, K. P., R. Greenwald, J. Esfandiari, J. H. Olsen, R. Ball, G. Dumonceaux, F. Dunker, C. Buckley, M. Richard, S. Murray, J. B. Payeur, P. Andersen, J. M. Pollock, S. Mikota, M. Miller, D. Sofranko, and W. R. Waters. 2006. Tuberculosis in elephants: antibody responses to defined antigens of *Mycobacterium tuberculosis*, potential for early diagnosis and monitoring of treatment. Clin. Vaccine Immunol. 13: 722–732.

13. Lyashchenko, K. P., M. Singh, R. Colangeli, and M. L. Gennaro. 2000. A multiantigen print immunoassay for the serological diagnosis of infectious diseases. J. Immunol. Meth. 242: 91–100.

14. Lyashchenko, K., A. O. Whelan, R. Greenwald, J. M. Pollock, P. Andersen, R. G. Hewinson, and H. M. Vordermeier. 2004. Association of tuberculin-boosted antibody responses with pathology and cell-mediated immunity in cattle vaccinated with *Mycobacterium bovis* BCG and infected with *M. bovis*. Infect. Immunol. 72: 2462–2467.

15. Michel, A. L., L. Venter, I. W. Espie, and M. L. Coetzee. 2003. *Mycobacterium tuberculosis* infections in eight species at the National Zoological Gardens of South Africa, 1991–2001. J. Zoo Wildl. Med. 34: 364–370.

16. Miller, M.A. Current diagnostic methods for tuberculosis in zoo animals. *In:* Fowler, M. E., and R. E. Miller (eds.). Zoo and Wild Animal Medicine Current Therapy. Saunders, St. Louis, Missouri. Pp. 10–19.

17. Moisson, P., F. Lacroix , C. Manson, G. André-Fontaine, D. Bakker, L. Fiette, M. F. Thorel, and J.-M. Lernould. 1998. Tuberculosis outbreak in some felid species and southern sea lions in Mulhouse Zoo from 1992 to 1996: diagnostic perspectives. Proc. Eur. Assoc. Zoo Wildl. Vet. 1998: 98–112.

18. Moser, I., W. M. Prodinger, H. Hotzel, R. Greenwald, K. P. Lyashchenko, D. Bakker, D. Gomis, T. Seidler, C. Ellenberger, U. Hetzel, K. Wuennemann, and P. Moisson. 2007. *Mycobacterium pinnipedii*: transmission from South American sea lions (*Otaria byronia*) to Bactrian camel (*Camelus bactrianus bactrianus*) and Malayan tapirs (*Tapirus indicus*). Vet. Microbiol. 127: 399–406.

19. Ramsay, E. C., and Z. Zainuddin. 1993. Infectious diseases of the rhinoceros and tapir. *In:* Fowler, M. E. (ed.). Zoo and Wild Animal Medicine, 3rd ed. W.B. Saunders, Philadelphia, Pennesylvania. Pp. 459–466.

20. Sternberg, S., K. Bernodt, A. Hollström, and B. Röken. 2002. Survey of tuberculin testing in Swedish zoos. J. Zoo Wildl. Med. 33: 378–380.

21. Thoen, C. O. Tuberculosis and other mycobacterial diseases in captive wild animals. *In:* M. E. Fowler (ed.). Zoo and Wild Animal Medicine. W. B. Sanders, Denver, Colorado. Pp. 45–49.

22. Thoen, C. O., and E. D. Ebel. 2006. Diagnostic tests for bovine tuberculosis, chap. 6. *In:* Thoen, C. O., J. H. Steele, and M. J. Gilsdorf (eds.). *Mycobacterium bovis* Infection in Animals and Humans. Blackwell Publishers, Boston, Massachusetts. Pp. 49–53.

23. Thompson, P. J., D. V. Cousins, B. L. Gow, D. M. Collins, B. H. Williamson, and H. T. Dagnia. 1993. Seals, seal trainers and mycobacterial infection. Am. Rev. Respir. Dis. 147: 164–167.

24. Thorel, M.-F., C. Karoui, A. Varnerot, C. Fleury, and V. Vincent. 1998. Isolation of *Mycobacterium bovis* from baboons, leopards and a sea lion. Vet. Res. 29: 207–212.

25. Waters, W. R., M. V. Palmer, J. P. Bannantine, R. Greenwald, J. Esfandiari, P. Andersen, J. McNair, J. M. Pollock, and K. P. Lyashchenko. 2005. Antibody responses in reindeer (*Rangifer tarandus*) infected with *Mycobacterium bovis*. Clin. Diagn. Lab. Immunol. 12: 727–735.

26. Waters, W. R., M. V. Palmer, J. P. Bannantine, D. L. Whipple, R. Greenwald, J. Esfandiari, P. Andersen, J. McNair, J. M. Pollock, and K. P. Lyashchenko. 2004. Antigen recognition by serum antibodies in white-tailed deer (*Odocoileus virginianus*) experimentally infected with *Mycobacterium bovis*. Clin. Diagn. Lab. Immunol. 11: 849–855.

27. Woods, R., D. V. Cousins, R. Kirkwood, and D. L. Obendorf. 1995. Tuberculosis in wild Australian fur seal (*Arctocephalus pusillus doriferus*) from Tasmania. J. Wildl. Dis. 31: 83–86.

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3.2 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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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 noninvasive 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®

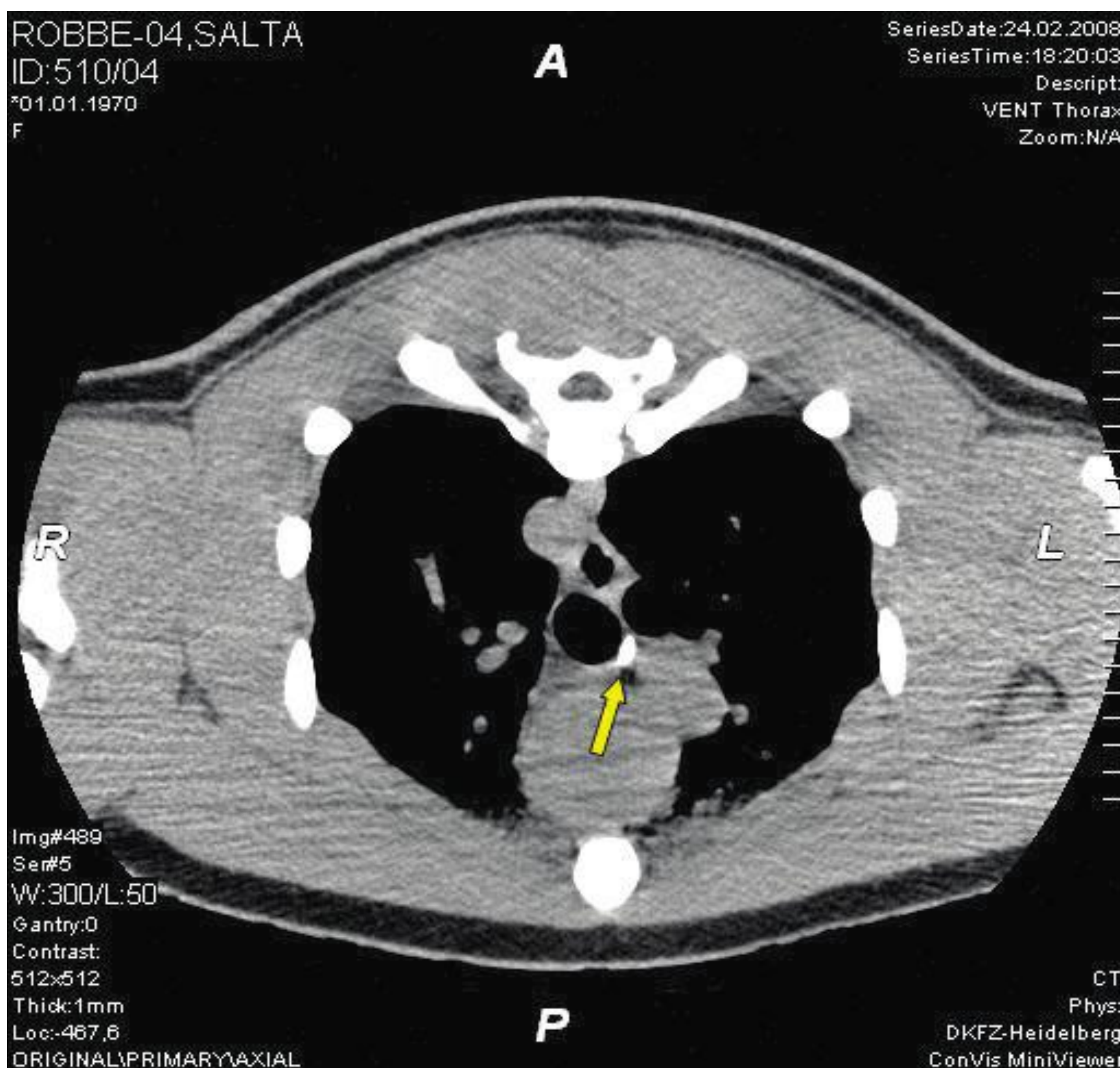

Figure 1. CT scan of female sea lion; yellow arrow is pointing to calcified mediastinal lymph node.

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® 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.

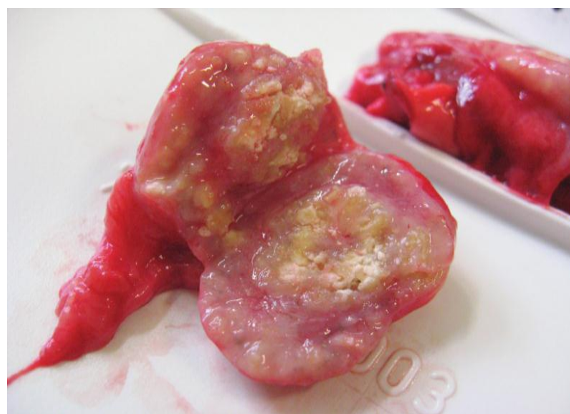

Figure 2. Mediastinal lymph node showing miliary calcifications

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.

