Mycobacterial infections in zoo animals: relevance, diagnosis and management*

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While the world prevalence of tuberculosis (TB) is increasing in the human population, TB infection remains a real concern in some animal populations all around the globe. Most mycobacteria of the TB complex are able to infect zoo and wildlife species, in which the pathogenesis, receptivity and immune responses vary widely. The diagnostic tools usually applied in domestic animals show limited performance in zoo species, especially when prevalence is low. Conversely, investigations of cell-mediated immunity through in vitro assay of γ-interferon may have numerous advantages, as long as the technical limits are known and can be improved upon. Furthermore, recent tools based on the investigation of humoral immunity seem very promising for the detection of antibodies directed against certain immunogenic mycobacterial antigens in a wide range of species. All these methods are currently evaluated in field studies, despite the difficulties to ensure rigorous validation. The development of these diagnostic tools is also impaired by the prevalence of mycobacteria other than TB also able to infect and create relevant disease in their host. Thus, decisions on positive and suspicious-animals issues should be taken based on the evaluation of the risk of transmission to the rest of the zoological collection, the possible treatment options, animal welfare, conservation considerations and, of course, the zoonotic potential of this pathogen.

Key-words: interferon-γ; mycobacteria; non-tuberculosis mycobacteria; serology; tuberculosis; zoo.

INTRODUCTION

Tuberculosis (TB) is the common name of the disease that could be caused by different species of bacteria belonging to the Mycobacterium tuberculosis Complex (MTC) (Table 1). One-third of the human population is currently infected by TB (World Health Organization, 2010) and in 2008 1·8 million people died from the disease (United Nations, 2010). Recent increases of Multi Drug Resistance (MDR-TB) or even Extreme Drug Resistance (XDR-TB) strains, and co-infection with human immunodeficiency virus (HIV), make the battle against TB more difficult (Hanekom et al., 2010). TB is now placed on the ‘most relevant diseases’ list in the health-related Millennium Development Goal 6 by the World Health Organization (WHO) and the United Nations (UN), together with acquired immune deficiency syndrome (AIDS) and malaria (United Nations, 2001, 2005; World Health Organization, 2010). Vigorous action plans are applied to fight the disease in developing countries, although these countries are also facing notable incidences of TB within the animal population.

The majority of the mycobacteria from the MTC have the ability to infect wild animals (Table 1), whereas the susceptibility, pathogenicity and immune responses towards mycobacterial infection vary widely between mycobacteria (Mostowy et al., 2005) and host-animal species. Although some mammals species seem to show a lower incidence rate (e.g. equids, New World monkeys), the predictability of infection outcome is still
hard to guess and, therefore, the eradication of TB is potentially linked to the capacity of early diagnosis in domestic and wild host species. Genetic key factors [e.g. interferon-γ (IFN-γ) receptor or vitamin D genotypes] are likely contributing towards these differences (Rhodes et al., 2003; Schluger, 2005). The course of the disease and occurrence of latent infection versus active infection are variable among species, from extremely sensitive Old World monkeys to apparently more resistant equids.

Species of mycobacteria out of the MTC and other than Mycobacterium leprae (causing leprosy) are known as mycobacteria other than tuberculosis (MOTT), non-tuberculous mycobacteria (NTM) or atypical mycobacteria. These are mainly environmental mycobacteria found in water and soil, but they are also able to contaminate vertebrates, although the occurrence of disease seems to be more related to the immune status of host or to very close or repeated contact.

Except for M. leprae, all mycobacteria are facultative intracellular pathogens, often invading macrophages as the first host cell. Thus, a direct diagnostic will require cellular samples and techniques to highlight the presence of mycobacteria (stains) or their DNA. For their survival, mycobacteria are driving immunopathological mechanisms of lymphocytes in a way that allows them to stay within the macrophage they infect. There are four groups of T-helper lymphocytes (Th). The Th1 group participates in cell-mediated immunity (CMI) by producing cytokines such as IFN-γ, and then activating other immune cells participating in the fight against the mycobacteria. The Th2 group provides help to B lymphocytes, which are in charge of producing humoral responses (immunoglobulins). During the course of the disease, the Th2 pathway is also activated, although humoral protection seems to be poorly effective against this intracellular pathogen. These immunological mechanisms normally lead to the eradication or the containment of the mycobacteria, and their systemic local effects could be tracked to assess previous or current presence of mycobacteria. When the Th1 pathway fails, it could lead to active disease with active replication of mycobacteria in the surrounding cells (macrophage, dendritic cells) or, more often, to containment within a granuloma, which is the specific latency phase of mycobacterial infection that is able to last for years. The probability of latency depends on the host species, the mycobacteria species, the infection dose and the route of transmission.