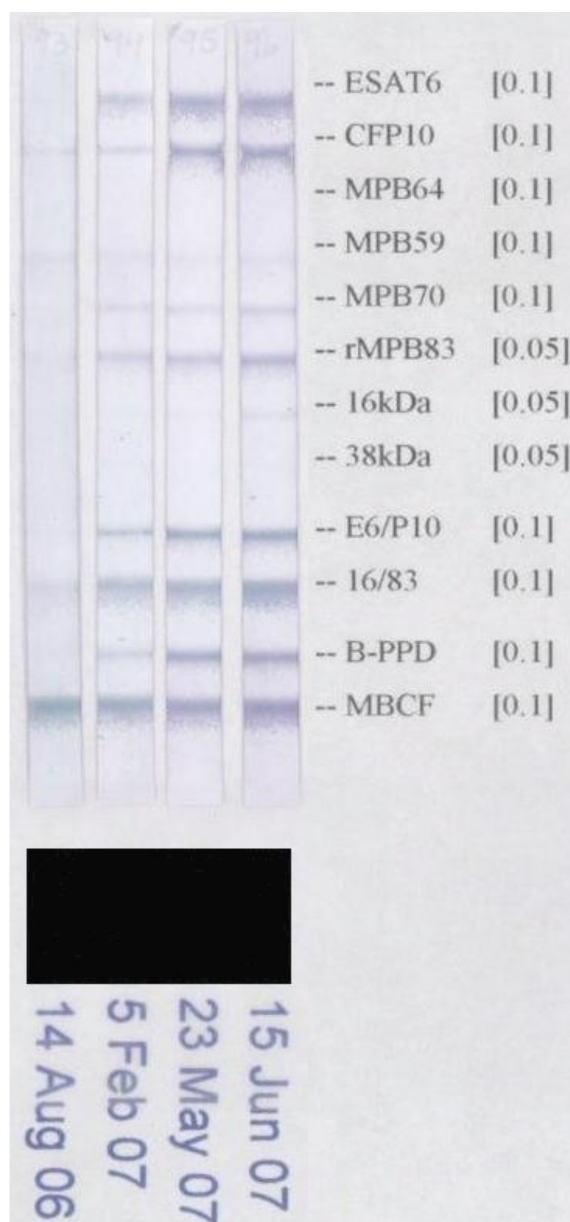

Figure 4. Seroconversion of Case 10 shown in a series of MAPIAs

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

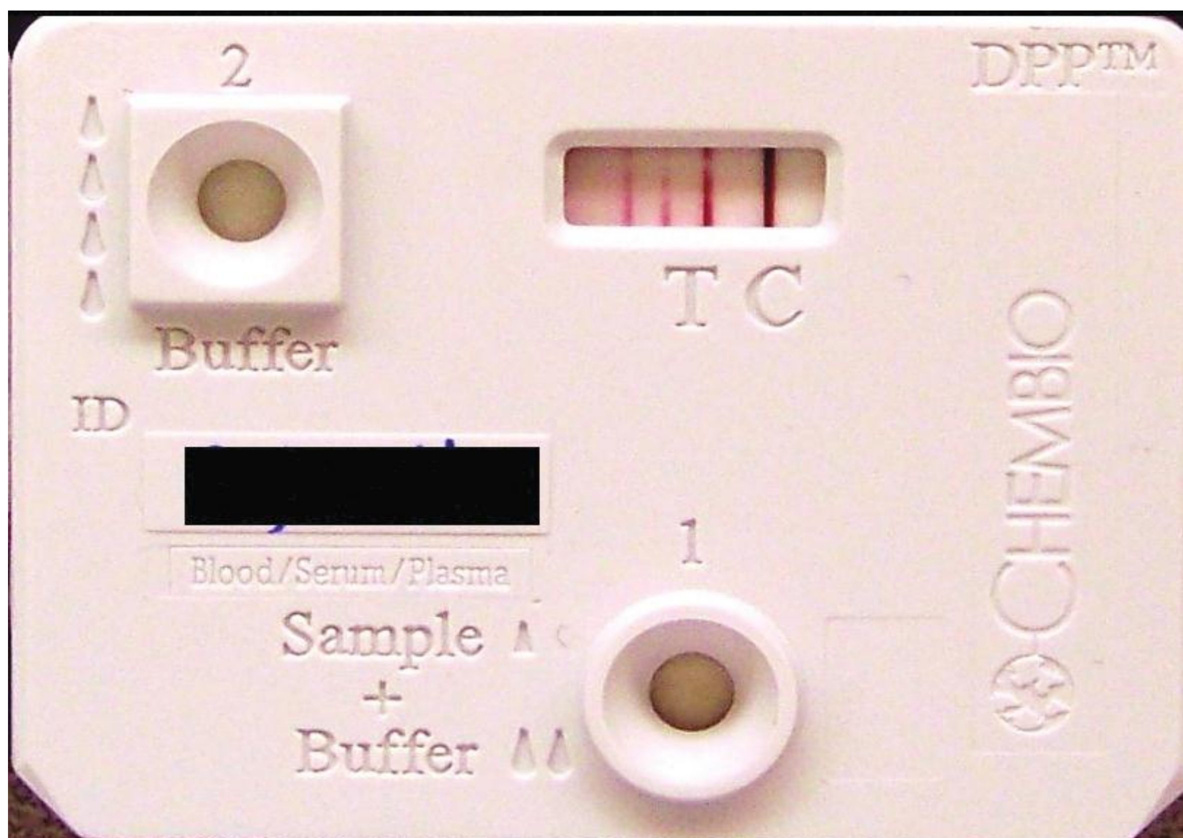

Figure 5. A positive DPP test, showing antibodies against MPB83, E6/P10, and F10

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 offe no sufficient penetration.

Treatment has been carried out after antibioti sensitivity testing. One animal (Case 10) that sero converted within a few months of infection, and i which *M. pinnipedii* has been identified by sputun culture ante-treatment, has been negative in mul tiple CT scans and sputum cultures since then. I needs to be considered that treatment of expose animals, if initiated early enough, may preven manifestation of infection. Post-treatment eutha nized animals revealed detection of mycobacteria i the calcified lymph nodes but only by PCR. Cultur remained negative in all these animals, wherea *M. pinnipedii* was cultured out of the lesions fron the nontreated animals. Therefore, at least up t now, there is a strong indication that treatment ha been successful in eliminating the bacteria.

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

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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Literature Cited

Anonymous. (2006, October). *Workshop of Co-ordinated Action SSPE-CT-2004-501903 – Veterinary network of laboratories researching into improved diagnosis and epidemiology of mycobacterial diseases*. Toledo, Spain.

Bengis, R. G. (1999). Tuberculosis in free-ranging mammals. In M. E. Fowler & R. E. Miller (Eds.), *Zoo & wild animal medicine* (4th ed., pp. 101-113). Philadelphia, PA: W. B. Saunders & Company.

Castro Ramos, M., Ayala, M., Errico, F., & Silvera, F. V. (1998). Aislamiento de *Mycobacterium bovis* en pinnípedos *Otaria byronia* (Lobo marino común) en Uruguay. [Isolation of *Mycobacterium bovis* in pinnipeds *Otaria byronia* (South American sea lion) in Uruguay]. *Revista de Medicina Veterinaria*, *79*(3), 197-200.

Cousins, D. (2006). Tuberculosis in fur seals and sea lions caused by *Mycobacterium pinnipedii*. In C. O. Thoen, J. H. Steele, & M. J. Gilsdorf (Eds.), *Mycobacterium bovis infection in animals and humans* (2nd ed., pp. 258-270). Ames, IA: Blackwell Publishing. http://dx.doi.org/10.1002/9780470344538.ch26

Cousins, D. V., Francis, B. R., Gow, B. L., Collins, D. M., McGlashan, C. H., Gregory, A., & Mackenzie, R. M. (1990). Tuberculosis in captive seals: Bacteriological studies on an isolate belonging to the *Mycobacterium tuberculosis* complex. *Research in Veterinary Science*, *48*, 196-200.

Cousins, D. V., Williams, S. N., Reuter, R., Forshaw, D., Chadwick, B., Coughran, D., . . . Gales, N. (1993). Tuberculosis in wild seals and characterisation of the seal bacillus. *Australian Veterinary Journal*, *70*, 92-97. http://dx.doi.org/10.1111/j.1751-0813.1993.tb03284.x

Cousins, D.V., Bastida, R., Cataldi, A., Quse, V., Redrobe, S., Dow, S., . . . Bernardelli, A. (2003). Tuberculosis in seals caused by a novel member of the *Mycobacterium tuberculosis* complex: *Mycobacterium pinnipedii* sp. *nov*. *International Journal of Systematic and Evolutionary Microbiology*, *53*, 1305-1314. http://dx.doi.org/10.1099/ijs.0.02401-0

Ehlers, K. (1965). Records of the hooded seal, *Cystophora cristata Erxl*, and other animals at Bremerhaven Zoo. *International Zoological Yearbook*, *5*, 148-149. http://dx.doi.org/10.1111/j.1748-1090.1965.tb01618.x

Forshaw, D., & Phelps, G. R. (1991). Tuberculosis in a captive colony of pinnipeds. *Journal of Wildlife Diseases*, *27*(2), 288-295.

Gomis, D., Moisson, P., Lyashchenko, K. P., Moser, I., Bakker, D., Gutiérrez, M. C., . . . Gravet, A. (2008, May). *Tuberculosis outbreaks in Mulhouse Zoo, 1992-96 and 2005-06: Diagnostic perspectives*. Proceedings of the Seventh Conference of the European Association of Zoo and Wildlife Veterinarians, Leipzig, Germany.

Greenwald, R., Lyashchenko, O., Esfandiari, J., Miller, M., Mikota, S., Olsen, J. H., . . . Lyashchenko, K. P. (2009). Highly accurate antibody assays for early and rapid detection of tuberculosis in African and Asian elephants. *Clinical and Vaccine Immunology*, *16*, 605-612. http://dx.doi.org/10.1128/CVI.00038-09

Harboe, M., Wiker, H. G., Duncan, J. R., Garcia, M. M., Dukes, T. W., Brooks, B. W., . . . Nagai, S. (1990). Protein G-based enzyme-linked immunosorbent assay for anti-MPB70 antibodies in bovine tuberculosis. *Journal of Clinical Microbiology*, *28*(5), 913-921.

Jurczynski, K., Lyashchenko, K. P., Gomis, D., Moser, I., Greenwald, R., & Moisson, P. (2011). Pinniped tuberculosis in Malayan tapirs (*Tapirus indicus*) and its transmission to other terrestrial mammals. *Journal of Zoo and Wildlife Medicine*, *42*(2), 222-227. http://dx.doi.org/10.1638/2009-0207.1

Kaandorp, J. (1998, May). *Diagnosis in mycobacteriosis*. Proceedings of the Second Conference of the European Association of Zoo and Wildlife Veterinarians, Chester, UK.

Kamerbeek, J., Schouls, L., Kolk, A., van Agterveld, M., van Soolingen, D., Kuijper, S., . . . van Emden, J. (1997). Simultaneous detection and strain differentiation of *Mycobacterium tuberculosis* for diagnosis, and epidemiology. *Journal of Clinical Microbiology*, *35*, 907-914.

Kaneene, J. B., & Thoen, C. O. (2004). Tuberculosis. *Journal of the American Veterinary Medical Association*, *224*(5), 685-691. http://dx.doi.org/10.2460/javma.2004.224.685

Lacave, G., Maillot, A., Alerte, V., Boschiroli, M. L., & Lecu, A. (2009, May). *Atypical case of* Mycobacterium pinnipedii *in a Patagonian sea lion (*Otaria flavescens*) and tuberculosis cases history review in captive pinnipeds*. Proceedings of the Fortieth Conference of the International Association for Aquatic Animal Medicine, San Antonio, TX.

Lange, C., Schaberg, T., Diel, R., & Greinert, U. (2006). *Aktueller stand der tuberkulosediagnostik* [Current status in diagnostics of tuberculosis]. *Deutsche Medizinische Wochenschrift*, *131*, 341-347. http://dx.doi.org/10.1055/s-2006-932523

Lecu, A., & Ball, R. (2011). Mycobacterial infections in zoo animals: Relevance, diagnosis and management. *International Zoo Yearbook*, *45*, 183-202. http://dx.doi.org/10.1111/j.1748-1090.2011.00141.x

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Lyashchenko, K. P., Singh, M., Colangeli, R., & Gennaro, M. L. (2000). A multi-antigen print immunoassay for the development of serological diagnosis of infectious diseases. *Journal of Immunological Methods*, *242*, 91-100. http://dx.doi.org/10.1016/S0022-1759(00)00241-6

Lyashchenko, K. P., Greenwald, R., Esfandiari, J., Olsen, J. H., Ball, R., Dumonceaux, G., . . . Waters, W. R. (2006). Tuberculosis in elephants: Antibody responses to defined antigens of *Mycobacterium tuberculosis*, potential for early diagnosis, and monitoring of treatment. *Clinical Vaccine Immunology*, *13*, 722-732. http://dx.doi.org/10.1128/CVI.00133-06

Mikota, S. K., & Maslow, J. (1997, October). *Theoretical and technical aspects of diagnostic techniques for mammalian tuberculosis*. Proceedings of the Conference of the American Association of Zoo Veterinarians, Houston, TX.

Miller, M. A. (2008). Current diagnostic methods for tuberculosis in zoo animals. In M. E. Fowler & R. E. Miller (Eds.), *Zoo and wild animal medicine* (6th ed., pp. 10-19). St. Louis, MO: Saunders Elsevier. http://dx.doi.org/10.1016/B978-141604047-7.50005-1

Moser, I., Prodinger, W. M., Hotzel, H., Greenwald, R., Lyashchenko, K. P., Bakker, D., . . . Moisson, P. (2008). *Mycobacterium 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. http://dx.doi.org/10.1016/j.vetmic.2007.08.028

Rodriguez, J., Fissanoti, G. J. C., del Portillo, P., Patarroyo, M. E., Romano, M. L., & Cataldi, A. (1999). Amplification of a 500bp fragment from culture isolates of *Mycobacterium bovis*. *Journal of Clinical Microbiology*, *37*, 2330-2332.

Supply, P., Allix, C., Lesjean, S., Cardoso-Oelemann, M., Rusch-Gerdes, S., Willery, E., . . . van Soolingen, D. (2006). Proposal for standardization of optimized mycobacterial interspersed repetitive unit-variable-number tandem repeat typing of *Mycobacterium tuberculosis*. *Journal of Clinical Microbiology*, *44*, 4498-4510. http://dx.doi.org/10.1128/JCM.01392-06

Thoen, C. O. (1993). Tuberculosis and other mycobacterial diseases in captive wild animals. In M. E. Fowler (Ed.), *Zoo and wild animal medicine* (3rd ed., pp. 45-49). Philadelphia, PA: W. B. Saunders.