Table 1. Tuberculosis complex Mycobacteria and their reported hosts.

<table>
<thead>
<tr>
<th>Mycobacteria of the Tuberculosis Complex</th>
<th>Major Historical Known Host or Burden</th>
<th>Reported Wild and Zoo Host</th>
</tr>
</thead>
<tbody>
<tr>
<td>M. tuberculosis</td>
<td>human, non-human primates</td>
<td>elephant, non-human primates, beisa oryx, addax, goats, birds, lowland tapir, giraffes, springboks, mongoose, rhinoceros, addra gazelle</td>
</tr>
<tr>
<td>M. bovis</td>
<td>cattle (+buffalo, bison)</td>
<td>all ruminants, badgers, possums, meerkats, big cats, canids, rodents, non-human primates, wild boars elephants, camelids, rhinoceros, onager, horse, birds</td>
</tr>
<tr>
<td>M. africanum</td>
<td>human</td>
<td>cattle, swine, non-human primates</td>
</tr>
<tr>
<td>M. microti</td>
<td>vole, camelids</td>
<td>New World monkeys, big cats</td>
</tr>
<tr>
<td>M. pinnipedii</td>
<td>pinnipeds</td>
<td>camel, tapir, big cats</td>
</tr>
<tr>
<td>M. caprae</td>
<td>goat, sheep, swine</td>
<td>swine, cattle wild boars, red deer, white-tailed deer, camel, bison</td>
</tr>
<tr>
<td>M. canetti</td>
<td>human</td>
<td>?</td>
</tr>
<tr>
<td>Dassie bacillus variant</td>
<td>hyraxes</td>
<td>meerkats</td>
</tr>
</tbody>
</table>
TRANSMISSION AND ZOONOTIC RISK FROM ZOO ANIMALS

Interspecific transmission can occur between taxonomically distant species as has been recorded in the literature (Lewerin et al., 2005; Jurcynski et al., 2007; Moser et al., 2008). Thus, silent extension of TB into a zoological collection owing to an increase of latent-form cases should be a real concern for zoo managers. Transmission routes between animals are directly associated with the localization of granulomas in shedding individuals: pulmonary lesions generally lead to airborne transmission, whereas mesenteric lymph-node lesions may lead to intestinal excretion of mycobacteria. Horizontal transmission is the most significant means of contamination but vertical transmission could also occur through placental or umbilical infection (Kaneene & Pfeiffer, 2006).

The potential for a human to acquire any mycobacterial disease from a zoo animal requires a combination of several events. First, the animal that is actively infected with a mycobacterial disease must be shedding and it must be in a form that has a good potential for infection. This is most likely in the form of aerosolized droplets from respiratory secretions. Sputum from non-human primates that have acquired the habit of spitting at people is also a potential source of infection. Other potential sources of infection include urogenital or gastrointestinal shedding and the aerosolization of these materials during routine husbandry and cleaning, such as the use of a high-pressure hose (Dalovisio et al., 1992).

Second, the species of mycobacteria and the pathogenicity it has for humans must be considered. Among the MTC mycobacteria, Mycobacterium bovis is likely to be the one with the more relevant zoonotic burden (Cosivi et al., 1998). Mycobacterium tuberculosis (Sternberg Hewerin et al., 2005), M. bovis (Stetter et al., 1995), Mycobacterium pinnipedii (Moser et al., 2008), Mycobacterium caprae (Pate et al., 2006) and Mycobacterium microti (Pattyn et al., 1970; A. Lécu, unpubl. data) are examples of known zoonotic MTC mycobacteria already reported within zoological collections, whereas ‘dassie bacillus’ was found in Meerkats Suricata suricatta and hyraxes Procavia sp but never reported in humans. Atypical mycobacteria found in zoo species may also infect humans (especially if immunosuppressed) but they cannot be named as truly ‘zoonotic’ as they are mainly acquired from the environment.