Thoen, C. O., & Barletta, R. G. (2006). Pathogenesis of *Mycobacterium bovis*. In C. O. Thoen, J. H. Steele, & M. J. Gilsdorf (Eds.), Mycobacterium bovis *infection in animals and humans* (pp. 18-33). Boston: Blackwell Publishers. http://dx.doi.org/10.1002/9780470344538.ch4

Thompson, P. J., Cousins, D. V., Gow, B. L., Collins, D. M., Williamson, B. H., & Dagnia, H. T. (1993). Seals, seal trainers and mycobacterial infection. *American Review of Respiratory Disease*, *147*, 164-167. http://dx.doi.org/10.1164/ajrccm/147.1.164

Thomson, B. (2006). Polymerase chain reaction detection of *Mycobacteria tuberculosis* complex in formalin-fixed tissues. In C. O. Thoen, J. H. Steele, & M. J. Gilsdorf (Eds.), Mycobacterium bovis *infection in animals and humans* (pp. 63-67). Boston: Blackwell Publishers. http://dx.doi.org/10.1002/9780470344538.ch8

West, G. (2006). Tuberculosis in captive exotic animals. In C. O. Thoen, J. H. Steele, & M. J. Gilsdorf (Eds.), Mycobacterium bovis *infection in animals and humans* (pp. 248-257). Boston: Blackwell Publishers. http://dx.doi.org/10.1002/9780470344538.ch25

3.3 Computed tomographic examination of South American sea lions (*Otaria flavescens*) with suspected *Mycobacterium pinnipedii* infection

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Papers

Computed tomographic examination of South American sea lions (*Otaria flavescens*) with suspected *Mycobacterium pinnipedii* infection

K. Jurczynski, J. Scharpegge, J. Ley-Zaporozhan, S. Ley, J. Cracknell, K. Lyashchenko, R. Greenwald, J. P. Schenk

Ten South American sea lions (*Otaria flavescens*) were presented for clinical evaluation and diagnosis of tuberculosis following known exposure to *Mycobacterium pinnipedii*. CT was used to determine whether foci of calcification in mediastinal lymph nodes, typically associated with pinniped tuberculosis, could be detected and whether CT was a useful diagnostic modality, in conjunction with other tests, for the diagnosis of tuberculosis in this species. Blood was collected from the caudal gluteal vein of each animal for serological testing using commercially available serological tests (ElephantTB STAT-PAK and DPP Vet; Chembio Diagnostic Systems) and a multiantigen print immunoassay (MAPIA), carried out at Chembio to verify the in-house kits. In four of nine animals that underwent CT scanning, lesions consistent with pinniped tuberculosis were apparent and these were confirmed at subsequent postmortem examination. The five remaining animals did not show any abnormalities on CT, with three being negative on serological tests, which were considered to be normal and potentially used as reference images for healthy sea lions. One animal could not be CT scanned due to its large size and weight (510 kg).

PINNIPED tuberculosis has occurred in a variety of wild and captive sea lions and fur seals (Cousins 2006, Gomis and others 2008). The bacteria responsible, *Mycobacterium pinnipedii*, belongs to the *Mycobacterium tuberculosis* complex (MTC) and although it primarily affects pinnipeds, it has shown pathogenicity in other species including human beings (Thompson and others 1993, Cousins 2006). Clinical signs are usually absent or non-specific despite massive tissue involvement (Gomis and others 2008). In general, postmortem findings include calcified, granulomatous lesions in thoracic and mediastinal lymph nodes and lungs (Cousins 2006). These are often difficult to identify at postmortem examination as lesions may be limited to a single deep lymph node (Cousins and others 2003, Kaneene and Thoen 2004).

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In the diagnosis of tuberculosis in animals, as well as in human beings, thoracic radiography is an extremely useful diagnostic modality (Kaandorp 1998). The techniques and radiographs produced when imaging pinniped thoraxes are similar in appearance to those of large canids (Van Bonn and others 2001). However, in human beings and animals, CT imaging is considered to be superior to thoracic radiology, especially in identifying the initial stages of tuberculosis (Bosch-Marcet and others 2004, Lange and others 2006, Sonntag and Mihaljevic 2009). Delacourt and others (1993) found that in 60 per cent of children with tuberculosis, no abnormalities were detected on thoracic radiography, yet enlarged lymph nodes were identified with CT. Although various species of pinnipeds have been kept in zoological collections for decades, there are no documented normal or pathological CT images of the thorax published in the literature. Even in the very popular California sea lion (*Zalophus californianus*), published literature is limited to CT scans of the skull (Sherrill and others 2004, Dennison and Schwarz 2008). The Heidelberg Zoo has been managing cases of pinniped tuberculosis since 2000 within its collection of South American sea lions (*Otaria flavescens*). A combination of diagnostic methods was used as part of the clinical evaluation of the animals with a known history of exposure. These were complemented by diagnostic imaging techniques in an attempt to achieve a greater sensitivity in rapid antemortem detection.

The aim of the study was to describe the normal CT thoracic anatomy of South American sea lions and to assess the ability of CT imaging to detect the presence of *M pinnipedii*-related pathology. In addition, a comparison between CT pathology and serological testing was undertaken to assess the use of CT as an additional modality in the diagnosis of tuberculosis in this species.

Materials and methods

The South American sea lions in this study were kept at the Heidelberg Zoo in Heidelberg, Germany. The group consisted of 10 animals and nine were examined with CT at the German Cancer Research Institute

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TABLE 1: Summary of the results of the diagnostic methods and outcome for each of the South American sea lions

Case*	August 2006	October 2006	February 2007	May 2007	June 2007	February 2008	April 2008	May 2008	July 2009	October 2009	August 2010	PM findings**
Case 1: 11-year-old female		RT: neg MAPIA: neg	RT: neg MAPIA: susp	RT: neg MAPIA: pos		CT: pos	RT: pos MAPIA: pos DPP: pos, euthanasia					AFB+ve PCR-ve Culture-ve
Case 2: 1-year-old female					RT: neg MAPIA: neg DPP: neg		RT: neg MAPIA: neg DPP: pos	CT: pos, euthanasia				AFB-ve PCR+ve Culture-ve
Case 3: 1-year-old female							RT: neg MAPIA: neg DPP: pos	CT: pos, euthanasia				AFB-ve PCR+ve Culture-ve
Case 4: 12-year-old female					RT: neg MAPIA: neg DPP: neg	CT: pos	RT: neg MAPIA: neg DPP: neg, euthanasia					AFB-ve PCR+ve Culture-ve Pregnant
Case 5: 10-year-old male	RT: neg	RT: neg MAPIA: neg	RT: neg MAPIA: neg				RT: neg MAPIA: neg DPP: pos x-ray: neg		Died of pneumonia combined with suspect MTB lesions	No CT undertaken due to size of animal (510 kg) too large for CT scanner gantry		AFB-ve PCR+ve Culture-ve
Case 6: 8-year-old female	RT: neg MAPIA: neg		RT: pos MAPIA: pos DPP: pos		RT: pos MAPIA: pos DPP: pos	CT: neg				CT: neg RT: pos	CT: neg RT: pos	NA
Case 7: 2-year-old female			RT: neg MAPIA: neg				CT: neg RT: pos MAPIA: pos DPP: pos			CT: neg RT: neg		NA
Case 8: 2-year-old male		RT: neg MAPIA: neg	RT: neg MAPIA: neg		RT: neg MAPIA: neg DPP: neg		CT: neg RT: neg DPP: neg			CT: neg RT: neg		NA
Case 9: 13-year-old female		RT: neg MAPIA: neg	RT: neg MAPIA: neg			CT: neg				CT: neg RT: neg		NA
Case 10: 8-year-old female		RT: neg MAPIA: neg DPP: neg	RT: neg MAPIA: neg				CT: neg RT: neg DPP: neg			Not scanned due to pregnancy	CT: neg RT: neg	NA

*Age at start of testing in 2006, **Gross and histopathology consistent with tuberculosis
RT Rapid test, MAPIA Multiantigen print immunoassay, DPP Dual path platform, AFB Acid-fast bacteria, MTB Mycobacterium tuberculosis, pos positive, neg negative, susp suspect, PM Postmortem findings

in Heidelberg in 2008. Due to very large weight (510 kg), one adult male sea lion (case 5) did not fit via the CT scanner. Digital radiographs of the thorax were performed on this animal using a computed radiography system (Fuji FCR 5000; Fuji Medical Systems). The remaining single male and eight females were one to 13 years old and either wild or captive born, weighing 40 to 150 kg. The sea lions were transported to the imaging suite in a conscious state and were anaesthetised upon arrival. Each animal was premedicated with a combination of 0.01 mg/kg medetomidine (Domitor; Pfizer Animal Health, 1 mg/ml), 0.23 mg/kg midazolam (Midazolam-ratiopharm; Ratiopharm, 5 mg/ml) and 0.08 mg/kg butorphanol (Alvegesic; Virbac, 10 mg/ml) administered via remote chemical immobilisation using a blowpipe. The sea lion was then assessed for responsiveness, jaw tone and reflexes, and once considered safe to approach a face mask was applied over the mouth and nares and a 5 per cent concentration of isoflurane (Isoba; Essex) was used to induce anaesthesia. Each animal was intubated and maintained on isoflurane (1 to 1.5 per cent) using a circle system (Sulla 808; Draeger) and oxygen. Monitoring consisted of visual assessment of respiratory rate, oesophageal stethoscope-auscultated assessment of heart rate and rectal pulse oximetry (Nellcor NPB-40) using a linear probe or tongue clip. The rectal temperature was assessed at regular intervals approximately every 10 minutes using a digital thermometer. Full clinical examinations were undertaken. At the completion of the procedure, the sea lions were returned to their crate and antagonists were given intramuscularly: 0.01 mg/kg flumazenil (Anexate; Roche Pharma, 0.1 mg/ml), 0.05 mg/kg atipamezole (Antisedan; Pfizer Animal Health, 5 mg/ml) and 0.1 mg/kg naltrexone (Naltrexone; Game Capture Products, 50 mg/ml).

The sea lions were placed in sternal recumbency in the CT gantry. The pectoral flippers were placed in a caudal direction adjacent to the thoracic wall, with the pelvic flippers extended caudally, ensuring that the animal was symmetrical in a dorsoventral plane. Wedges and duct tape were used as positioning aids.

The animals were examined using a clinical 16-detector CT scanner (Toshiba Aquilion 16; Toshiba Medical Systems). Acquisition parameters were 120 kV, 0.5 second rotation speed, 200 mAs (current intensity x rotation time), pitch 1.5, slice thickness 1 mm, increment 0.8 mm. Size of the scan field was 400 mm in diameter and scan length was 800 mm and images were achieved with a duration of 15 to 17 seconds. All images were acquired during an inspiratory breath-hold. Image analysis was performed on transversal source data as well as on secondary reformatted planes in frontal view. Bone windows were preferred to detect calcifications and lung windows were examined to identify any changes of the lung parenchyma. For evaluation of the lung parenchyma and mediastinum, the standard kernels were used (FC51 and FC13, respectively).

Animals that showed calcified granulomatous lesions on CT, in combination with positive serological data, were euthanased using 40 mg/kg embutramide (T61; Intervet, 200 mg/ml) intravenously and postmortem examinations were performed within 12 hours at the Chemisches and Veterinaeruntersuchungsamt Heidelberg. Full gross postmortem examinations were undertaken with tissues submitted for histopathology. Any lesions consistent with tuberculosis were swabbed with swabs submitted in Amies transport medium, with whole tissues submitted for bacteriological culture and PCR at Friedrich-Loeffler-Institute, the Federal Research Institute for Animal Health in Germany.

Results from serological testing for these animals were compared with those of the CT, postmortem and PCR findings. The serological test consisted of the rapid test (RT) (ElephantTB STAT-PAK;

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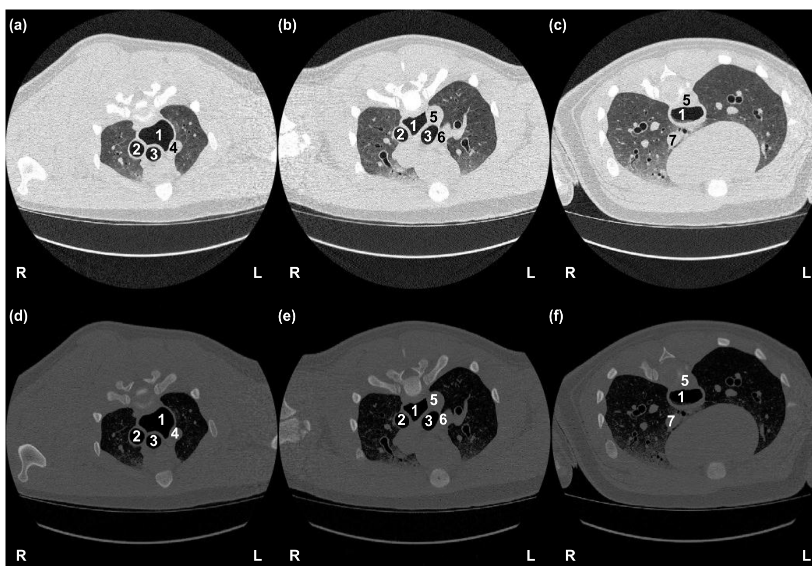

FIG 1: Lung window images in the transverse plane of a healthy South American sea lion. a to c in craniocaudal progression show oesophagus (1), principal bronchus (dexter) (2), principal bronchus (sinister) (3), subclavian artery (4), thoracic aorta (5), pulmonary artery (6) and caudal vena cava (7). d to f corresponding bone window images to a to c

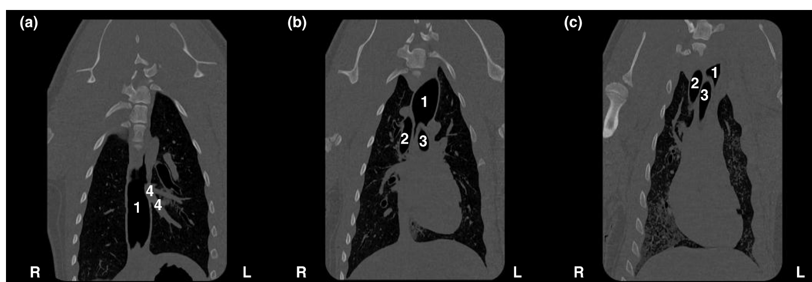

FIG 2: Reconstruction of bone window images in the dorsal plane of a healthy South American sea lion. a to c show oesophagus (1), principal bronchus (dexter) (2), principal bronchus (sinister) (3), pulmonary vein (4)

Chembio), a multiantigen print immunoassay (MAPIA; Chembio) and the Dual path platform (DPP) assay (DPP Vet; Chembio).

Results

Nine sea lions were successfully anaesthetised and underwent full clinical examination and CT scanning, which were compared with previous serological tests for each animal (Table 1). Four of the nine sea lions showed calcifications in mediastinal lymph nodes (cases 1 to 4); the remaining five animals did not exhibit calcifications of lymph nodes or other detectable pathology on CT. Of the five animals without CT signs that would be considered consistent with tuberculosis, three were also negative on all of the serological tests (cases 8 to 10). Due to the lack of pathology and negative results on all three of the serological tests, these three are considered to represent normal, non-pathological thoracic anatomy of the South American sea lion. The remaining two were positive on all three serological tests but were found to have no detectable lesions on CT (cases 6 and 7). Representative CT images from case 8, consistent with those found in cases 8 to 10 and considered to represent the normal thorax of the sea lion, are shown in Fig 1. The transverse plane, lung window (Fig 1a, b, c) and bone window (Fig 1d, f) are complemented by the images from the dorsal plane, bone window (Fig 2a, b, c).

Representative CT images taken from case 3 (Figs 3 and 4) are in a similar plane to the normal views shown in Figs 1 and 2. Fig 3 (transverse plane, bone window) shows a radiodense lesion considered to be consistent with calcification, located at the site of the mediastinal lymph node. This was seen in each of the sea lions 1 to 4 and was considered to be consistent with potential pathology related to pinniped tuberculosis. These were defined as positive lesions on CT. The transverse plane, bone window (Fig 3a, b) are complemented by dorsal plane, bone window views taken from the same animal (Fig 4a, b, c).

The three cases with both positive DPP and positive lesions on CT were euthanased. One animal (case 4), with a negative DPP and positive CT, was euthanased as well following correlation with the CT lesions and the postmortem findings in the other cases combined with a history of known exposure. In all four CT positive cases, the mediastinal lymph nodes seen on CT were examined at postmortem, where they were found to be enlarged in size with multifocal miliary calcified areas (Fig 5). Histopathology confirmed a granulomatous lymphadenitis with central dystrophic calcification in each of the four animals. Case 5, which died suddenly from *Streptococcus equi* subspecies *zooepidemicus* bronchopneumonia during the study period, was also found to have a similar lymph node pathology at gross and histological postmortem examination.

Ziehl-Neelsen staining was performed on all lesions but yielded acid-fast bacteria in one animal only (case 1).

PCR was positive for MTC organisms in all but one animal (case 1). Culture was negative in all of the five animals (cases 1 to 5).

Discussion

This study provides reference images on what can be considered to be the normal CT anatomy of the South American sea lion thoracic cavity. The air-filled radiolucent trachea is identified easily and is a valuable landmark for orientation. In dogs, the trachea branches into the bronchial tree caudal to the aortic arch at the fifth or sixth intercostal space whereas in otariids, the bifurcation of the trachea into the main-stem bronchi takes place at the thoracic inlet (Rommel and Lowenstine 2001, Burk and Feeney 2003). The knowledge of species-specific anatomic features facilitates orientation and assessment of images of healthy animals while enabling detection of abnormalities when pathology occurs. Thoracic CT imaging can be complicated by artifacts created by respiratory motion (Sonntag and Mihaljevic 2009). Therefore, in general, it is preferred to acquire images in animals that have been hyperventilated immediately before imaging to stop respiration for the duration of the scan. In sea lions, however, anaesthesia always poses an increased risk. Marine mammals are well adapted to deep dives and have a high tolerance for high carbon dioxide levels and acidosis. These adaptations can activate a dive response under anaesthesia, which has been the cause of several deaths (Haulena and Heath 2001). In these cases, it was felt that the benefit for imaging by hyperventilating the animals was outweighed by the potential risk that hyperventilation may lead to unacceptable levels of apnoea and subsequent reduction in uptake of volatile anaesthetic agents.

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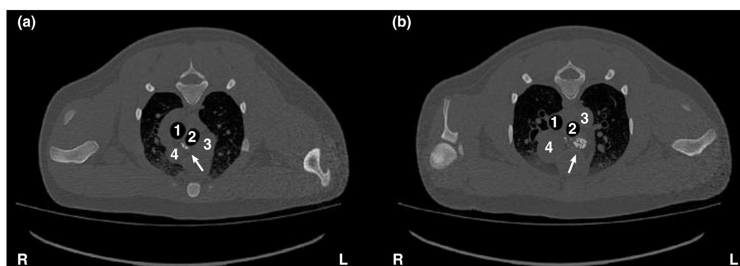

FIG 3: Bone window images in the transverse plane of a South American sea lion showing calcified mediastinal lymph nodes (arrow). a to b: principal bronchus (dexter) (1), principal bronchus (sinister) (2), thoracic aorta (3), cranial vena cava (4)

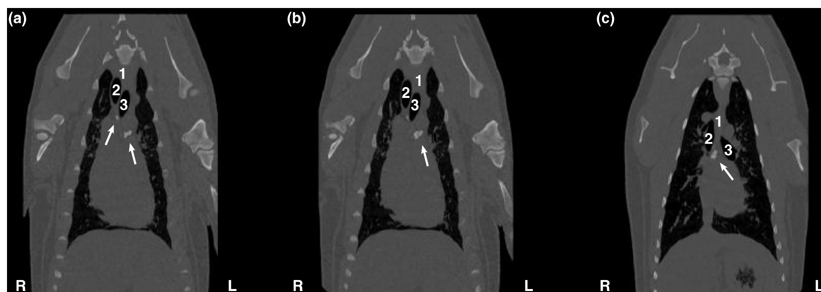

FIG 4: Reconstruction of bone window images in the dorsal plane of a South American sea lion showing calcified mediastinal lymph nodes (arrow). a to c: oesophagus (1), principal bronchus (dexter) (2), principal bronchus (sinister) (3)

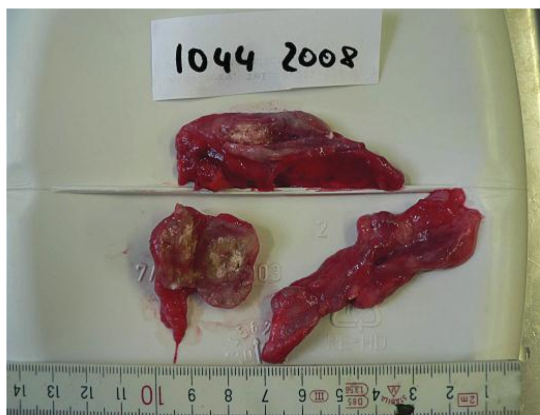

FIG 5: Calcified mediastinal lymph nodes from case 1, South American sea lion

In addition to developing normal thoracic CT imaging data, this study has demonstrated the ability of CT to detect calcified lesions in the lymph nodes of the thoracic cavity. With the special focus to target mineralisation of lymph nodes, in the present CT study protocol, the authors used bone and lung reconstruction algorithms. For the purposes of the study, it seems adequate, but for future investigations and to account for the possibility of non-tuberculosis findings, this protocol should be completed by soft tissue reconstruction. Because other pathology could be present in the head and neck (which the cranial mediastinal lymph nodes also drain), CT protocols must be adapted to future clinical requirements. During the investigation of pinniped tuberculosis in this study, several diagnostic methods were applied. CT has proven to be a useful diagnostic imaging method for the detection of calcified lymph nodes in sea lions weighing less than 150 kg. Standard radiographic techniques offer more technical difficulties due to the size of the animals, their blubber layer and resulting insufficient x-ray penetration. It is unknown whether the adult male (case 5) had lymph node calcifications in the year before its death; the extent of the lesions means this could have been possible, yet these were not detected using digital thoracic radiography. A final diagnosis of tuberculosis cannot be made on the presence of calcified mediastinal lymph nodes alone as there are other differential diagnoses that have the potential to produce similar pathology. Pulmonary granulomas in sea lions may also result from infection with *Coccidioides immitis*, *Cryptococcus neoformans* or *Nocardia* species (Gulland and others 2001, Frost 2006). However, CT is a useful modality when utilised in conjunction with additional diagnostic tests, especially in the face of known exposure to MTC bacteria and may facilitate detection of infected animals. All of the animals examined in this study had been tested by a variety of serological tests (ElephantTB Stat-Pak; MAPIA and DPP), none of which have a sensitivity of 100 per cent nor have been validated for the use in this species. There were some discrepancies between the serological findings and those of the CT and pathological findings, which is consistent with that seen in other species (Greenwald and others 2009, Schmitt and others 2010, Waters and others 2011). Case 4 had contradictory serological versus CT test results, being negative on serological testing but demonstrating a positive CT and subsequent positive PCR. The reason for this is unknown, however it is likely that this is a result of the variation in sensitivities and specificities of each of the different tests, even though they use similar antigens. The RT, MAPIA and DPP each use different methods of antibody detection; therefore, it is not unusual to find discordant results between these tests, especially if they are not optimised for a particular species, in this case sea lions (Lyashchenko, personal communication). Other hypotheses include a failure of the animal to generate a measurable serological response in this individual via homozygotic influences on the immune response (Acevedo-Whitehouse and others 2009) or the animal being in an anergic phase of the humoral response (Vordermeier and others 2004). An alternative consideration was that the infection was in the early stages, with cell-mediated immunity dominating, however, the degree of calcification and pathology present does not support this. It was considered that the antigen profile was suitable for this species due to the high incidence of positive results in the MTC-positive animals. Cases 6 and 7 showed positive serological tests yet were negative on CT. It is hypothesised that these animals were in the early stages of tuberculosis infection and had not progressed to developing caseous lymph nodes before instigation of therapy. Follow-up CT in these animals shows that the suspected tuberculosis infection did not progress any further. Serological profiles would have been beneficial in these two cases: measurement of changes in serological profiles in response to treatment would have been useful to assess and monitor the efficacy of treatment as has been done in other species. Multiple sputum samples were collected, but not reported here, to detect shedding of mycobacteria by microscopy, PCR and culture (Jurczynski and others 2011). Every animal had received prophylactic treatment with rifampicin (7.5 mg/kg) and isoniazid (5 mg/kg) for a minimum of eight months before imaging was performed (see Table 1). This is a possible explanation for the negative culture results. Another animal, separate to this study but previously from this group, was kept at another institution and did not receive treatment. This sea lion showed calcification in the mediastinal lymph node on CT

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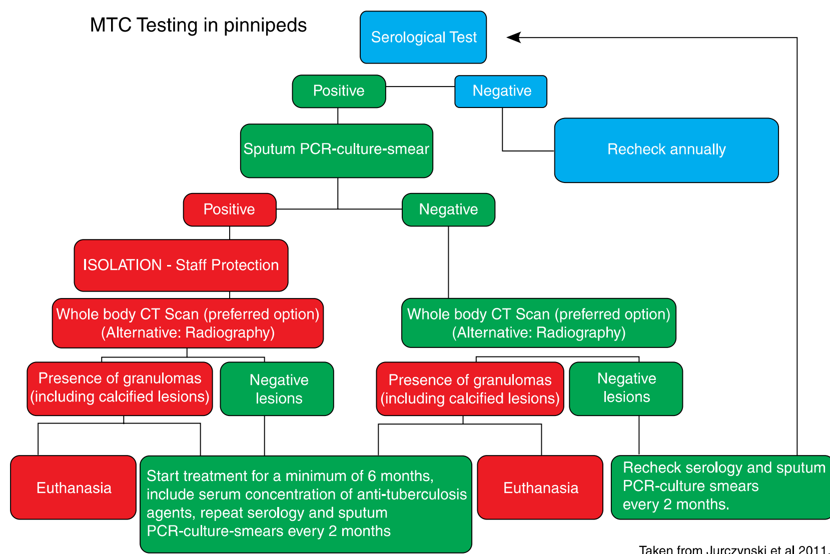

FIG 6: Proposed flow chart to help decision-making process when faced with sea lions with suspected *M pinnipedii* infection (Jurczynski and others 2011)

scans and at postmortem examination. Subsequent culture of the lesion identified *M pinnipedii* and spoligotyping confirmed the identical strain which was recovered in the original group (Jurczynski and others 2011). As a result of this and other studies, the following decision-making tree has been proposed to facilitate decision making with regard to suspected *M pinnipedii* infection for institutions keeping pinnipeds (Fig 6).

In conclusion, this paper demonstrates normal thoracic images of healthy sea lions for comparison with those of infected sea lions allowing CT imaging detection of calcified lymph nodes located in the mediastinum. In animals with suspected tuberculosis infection, CT scans are a valuable addition to other testing methods. The use of CT in isolation does not provide a definitive diagnosis of tuberculosis due to other infectious agents causing similar pathology. However, when used in conjunction with other modalities, such as serology, CT allows rapid detection of lesions consistent with tuberculosis which, in turn, allows informed, evidence-based decisions on whether to treat or to euthanase an animal within a collection that would not be possible when relying on serology alone. This is especially important with a known history of exposure. In times of breeding programmes and worldwide animal exchange, CT imaging can be included in preshipment testing of sea lions to enable prevention of spread of disease to other collections. In addition, the zoonotic nature of this disease has to be taken into account, especially if zoological institutions offer close-contact programmes with their sea lions; it is essential to protect staff as well as the public by examining animals on a regular basis.

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References

ACEVEDO-WHITEHOUSE, K., PETETTI, L., DUIGNAN, P. & CASTINEL, A. (2009) Hookworm infection, anaemia and genetic variability of the New Zealand sea lion. *Proceedings of the Royal Society* **276,** 3523-3529

BURK, R. L. & FEENEY, D. A. (2003) Small animal radiology and ultrasonography. In A Diagnostic Atlas and Text. 3rd edn. Eds R. L. Burk, D. A. Feeney. St. Louis, Elsevier Science. pp 44

BOSCH-MARCET, J., SERRES-CRÉIXAMS, X., ZUASNABAR-COTRO, A., CODINA-PUIG, X., CATALÀ-PUIGBÓ, M. & SIMON-RIAZUELO, J. L. (2004) Comparison of ultrasound with plain radiography and CT for the detection of mediastinal lymphadenopathy in children with tuberculosis. *Pediatric Radiology* **34,** 895-900

COUSINS, D. (2006) Tuberculosis in fur seals and sea lions caused by *Mycobacterium pinnipedii*. In *Mycobacterium bovis* Infection in Animals and Humans. Eds C. O. Thoen, J. H. Steele, M. J. Gilsdorf. Ames, Blackwell Publishing. pp 258-270

COUSINS, D.V., BASTIDA, R., CATALDI, A., QUSE, V., REDROBE, S., DOW, S., DUIGNAN, P., MURRAY, A. & OTHERS (2003). Tuberculosis in seals caused by a novel member of the *Mycobacterium tuberculosis* complex: *Mycobacterium pinnipedii* sp. nov. *International Journal of Systematic and Evolutionary Microbiology* **53**, 1305-1314.

DELACOURT, C., MANI, T. M., BONNEROT, V., DE BLIC, J., SAYEG, N., LALLEMAND, D. & SCHEINMANN, P. (1993) Computed tomography with normal chest radiograph in tuberculous infection. *Archives of Disease in Childhood* **69,** 430-432

DENNISON, S. E. & SCHWARZ, T. (2008) Computed tomographic imaging of the normal immature California sea lion head (*Zalophus californianus*). *Veterinary Radiology & Ultrasound* **49 (6),** 557-563

FROST, P. A. (2006) Tuberculosis in non-human primates with an emphasis on *Mycobacterium bovis*. In *Mycobacterium bovis* Infection in Animals and Humans. Eds C. O. Thoen, J. H. Steele, M. J. Gilsdorf. Blackwell Publishing. pp 271-284

GOMIS, D., MOISSON, P., LYASHCHENKO, K.P., MOSER, I., BAKKER, D., GUTIÉRREZ, M.C., BOSCHIROLI, M.L., GRAVET, A. & OTHERS (2008) Tuberculosis outbreaks in Mulhouse Zoo, 1992-96 and 2005-06: diagnostic perspectives. Proceedings of the European Association of Zoo and Wildlife Veterinarians Conference. Leipzig, Germany, May 1 to 4, 2008. pp 93-111

GREENWALD, R., LYASHCHENKO, O., ESFANDIARI, J., MILLER, M., MIKOTA, S., OLSEN, J.H., BALL, R., DUMONCEAUX, G. & OTHERS (2009) Highly accurate antibody assays for early and rapid detection of tuberculosis in African and Asian elephants. *Clinical and Vaccine Immunology* **16,** 605-612

GULLAND, F. M. D., HAULENA, M. & DIERAUF, L. A. (2001) Seals and sea lions. In CRC Handbook of Marine Mammal Medicine. 2nd edn. Eds L. A. Dierauf, F. M. D. Gulland. Boca Raton, CRC Press. pp 907-926

HAULENA, M. & HEATH, R. B. (2001) Marine mammal anesthesia. In CRC Handbook of Marine Mammal Medicine. 2nd edn. Eds L. A. Dierauf, F. M. D. Gulland. Boca Raton, CRC Press. pp 655-688

JURCZYNSKI, K., LYASHCHENKO, K. P., SCHARPEGGE, J., FLUEGGER, M., LACAVE, G., MOSER, I., TORTSCHANOFF, S. & GREENWALD, R. (2011) Use of multiple diagnostic tests to detect *Mycobacterium pinnipedii* infections in a large group of South American sea lions (*Otaria flavescens*). *Aquatic Mammals* (In press)

KANEENE, J. B. & THOEN, C. O. (2004) Tuberculosis. *Journal of the American Veterinary Medical Association* **224,** 685-691

KAANDORP, J. (1998) Diagnosis in mycobacteriosis. Proceedings of the European Association of Zoo and Wildlife Veterinarians Conference. Chester, UK, May 21 to 24, 1998. pp 85-91

LANGE, C., SCHABERG, T., DIEL, R. & GREINER, U. (2006) Aktueller Stand der Tuberkulosediagnostik. *Deutsche Medizinische Wochenschrift* **131,** 341-347

ROMMEL, S. A. & LOWENSTINE, L. J. (2001) Gross and microscopic anatomy. In CRC Handbook of Marine Mammal Medicine. 2nd edn. Eds L. A. Dierauf, F. M. D. Gulland. Boca Raton, CRC Press. pp 129-164

SCHMITT, D. L., OLSON, D. & RIDDLE, H. S. (2010) Summary of fourth annual tuberculosis and elephants workshop. *Gajah* **33,** 68-70

SHERRILL, J., PEAVY, G.M., KOPIT, M.J., GARNER, M.M., GARDINER, C.H. & ADAMS, L.M. (2004) Use of laser rhinoscopy to treat a nasal obstruction in a captive California sea lion (*Zalophus californianus*). *Journal of Zoo and Wildlife Medicine* **35 (2),** 232-241

SONNTAG, F. & MIHALJEVIC, M. (2009) Thorax. In CT- und MRT-Atlas: Transversalanatomie des Hundes. Eds M. Mihaljevic, M. Kramer, H. Gomercic. Berlin, Parey Verlag. pp 76-99

THOMPSON, P.J., COUSINS, D.V., GOW, B.L., COLLINS, D.M., WILLIAMSON, B.H. & DAGNIA, H.T. (1993) Seals, seal trainers and mycobacterial infection. *American Review of Respiratory Disease* **147,** 164-167

VAN BONN, W., JENSEN, E. D. & BROOK, F. (2001) Radiology, computed tomography, and magnetic resonance imaging. In CRC Handbook of Marine Mammal Medicine. 2nd edn. Eds L. A. Dierauf, F. M. D. Gulland. Boca Raton, CRC Press. pp 557-592

VORDERMEIER, M., GOODCHILD, A., CLIFTON-HADLEY, R. & DE LA RUA, R. (2004) The interferon-gamma field trial: background, principles and progress. *Veterinary Record* **155,** 37-38

WATERS, W. R., STEVENS, G. E., SCHENBAUM, M. A., ORLOSKI, K. A., ROBBE-AUSTERMAN, S., HARRIS, N. B., HALL, S. M., THOMSEN, B. V. & OTHERS (2011) Bovine tuberculosis in a Nebraska herd of farmed Elk and Fallow Deer: a failure of the tuberculin skin test and opportunities for serodiagnosis. *Veterinary Medicine International* www.ncbi.nlm.nih.gov/pmc/articles/PMC3087612/pdf/VMI2011-953985.pdf. Accessed July 2, 2011

4 DISCUSSION

In the present study a multi-species outbreak of tuberculosis caused by Mycobacterium pinnipedii is investigated. Although pinnipeds appear to be the natural host, *M. pinnipedii* is pathogenic to other animals as well (COUSINS et al. 2003). At the Heidelberg zoo the transmission from sea lions to Malayan tapirs, Bactrian camels and a Crested porcupine was confirmed. Several diagnostic methods were applied.

In general, isolation of bacteria is necessary to confirm an infection with the *M. tuberculosis complex*-organisms. This method, however, can only identify extensive shedders (LYASHCHENKO et al. 2000). PCR is a more sensitive method of detection and can be used on faeces, sputum, or tracheal lavage samples (LARSEN and SALMAN 2001). However, only a few animals that were extensively shedding could be identified *ante mortem* using this method on sputum samples in the present study. Faecal samples were always negative, even in a sea lion that was shedding great amounts of mycobacteria.

The TST used for domestic ruminants has been applied in many exotic species, but it requires double injections for the execution and reading of the test (BENGIS 1999). In most exotic species this means double anaesthesia. In addition, it detects only infection, not necessarily active disease (KAANDORP 1998). In pinnipeds, tuberculin skin tests have not been validated and false-negative results have occured (FORSHAW and PHELPS 1991). Preceding the present study the author has seen some nonspecific reactions including the loss of parts of the flipper of a sea lion and it was decided not to use this test. Tapirs are known to have nonspecific reactions (MILLER 2008). In one of the tapirs of this study, the TST proved useful in the post-skin test antibody response test which had been reported to increase humoral antibody levels in animals infected with tuberculosis (BERNARDELLI et al. 1996). In this test, a blood sample was taken before performing a TST and 2-3 wk later. The expected antibody boost was clearly confirmed by the MAPIA. No reaction in TB-negative Malayan tapirs suggests high diagnostic specificity (GOMIS et al. 2008).

The serological results helped identify most of the infected sea lions and all of the tapirs. ElephantTB STAT-PAK assay, MAPIA and DPP were used to detect seroconversions and identify seroreactive antigens. In one of the tapirs and two of the sea lions, the ElephantTB STAT-PAK and the MAPIA were positive at a very early stage of the infection, demonstrating a gradual change over time, and therefore, indicating the differential emergence of mycobacterial antigens in the course of infection. In the tapir, the results of both tests were confirmed at necropsy. According to the necropsy findings in sea lions, the DPP assay showed the highest sensitivity compared to that of ElephantTB STAT-PAK assay and MAPIA. Most probably this is a result of the variation in sensitivities and specificities of each of the different tests in species in which they have not been validated for.

ELISA has not been carried out in this study. With an increasing number of antigens the sensitivity of the test decreased. MAPIA was chosen because the protein binding capacity is higher for nitrocellulose used in MAPIA than for polystyrene used in ELISA. Less interference among antigens used was expected.

In addition to the serological tests, CT has proven to be a useful diagnostic imaging method for the detection of calcified lesions in the lymph nodes of the thoracic cavity of sea lions weighing less than 150 kg. This weight limitation and the size of the available gantry excluded tapirs, camels, as well as the adult male sea lions from this examination. Standard radiographic techniques might be used but insufficient x-ray penetration has to be considered.

A final diagnosis of tuberculosis cannot be made on the presence of calcified mediastinal lymph nodes alone as there are other differential diagnoses that have the potential to produce similar pathology (FROST 2006; GULLAND et al. 2001).

Still, CT is a useful modality when utilized in conjunction with additional diagnostic tests, especially in the face of known exposure to mycobacteria and may facilitate detection of infected animals.

In this study it was used to evaluate possible candidates for treatment. It is unknown how long calcification of the lymph nodes takes to develop, but its presence in combination with positive serological tests is a strong indicator of infection. Here it was hypothesized that the encapsulation within the lymph nodes might protect the dormant viable bacilli from treatment with anti-tuberculosis drugs. However, on necropsy, from all of the post-treatment euthanized animals live mycobacteria were not recovered, whereas *M. pinnipedii* was cultured out of the lesions from the non-treated animals. In the culture-negative animals histopathology results and positive PCR results the involvement of *M. pinnipedii* was more than likely.

Therefore, there is a strong indication that treatment has been successful in killing the bacteria even within the calcified lesions. Still, it was decided not to take a risk and keep animals that might start shedding under certain circumstances within the collection.

The success of treatment was assumed when one sea lion 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. Another showed a positive serological test and was negative on CT as well. It is hypothesized that these animals were in the early stages of tuberculosis infection and had not progressed to developing calcified lymph nodes before initiation of treatment. It would have been beneficial to follow drug concentration in the serum to monitor efficacy of treatment but this was impossible because none of the animals had been trained for venepuncture.

Spoligotyping conducted on the various isolates determined the suspected origin of the *M. pinnipedii* strain. The spoligotyping patterns of all four tapir isolates were compared with those from the Bactrian camel and South American sea lion identified at the Heidelberg Zoo, the strains appeared identical and characteristic for *M. pinnipedii* (MOSER et al. 2007). This finding confirmed that one tapir likely brought the infectious agent from Heidelberg to Mulhouse, where the other tapirs became infected by direct contact or sharing of the exhibit and then subsequently developed clinical disease. The spoligotyping pattern of the sea lion in Hamburg, born in Heidelberg, was identical to the one in Heidelberg. It is assumed that the animal in Hamburg harboured the infectious agent since its arrival from Heidelberg years earlier. A comparison to the strain recovered from the sea lions of Le Pal Zoo identified a different strain. The spoligotyping pattern of the porcupine revealed *M. pinnipedii*, but the exact strain was never identified because the animal died 7 yr before.

In *post mortem* examinations lesions associated with tuberculosis have been found in lungs and surrounding lymph nodes predominantly. This supports 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, Bactrian camel, and Indian crested porcupine. These three species were housed in enclosures adjacent to the sea lion pool and were cared for by the same keepers. Fomites could be another source of infection.

In this study it was not possible to determine the sensitivity and specificity for the testing modalities described. Further evaluation and validation requires additional animals, which will be challenging due to the limited number of animals in captivity. In addition, a true negative status could not be confirmed without *post mortem* examination. The tests are not specifically designed for the species in question and 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. There are several unknown factors here that are impossible to determine other than by 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.

In conclusion, no *ante mortem* test that is 100% reliable for detecting tuberculous infections 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. In this study, RT and MAPIA showed promising results in identifying tuberculosis due to *M. pinnipedii* in tapirs. Depending on interpretation and in combination, RT, MAPIA, DPP, and CT scans can be utilised to provide strong evidence of infection of *M. pinnipedii* related disease in South American sea lions.

The serological assays evaluated in this study showed encouraging results and should be considered as important tools to detect, *ante mortem*, tapirs and sea lions infected with *M. pinnipedii*. This of extreme importance for subsequent decisions in regard to particular populations in captivity. Pre-shipment screenings of animals during exchanges or imports of wild animals of unknown disease status, with improved diagnostic tests can help with disease eradication in zoos. In addition, the zoonotic nature of this disease has to be taken into account, especially if zoological institutions offer close contact to their sea lions; it is essential to protect staff as well as the public by examining animals on a regular basis.
The spoligotyping confirmed the origin of infection. This method remains an important tool for epidemiology of zoonotic infectious diseases.
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.

5 SUMMARY

Kerstin Jurczynski

Tuberculosis in South American sea lions (*Otaria flavescens*) – diagnostic options and its epidemiologic importance for other mammals within the zoological garden

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57 pages, 10 figures, 1 Table, 3 publications, 110 references

Keywords: *Mycobacterium pinnipedii*, South American sea lion, tuberculosis

Tuberculosis is a widely spread zoonotic disease caused by acid-fast bacteria of the *Mycobacterium tuberculosis* complex in a variety of mammalian species. In pinnipeds, tuberculosis has been reported in different captive and wild sea lions and fur seals.
The causative agent, *Mycobacterium pinnipedii*, is part of the *M. tuberculosis* complex and has shown pathogenicity in other mammalian species including human beings.
Since 2000 the Heidelberg zoo has been dealing with tuberculosis in its collection of South American sea lions (*Otaria flavescens*). After a Malayan tapir (*Tapirus indicus*) was transferred to a zoological institution in France it transmitted the disease to the other tapirs that succumbed to tuberculosis. Culturing and spoligotyping confirmed the origin, the sea lions at the Heidelberg zoo.
An investigation of the sea lion group housed at Heidelberg in addition to different species of mammals living in adjacent exhibits as well as a sea lion, born in Heidelberg but then living in Hamburg, revealed multiple cases of pinniped tuberculosis.
In the study several diagnostic methods were applied. All of the sea lions were premedicated using medetomidine (0.01 mg/kg), midazolam (0.23 mg/kg) and butorphanol (0.08 mg/kg) administered via blowpipe and anesthesia was induced and maintained with isoflurane. Venepuncture was performed on the caudal gluteal vein or the veins of the hind flippers. Except for the animals, which have died prior to the study and additionally two very large (> 500 kg) males, the animals were examined through the help of computed tomography under anaesthesia.
The serological assays, ElephantTB STAT-PAK assay, MAPIA and DPP, were useful point-of-care tools to detect seroconversions and identify seroreactive antigens. In sea lions, 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. The spoligotyping patterns of Mulhouse and Hamburg were identical to that of the strain identified at the Heidelberg Zoo. This was considered to be a strong indication that the sea lion in Hamburg as well as the tapir in Mulhouse has harboured the infectious agent since their arrival from Heidelberg.

The tapir developed fulminant pathology and managed to transmit the disease within a few months whereas the sea lion in Hamburg showed calcified lesions in the mediastinal lymph nodes only.

In addition to the serological tests, CT has proven to be a good diagnostic imaging method for the detection of calcified lymph nodes in animals up to 150 kg. This does not provide a definitive diagnosis of tuberculosis due to other infectious agents causing similar pathology. However, when used in conjunction with other modalities, such as serology, CT allows rapid detection of lesions consistent with tuberculosis which, in turn, allows informed, evidence-based decisions on whether to treat or to euthanize an animal within a collection that would not be possible when relying on serology alone.

Treatment has been carried out after antibiotic sensitivity testing. There is a strong indication that treatment of exposed animals, if initiated early enough, may prevent manifestation of infection. In animals with calcified lesions post-treatment necropsies did not reveal live mycobacteria, but until now it is hypothesized that treatment does not inactivate dormant mycobacteria, especially as long as no pharmacokinetic study has confirmed the efficacy of treatment.

The study revealed a great potential of various diagnostic methods in the diagnosis of pinniped tuberculosis in different animal species and emphasizes the importance of a testing regime that consists of multiple tests rather than a single one.

6 ZUSAMMENFASSUNG

Kerstin Jurczynski

Die Tuberkulose der Südamerikanischen Seelöwen (*Otaria flavescens*) – diagnostische Möglichkeiten und die epidemiologische Bedeutung für andere Säugetiere im zoologischen Garten

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Schlüsselwörter: *Mycobacterium pinnipedii*, Südamerikanischer Seelöwe, Tuberkulose

Tuberkulose ist eine weit verbreitete Zoonose, die von säurefesten Bakterien aus dem *Mycobacterium tuberculosis*-komplex bei verschiedenen Säugetierspezies verursacht wird. Bei Robben wurde die Tuberkulose bei unterschiedlichen Seelöwen und Seebären im Freiland sowie in Menschenhand beschrieben.
Der verantwortliche Erreger, *Mycobacterium pinnipedii*, ist ein Teil des *M. tuberculosis*-komplex und Pathogenität wurde auch bei anderen Säugetieren, einschließlich des Menschen, nachgewiesen.
Seit dem Jahr 2000 ist die Tuberkulose im Bestand der Südamerikanischen Mähnenrobben (*Otaria flavescens*) des Heidelberger Zoos präsent. Der Transfer eines Schabrackentapirs (*Tapirus indicus*) in einen französischen zoologischen Garten führte zur Infektion anderer Tapire, welche letztendlich der Tuberkulose erlagen. Kulturelle Anzucht und Spoligotypisierung bestätigten den Ursprung der Krankheit, die Seelöwen des Heidelberger Zoos.
Eine Untersuchung des Heidelberger Seelöwenbestandes und darüber hinaus weiterer Säugetierarten aus den umliegenden Gehegen zusätzlich zu einem in Heidelberg geborenen und zum Untersuchungszeitpunkt in Hamburg lebenden Seelöwen offenbarte zahlreiche Fälle von Robbentuberkulose.
In dieser Studie wurden verschiedene Diagnostikmethoden angewandt. Um diese durchzuführen, wurden alle Seelöwen mit Medetomidin (0.01 mg/kg), Midazolam (0.23 mg/kg) und Butorphanol (0.08 mg/kg) via Blasrohr prämediziert. Die Narkose wurde mit Isofluran eingeleitet und aufrecht erhalten. Die Blutentnahmen wurden an der kaudalen Glutealvene oder den Venen der Hinterflossen durchgeführt. Außer den Tieren, die vor der Studie verstorben waren und zusätzlich zwei sehr große (> 500 kg) männliche Tiere, wurden alle Tiere durch computertomographische Diagnostik unter Narkose untersucht.

Die serologischen Verfahren, ElephantTB STAT-PAK assay, MAPIA und DPP, erwiesen sich als nützliche point-of-care Diagnostikmethoden, um Serokonversionen nachzuweisen und seroreaktive Antigene zu erkennen.
Der DPP bewies, im Vergleich zu dem ElephantTB STAT-PAK assay und dem MAPIA, bei den Seelöwen die größte Sensitivität. Dies konnte aus der besseren Übereinstimmung mit den pathologischen Befunden geschlossen werden.
Die Spoligotypisierung wurde zur Bestimmung der beteiligten *M. pinnipedii* Stämme genutzt.
Die Typisierungsmuster aus Mulhouse und Hamburg waren mit den im Heidelberger Zoo identifizierten Stämmen identisch. Dies kann als überzeugender Anhaltspunkt dafür betrachtet werden, dass der Seelöwe in Hamburg ebenso wie der Tapir in Mulhouse den Erreger bereits seit ihrer Ankunft aus Heidelberg in sich getragen haben.
Der Tapir entwickelte eine fulminante Pathologie und infizierte die anderen Tapire innerhalb weniger Monate. Der Seelöwe in Hamburg hingegen wies lediglich Kalzifizierungen in den Mediastinallymphknoten auf.
Zusätzlich zu den serologischen Tests erwies sich die Computertomographie als gutes diagnostisches bildgebendes Verfahren, um kalzifizierte Lymphknoten bei Robben mit einer Körpermasse bis zu 150 kg darzustellen. Sie liefert allerdings keine definitive Diagnose der Tuberkulose, da andere Erreger eine ähnliche Pathologie verursachen können. In Verbindung mit anderen Untersuchungsmethoden, wie zum Beispiel der Serologie, ermöglicht die Computertomographie einen schnellen Nachweis von im Einklang mit Tuberkulose stehenden Läsionen. Dies wiederum erlaubt auf Fakten basierende Entscheidungen, ob eine Behandlung oder die Euthanasie des Tieres innerhalb eines Bestandes erfolgen soll. Diese Entscheidungen sollten aufgrund von serologischen Resultaten allein nicht erfolgen.
Eine Behandlung wurde nach Resistenztest durchgeführt. Es wird vermutet, dass die Behandlung infizierter Tiere, wenn sie frühzeitig genug durchgeführt wird, eine Manifestation der Infektion verhindert. Bei zuvor behandelten Tieren mit kalzifierten Läsionen konnten bei pathologischen Untersuchungen keine lebenden Mykobakterien nachgewiesen werden. Dennoch wird bislang angenommen, dass eine Behandlung die ruhenden Bakterien nicht inaktivieren kann. Dies gilt zumindest solange, bis pharmakokinetische Studien die Effektivität der Antibiotika bestätigen.
Die vorliegende Studie zeigt das bedeutende Potenzial verschiedener Diagnostikverfahren zum Nachweis von Robbentuberkulose bei verschiedenen Tierarten und unterstreicht die Bedeutung der Anwendung mehrerer Test an Stelle eines Einzelnen.

7 REFERENCES

Acevedo-Whitehouse K, Petetti L, Duignan P, Castinel A. Hookworm infection, anaemia and genetic variability of the New Zealand sea lion. Proceedings of the Royal Society 2009; 276: 3523-9.

Ahmed N, Alam M, Majeed AA, Rahman SA, Cataldi A, Cousins D, Hasnain SE. Genome sequence based, comparative analysis of the fluorescent amplified fragment length polymorphisms (FAFLP) of tubercle bacilli from seals provides molecular evidence for a new species within the *Mycobacterium tuberculosis* complex. Infect Genet Evol 2003; 2: 193-9.

Alexander KA, Pleydell E, Williams MC, Lane EP, Nyange JFC, Michel AL. *Mycobacterium tuberculosis*: An Emerging Disease of free-ranging wildlife. Emerg Infect Dis 2002; 8 (6): 598-601.

Anonymous. Workshop of Co-ordinated Action SSPE-CT-2004-501903 - Veterinary network of laboratories researching into improved diagnosis and epidemiology of mycobacterial diseases. Toledo, Spain. 2006.

Bastida R, Loureiro J, Quse V, Bernadelli A, Rodriguez D, Costa E. Tuberculosis in a wild subantarctic fur seal from Argentina. J Wildlife Dis 1999; 35 (4): 796-8.

Bengis RG. Tuberculosis in free-ranging mammals. In: Fowler ME, Miller RE (Eds.), Zoo and Wild Animal Medicine, 4th ed. Philadelphia, PA: W. B. Saunders & Company, 1999; 101-13.

Bernardelli A, Bastida R, Loureiro J, Michelis H, Romano MI, Cataldi A, Costa E. Tuberculosis in sea lions and fur seals from the south-western Atlantic coast. Rev Sci tech Off int Epiz 1996; 15 (3): 985-1005.

Berta A. Pinnipedia, Overview. In: Perrin WF, Wursig B, Thewissen JGM (Eds.), Encyclopedia of Marine Mammals, 2nd ed. San Diego, CA: Academic Press, 2009; 878-885.

Bigi F, Garcia-Pelayo MC, Nunez-Garcia J, Peralta A, Caimi KC, Golby P, Hinds J, Cataldi A, Gordon SV, Romano MI. Identification of genetic markers of *Mycobacterium pinnipedii* through genome analysis. FEMS Microbiology Letters 2005; 248: 147-52.

Blair WR. Report of the veterinarian. In: 17th Annual Report of the New York Zoological Society. New York Zoological Society, New York, 1912: 74.

Bosch-Marcet J, Serres-Créixams X, Zuasnabar-Cotro A, Codina-Puig X, Català-Puigbó M, Simon-Riazuelo JL. Comparison of ultrasound with plain radiography and CT for the detection of mediastinal lymphadenopathy in children with tuberculosis. Pediatr Radiol 2004; 34: 895-900.

Burk RL, Feeney DA. Small animal radiology and ultrasonography. In: Burk RL, Feeney DA (Eds.), A Diagnostic Atlas and Text, 3rd ed. St. Louis: Elsevier Science, 2003; 44.

Cappozzo HL, Perrin WF. South American sea lion, *Otaria flavescens*. In: Perrin WF, Wursig B, Thewissen JGM (Eds.), Encyclopedia of Marine Mammals, 2nd ed. San Diego, CA: Academic Press, 2009; 1076-8.

Castro Ramos M, Ayala M, Errico F, Silvera FV. Aislamiento de *Mycobacterium bovis* en Pinnípedos *Otaria byronia* (Lobo marino común) en Uruguay. Rev Med Vet 1998; 79(3): 197-200.

Cousins D. Tuberculosis in fur seals and sea lions caused by *Mycobacterium pinnipedii*. In: Thoen CO, Steele JH, Gilsdorf MJ (Eds.), *Mycobacterium bovis* infection in Animals and Humans, 2nd ed. Ames, IA: Blackwell Publishing, 2006; 258-270.

Cousins DV, Bastida R, Cataldi A, Quse V, Redrobe S, Dow S, Duignan P, Murray A, Dupont C, Ahmed N, Collins DM, Butler WR, Dawson D, Rodriguez D, Loureiro J, Romano MI, Alito A, Zumarraga M, Bernardelli A. Tuberculosis in seals caused by a novel member of the *Mycobacterium tuberculosis* complex: *Mycobacterium pinnipedii sp.nov*. Int J Syst Evol Micr 2003; 53: 1305-14.

Cousins DV, Francis BR, Gow BL, Collins DM, McGlashan CH, Gregory A, Mackenzie RM. Tuberculosis in captive seals: bacteriological studies on an isolate belonging to the *Mycobacterium tuberculosis* complex. Res Vet Sci 1990; 48: 196-200.

Cousins DV, Williams SN, Reuter R, Forshaw D, Chadwick B, Coughran D, Collins P, Gales N. Tuberculosis in wild seals and characterisation of the seal bacillus. Aust Vet J 1993; 70 (3): 92-7.

Dean GS, Crawshaw TC, de la Rua-Domenech R, Farrant L, Greenwald R, Higgings RJ, Lyashchenko K, Vordermeier HM, Twomey DF. Use of serological techniques for diagnosis of *Mycobacterium bovis* infection in a llama herd. Vet Rec 2009; 165: 323-4.

De Blainville MHD. Sur quelque cranes de phoques. J Phys Chim Hist Nat Arts 1820; 91: 286-300.

Delacourt C, Mani TM, Bonnerot V, De Blic J, Sayeg N, Lallemand D, Scheinmann P. Computed tomography with normal chest radiograph in tuberculous infection. Arch Dis Child 1993; 69: 430-2.

De Lisle GW, Bengis RG, Schmitt SM, O´Brien JO. Tuberculosis in free-ranging wildlife: detection, diagnosis and management. Rev sci tech Off int Epiz 2002; 21(2): 317-34.

De Lisle GW, Collins DM, Loveday AS, Young WA, Julian AF. A report of tuberculosis in cats in New Zealand and the examination of strains of Mycobacterium bovis by DNA restriction endonuclease analysis. N Z Vet J 1990; 38: 10-3.

De Lisle GW, Crews K, de Zwart J, Jackson R, Knowles GJE, Patterson KD, MacKenzie RW, Waldrup KA, Walker R. Mycobacterium bovis infections in wild ferrets. N Z Vet J 1993; 41: 148-9.

Dennison SE, Schwarz T. Computed tomographic imaging of the normal immature California sea lion head (*Zalophus californianus*). Vet Radiol Ultrasoun 2008; 49(6): 557-63.

Dunn JL, Buck JD, Robeck TR. Bacterial diseases of cetaceans and pinnipeds. In: Dierauf LA, Gulland FMD (Eds.), CRC Handbook of Marine Mammal Medicine, 2nd ed. Boca Raton, Florida: CRC Press, 2001; 309-35.

Forshaw D, Phelps GR. Tuberculosis in a captive colony of pinnipeds. J Wildlife Dis 1991; 27 (2): 288-95.

Ehlers K. Records of the hooded seal Cystophora cristata Erxleben, and other animals at Bremerhaven Zoo. In: Int Zoo Yearbook, 1965; 5: 148-9.

Frost PA. Tuberculosis in Nonhuman Primates with and emphasis on *Mycobacterium bovis*. In: Thoen CO, Steele JH, Gilsdorf MJ (Eds.), *Mycobacterium bovis* infection in Animals and Humans, 2nd ed. Ames, IA: Blackwell Publishing, 2006; 271-84.

Gomis D, Moisson P, Lyashchenko KP, Moser I, Bakker D, Gutierrez MC, Boschiroli ML, Gravet A. Tuberculosis outbreaks in Mulhouse zoo, 1992-96 and 2005-06: diagnostic perspectives. Proceedings of the 7th conference of the European Association of Zoo and Wildlife Veterinarians, Leipzig, 2008; 157-71.

Greenwald R, Esfandiari J, Lesellier S, Houghton R, Pollock J, Aagaard C, Andersen P, Hewinson RG, Chambers M, Lyashchenko K. Improved serodetection of *Mycobacterium bovis* infection in badgers (*Meles meles*) using multiantigen test formats. Diagn Microbiol Infect Dis 2003; 46: 197-203.

Greenwald R, Lyashchenko O, Esfandiari J, Miller M, Mikota S, Olsen JH, Ball R, Dumonceaux G, Schmitt D, Moller T, Payeur JB, Harris B, Sofranko D, Waters WR,
Lecu A, Ball R. Mycobacterial infections in zoo animals: relevance, diagnosis and management. Int Zoo Yearbook, 2011; 45: 183-202.

Lyashchenko KP. Highly accurate antibody assays for early and rapid detection of tuberculosis in African and Asian elephants. Clin Vaccine Immunol 2009; 16: 605-12.

Gulland FMD, Haulena M, Dierauf LA. Seals and sea lions. In: Dierauf LA, Gulland FMD (Eds.), CRC Handbook of Marine Mammal Medicine, 2nd ed. Boca Raton, Florida: CRC Press, 2001; 907-26.

Haagsma J, Eger A. ELISA for diagnosis of tuberculosis and chemotherapy in zoo and wildlife animals. Proceedings of the Conference of the American Association of Zoo Veterinarians, South Padre Island, TX, 1990; 107-10.

Harboe M, Wiker HG, Duncan JR, Garcia MM, Dukes TW, Brooks BW, Turcotte C, Nagai S. Protein G-Based Enzyme-linked immunosorbent assay for anti-MPB70 antibodies in bovine tuberculosis. J Clin Microbiol 1990; 28(5): 913-21.

Harris NB. Molecular Techniques: Applications in Epidemiologic Studies. In: Thoen CO, Steele JH, Gilsdorf MJ (Eds.), *Mycobacterium bovis* infection in Animals and Humans, 2nd ed. Ames, IA: Blackwell Publishing, 2006; 54-62.

Haulena M, Heath RB. Marine mammal anesthesia. In: Dierauf LA, Gulland FMD (Eds.), CRC Handbook of Marine Mammal Medicine, 2nd ed. Boca Raton, Florida: CRC Press, 2001; 655-88.

Hunter JE, Duignan PJ, Dupont C, Fray L, Fenwick SG, Murray A. First report of potentially zoonotic tuberculosis in fur seals in New Zealand. N Z Med J 1998; 111 (1063): 130-1.

Janssen DL, Rideout BA, Edwards ME. Medical management of captive tapirs (*Tapirus spp.*). Proceedings of the Conference of the American Association of Zoo Veterinarians, Puerto Vallarta, Mexico, 1996; 1-11.

Jurczynski K, Lyashchenko K, Gomis D, Lecu A, Tortschanoff S, Klarenbeek A, Moser I. *Mycobacterium pinnipedii* infection in South American sea lions (*Otaria byronia*) in Europe. Proceedings of the 43rd Symposium on Diseases of Zoo and Wild Animals, Edinburgh, Scotland, 2007; 180.

Jurczynski K, Lyashchenko KP, Gomis D, Moser I, Greenwald R, Moisson P. Pinniped tuberculosis in Malayan Tapirs (*Tapirus indicus*) and its transmission to other terrestrial mammals. J Zoo Wildlife Med, 2011; 42(2): 222-7.

Jurczynski K, Lyashchenko K, Scharpegge J, Fluegger M, Lacave G, Moser I, Tortschanoff S, Greenwald R. Use of multiple diagnostic tests to detect *Mycobacterium pinnipedii* infections in a large group of South American sea lions (*Otaria flavescens*). Aq Mamm 2012; 38 (1): 43-55.

Kaandorp, J. Diagnosis in Mycobacteriosis. Proceedings of the 2nd Conference of the European Association of Zoo and Wildlife Veterinarians, Chester, 1998; 85-91.

Kamerbeek J, Schouls L, Kolk A, Van Agterveld M, van Soolingen D, Kuijper S, Bunschoten A, Molhuizen H, Shaw R, Goyal M, van Embden J. Simultaneous detection and strain differentiation of *Mycobacterium tuberculosis* for diagnosis and epidemiology. J Clin Microbiol 1997; 35: 907-14.

Kaneene JB, Thoen CO. Tuberculosis. Vet Med Today: Zoonosis Update. J Am Vet Med Assoc 2004; 224 (5): 685-91.

Kiers A, Klarenbeek A, Mendelts B, Van Soolingen D, Koeter G. Transmission of *Mycobacterium pinnipedii* to humans in a zoo with marine mammals. Int J Tuberc Lung Dis 2008; 12 (12): 1469-73.

Lacave G, Maillot A, Alerte V, Boschiroli ML, Lecu A. Atypical case of *Mycobacterium pinnipedii* in a Patagonian sea lion (*Otaria flavescens*) and Tuberculosis cases history review in pinnipeds. Proceedings of the 40th Conference of the International Association of Aquatic Animal Medicine, San Antonio, TX, 2009; 155.

Lange C, Schaberg T, Diel R, Greinert U. Aktueller Stand der Tuberkulosediagnostik. Dtsch Med Wochenschr 2006; 131: 341-7.

Larsen RS, Salman MD. Diagnostic testing for *M. bovis* in nondomestic hoofstock. Proceedings of the Conference of the American Association of Zoo Veterinarians, Orlando, FL, 2001; 203-8.

Larsen RS, Salman MD, Mikota SK, Isaza R, Triantis J. Validation and use of a multiple-antigen ELISA for detection of tuberculosis infection in elephants. Proceedings of the Conference of the American Association of Zoo Veterinarians, Orlando, FL, 2001; 231-3.

Lewin A, Sharbati-Tehrani S. Slow growth rate of mycobacteria. Possible reasons and significance for their pathogenecity. Bundesgesundheitsblatt Gesundheitsforschung Gesundheitsschutz 2005; 48(12): 1390-9.

Lyashchenko KP, Greenwald R, Esfandiari J, Olsen JH, Ball R, Dumonceaux G, Dunker F, Buckley C, Richard M, Murray S, Payeur JB, Andersen P, Pollock JM, Mikota S, Miller M, Sofranko D, Waters WR. Tuberculosis in elephants: antibody responses to defined antigens of *Mycobacterium tuberculosis*, potential for early diagnosis, and monitoring of treatment. Clin Vaccine Immunol 2006; 13: 722-32.

Lyashchenko KP, Singh M, Colangeli R, Gennaro ML. A multi-antigen print immunoassay for the development of serological diagnosis of infectious diseases. J Immunol Methods 2000; 242: 91-100.

Lyashchenko K, Whelan AO, Greenwald R, Pollock JM, Andersen P, Hewinson RG, Vordermeier HM. Association of tuberculin-boosted antibody responses with pathology and

cell-mediated immunity in cattle vaccinated with *Mycobacterium bovis* BCG and infected with *M. bovis*. Infect Immunol 2004; 72: 2462-7.

Maslow J. Tuberculosis and other mycobacteria as zoonoses. Proceedings of the Conference of the American Association of Zoo Veterinarians, Houston, TX, 1997; 110-5.

Metchock BG, Nolte FS, Wallace RJ. Mycobacterium. In: Murray PR, Baron EJ, Pfaller MA, Tenover FC, Yolken RH. (Eds.), Manual of Clinical Microbiology. Washington D.C.: ASM Press, 1999; 399-427.

Michel AL, Venter L, Espie IW, Coetzee ML. *Mycobacterium tuberculosis* infections in eight species at the National Zoological Gardens of South Africa, 1991-2001. J Zoo Wildl Med 2003; 34(4): 364-70.

Mikota SK, Maslow J. Theoretical and technical aspects of diagnostic techniques for mammalian tuberculosis. Proceedings of the Conference of the American Association of Zoo Veterinarians, Houston, TX, 1997; 162-5.

Mikota SK, Maslow J. Epidemiology and treatment of tuberculosis in elephants. Proceedings of the Conference of the American Association of Zoo Veterinarians, Milwaukee, WI, 2002; 384-7.

Miller J, Jenny A, Rhyan J, Saari D, Suarez D. Detection of *Mycobacterium bovis* in formalin-fixed, paraffin-embedded tissue of cattle and elk by PCR amplification of an IS6110 sequence specific for *Mycobacterium tuberculosis* complex organisms. J Vet Diagn Invest 1997; 9: 244-9.

Miller MA. Current Diagnostic Methods for Tuberculosis in Zoo Animals. In: Fowler ME, Miller RE (Eds.), Zoo and Wild Animal Medicine, 6th ed. St. Louis, MO: Saunders Elsevier, 2008; 10-9.

Moisson P, Lacroix F, Manson C, André-Fontaine G, Bakker D, Fiette L, Thorel MF, Lernould JM. Tuberculosis outbreak in some felid species and southern sea lions in Mulhouse zoo from 1992 to 1996: Diagnostic perspectives. Proceedings of the 2nd Conference of the European Association of Zoo and Wildlife Veterinarians, Chester, 1998; 98-112.

Moser I, Prodinger WM, Hotzel H, Greenwald R, Lyashchenko KP, Bakker D, Gomis D, Seidler T, Ellenberger C, Hetzel U, Wuennemann K, Moisson P. *Mycobacterium_pinnipedii*: transmission from South American sea lion (*Otaria byronia*) to Bactrian camel (*Camelus bactrianus bactrianus*) and Malayan tapirs (*Tapirus indicus*). Vet Microbiol 2007; 127: 399-406.

National Tuberculosis Working Group for Zoo and Wildlife Species.Tuberculosis Surveillance Plan for Non-domestic Hoofstock. 2001; 30 Pgs.

Needham DJ, Phelps GR. Interpretation of tuberculin tests in pinnipeds. Proceedings of the Conference of the American Association of Zoo Veterinarians, South Padre Island, TX, 1990; 126-7.

Olivia D, Crespo EA, Dans SL, Sepúlveda M. Workshop on the status of South American sea lions along the distribution range; 2009 June 15-17; Valparaíso, Chile.

Palmer MV, Waters WR, Whipple DL. Milk containing *Mycobacterium bovis* as a source of infection for white-tailed deer fawns. Tuberculosis 2002; 82: 161-5.

Ramsay EC, Zainuddin Z. Infectious diseases of the rhinoceros and tapir. In: Fowler ME (Ed.), Zoo and Wild Animal Medicine, 3rd ed. Philadelphia, Pennsylvania: W. B. Saunders, 1993; 459-66.

Redrobe S. Diagnostic investigation and epidemiology of tuberculosis at a British zoo – multispecies infection with a new form of Mycobacterium. Proceedings of the Conference of the American Association of Zoo Veterinarians, Minneapolis, MN, 2003; 119.

Rodriguez J, Fissanoti GJC, del Portillo P, Patarroyo ME, Romano ML, Cataldi A. Amplification of a 500bp fragment from culture isolates of *Mycobacterium bovis*. J Clin Microbiol 1999; 37: 2330-2.

Rodriguez DH, Bastida RO. The Southern sea lion, *Otaria byronia* or *Otaria flavescens*? Mar Mamm Sci 1993; 9 (4): 372-381.

Romano MI, Alito A, Bigi F, Fisanotti JC, Cataldi A. Genetic characterization of mycobacteria from South American wild seals. Vet Microbiol 1995; 47: 89-98.

Romeis B. Mikroskopische Technik. München, Wien: Verlag Urban und Schwarzenberg; 1989.

Rommel SA, Lowenstine LJ. Gross and microscopic anatomy. In: Dierauf LA, Gulland FMD (Eds.), CRC Handbook of Marine Mammal Medicine, 2nd ed. Boca Raton, Florida: CRC Press, 2001; 129-64.

Schaberg T, Forssbohm M, Kirsten D, Loddenkemper R, Rieder H, Urbanczik R. Richtlinien zur medikamentösen Behandlung der Tuberkulose im Erwachsenen- und Kindesalter. Pneumologie 2001; 55: 494-511.

Schmitt DL, Olson D, Riddle HS. Summary of fourth annual tuberculosis and elephants workshop. Gajah 2010; 33: 68-70.

Shaw G. Seals. In: Kerasly G (Ed.), General Zoology of Systematic Natural History. London: 1800; Vol 1 (2): 266-291.

Sherrill J, Peavy GM, Kopit MJ, Garner MM, Gardiner CH, Adams LM. Use of laser rhinoscopy to treat a nasal obstruction in a captive California sea lion (*Zalophus californianus*). J Zoo Wildlife Med 2004; 35(2): 232-241.

Sonntag F, Mihaljevic M. Thorax. In: Mihaljevic M, Kramer M, Gomercic H (Eds.), CT- und MRT-Atlas: Transversalanatomie des Hundes. Berlin: Parey Verlag, 2009; 76-99.

Steiger K, Ellenberger C, Schüppel KF, Richter E, Schmerbach K, Krautwald-Junghanns ME, Wünnemann K, Eulenberger K, Schoon HA. Ungewöhnliche Mykobakterien-Infektionen bei Haus-und Zootieren: eine Kasuistik unter besonderer Berücksichtigung der Pathologie. Dtsch Tierärztl Wochenschr 2003; 110 (9): 382-8.

Sternberg S, Bernodt K, Holmström A, Röken B. Survey of tuberculin testing in Swedish zoos. J Zoo Wildl Med 2002; 33(4): 378-80.

Stetter MD, Mikota SK, Gutter AF, Monterroso ER, Dalovisio JR, Degraw C, Forley T. Epizootic of *Mycobacterium bovis* in a zoologic park. J Am Vet Med Assoc 1995; 207 (12): 1618-21.

Supply P, Allix C, Lesjean S, Cardoso-Oelemann M, Rusch-Gerdes S, Willery E, Savine E, de Haas P, van Deutekom H, Roring S, Bifani P, Kurepina N, Kreiswirth B, Sola C, Rastogi N, Vatin V, Gutierrez MC, Fauville M, Niemann S, Skuce R, Kremer K, Locht C, van Soolingen D. Proposal for standardization of optimized mycobacterial interspersed repetitive unit-variable-number tandem repeat typing of *Mycobacterium tuberculosis*. J Clin Microbiol, 2006; 44: 4498-4510.

Thoen CO. Tuberculosis and other mycobacterial diseases in captive wild animals. In: Fowler ME (Ed.), Zoo and Wild Animal Medicine, 3rd ed. Philadelphia, PA: W. B. Saunders, 1993; 45-9.

Thoen CO, Barletta RG. Pathogenesis of *Mycobacterium bovis*. In: Thoen CO, Steele JH, Gilsdorf MJ (Eds.), *Mycobacterium bovis* infection in Animals and Humans, 2nd ed. Ames, IA: Blackwell Publishing, 2006; 18-33.

Thoen CO, Ebel ED. Diagnostic tests for Bovine Tuberculosis. In: Thoen CO, Steele JH, Gilsdorf MJ (Eds.), *Mycobacterium bovis* infection in Animals and Humans, 2nd ed. Ames, IA: Blackwell Publishing, 2006; 49-53.

Thoen CO, Himes EM. Mycobacterial infections in exotic animals. In: Montali RJ (Ed.),The comparative pathology of zoo animals. Washington, D. C.: Smithsonian Institute Press, 1980; 241-5.

Thompson PJ, Cousins DV, Gow BL, Collins DM, Williamson BH, Dagnia HT. Seals, Seal Trainers, and Mycobacterial Infection. Am Rev Respir Dis 1993; 147: 164-7.

Thomson B. Polymerase chain reaction detection of *Mycobacterium tuberculosis* complex in formalin-fixed tissues. In: Thoen CO, Steele JH, Gilsdorf MJ (Eds.), *Mycobacterium bovis* infection in Animals and Humans, 2nd ed. Ames, IA: Blackwell Publishing, 2006; 63-7.

Thorel MF, Karoui C, Varnerot A, Fleury C, Vincent V. Isolation of *Mycobacterium bovis* from baboons, leopards and a sea lion. Vet Res 1998; 29: 207-12.

Van Bonn W, Jensen ED, Brook F. Radiology, computed tomography, and magnetic resonance imaging. In: Dierauf LA, Gulland FMD (Eds.), CRC Handbook of Marine Mammal Medicine, 2nd ed. Boca Raton, Florida: CRC Press, 2001; 557-92.

Vordermeier M, Goodchild A, Clifton-Hadley R, De la Rua R. The interferon-gamma field trial: background, principles and progress. Vet Rec 2004; 155: 37-8.

Walter O. European Regional South American sea lion studbook, Twycross Zoo, United Kingdom; 2011.

Waters WR, Palmer MV, Bannantine JP, Greenwald R, Esfandiari J, Andersen P, McNair J, Pollock JM, Lyashchenko KP. Antibody responses in reindeer (*Rangifer tarandus*) infected with *Mycobacterium bovis*. Clin Diagn Lab Immunol 2005; 12: 727-35.

Waters WR, Palmer MV, Bannantine JP, Whipple DL, Greenwald R, Esfandiari J, Andersen P, McNair J, Pollock JM, Lyashchenko KP. Antigen recognition by serum antibodies in white-tailed deer (*Odocoileus virginianus*) experimentally infected with *Mycobacterium bovis*. Clin Diagn Lab Immunol 2004; 11: 849-55.

Waters WR, Stevens GE, Schenbaum MA, Orloski KA, Robbe-Austerman S, Harris NB, Hall SM, Thomsen BV, Wilson AJ, Brannian RE, Nelson JT, Schafer S, Esfandiari J, Dutton M, Greenwald R, Lyashchenko KP. Bovine tuberculosis in a Nebraska herd of farmed Elk and Fallow Deer: a failure of the tuberculin skin test and opportunities for serodiagnosis. Vet Med Int, 2011. Available from: www.ncbi.nlm.nih.gov/pmc/articles/PMC3087612/pdf/VMI2011-953985.pdf

West G. Tuberculosis in captive exotic animals. In: Thoen CO, Steele JH, Gilsdorf MJ (Eds.), *Mycobacterium bovis* Infection in Animals and Humans, 2nd ed. Ames, IA: Blackwell Publishing, 2006; 248-257.

Woods R, Cousins DV, Kirkwood R, Obendorf DL. Tuberculosis in a wild australian fur seal (*Arctocephalus pusillus doriferus*) from Tasmania. J Wildlife Dis 1995; 31 (1): 83-6.

Zumárraga MJ, Bernardelli A, Bastida R, Quse V, Loureiro J, Cataldi A, Bigi F, Alito A, Castro Ramos M, Samper S, Otal I, Martin C, Romano MI. Molecular characterization of mycobacteria isolated from seals. Microbiology 1999; 145: 2519-26.

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