Third, the route of absorption will have a bearing on the zoonotic risk of any mycobacterial disease. Respiratory shedding of any mycobacterial pathogens will have greater zoonotic potential than oral absorption of mycobacteria.

Fourth, exposure is another essential component of any zoonotic spread of mycobacteria. Prolonged exposure and close contact will increase the likelihood of zoonotic transmission. The risk for TB transmission from an animal with a case of active TB is higher for daily handlers than for people with only brief contact, such as members of the viewing public. Trained elephants (Furley, 1997) and sea lions (Kiers et al., 2008) are classic examples of the zoonotic potential of mycobacterial disease from zoo animals, mainly because of the close contact between keepers and animals during training sessions. Housing conditions will also play an important role in zoonotic potential and examples are reported of zoonotic transmission from rhinoceros species (Stetter et al., 1995). A surveillance programme that targets early detection in the more sensitive species (non-human primates, elephants, ungulates, sea lions) has the potential to identify risk and anticipate transmission to all people submitted to exposure. Improved diagnostics that raise the level of suspicion before actual shedding may prove to be extremely beneficial to prevent extension of TB within humans and non-human inhabitants of zoos.

An important zoonotic potential exists in post-mortem examinations of infected animals. It is critical to approach any suspect case with caution and use appropriate protective equipment. In some species, such as elephants, it may be advisable to have respiratory protective equipment in use during...
the entire examination. For the United States, specific guidelines can be found at http://www.osha.gov/SLTC/tuberculosis/standards.html, and the Centers for Disease Control and Prevention (CDC) for Guidelines for Preventing the Transmission of Mycobacterium tuberculosis in Health-Care Settings, 2005 (Jensen et al., 2005).

In addition to precautions in handling suspect post-mortem cases and a vigorous ante-mortem TB surveillance programme for zoo animals, an employee health plan is recommended. A regular TB survey of all zoo personnel (through a skin test or an IFN-γ test) should be set in place in coordination with local public-health authorities, likely associated with a vaccination schedule. Intervals between controls are set based on current national medical recommendations and local risk assessment. Mycobacterial infections are opportunistic pathogens and the transition from infection to disease depends on the host’s immune competency. Educating staff to identify the factors that will minimize the risk of infection and reduce the risk of developing active disease (cigarette smoking, excessive alcohol consumption, high-fat diets) will also help to reduce the zoonotic potential of any mycobacterial disease, in the same way that should be considered for all opportunistic pathogens in the zoo community.

It should be noted that *M. tuberculosis* is the main agent of TB found in humans, whereas *Mycobacterium africanum*, *M. bovis* and *Mycobacterium canetti* account for <1% of TB occurrence in humans (Olsen et al., 2010). The risk of reverse zoonosis, from humans to animals, also exists and has already been reported in several species, such as cattle (Ocepek et al., 2005), non-human primates (Michel & Huchzermeyer, 1998) and birds (Schmidt et al., 2008; Steinmetz et al., 2006).

**NATIONAL REGULATIONS AND ZOO CONCERNS**

In all countries, legislation relating to animal TB is implemented to achieve two main goals. The first is to eradicate TB from domestic-animal populations and to keep its prevalence below the ‘free of TB’ threshold. The second is to avoid contamination from outside (livestock importations, wildlife). Thus, the zoo community stands in a special position, often keeping both domestic and wild species, with a constant need for animal transfers and exchanges.

**European national programmes and zoo concerns**

All domestic species of cattle, buffalo and wood bison in zoos and safari parks should be subjected to routine tuberculin testing as often as the indicated testing interval for the area in which the zoo is located. As long as they stay within their original facility without any close TB outbreak, wild species in captivity are generally exempted from statutory TB testing and, currently, there is no officially approved screening test for TB in species other than bovines, deer and, occasionally, primates (Cousins & Florisson, 2005; Defra, 2008).

Several texts define sanitary policy for TB within-country members [European Union (EU) Council Directive 64/432/EEC, and all following modifications; Council Directive, 1964]. The EU policy towards TB in animal health mainly focuses on the eradication of bovine TB and is based on two fundamental principles: first, the Member States are primarily responsible for the eradication of bovine TB and may receive community financial support for the eradication programme; second, eradication of bovine TB in the EU must be the final target and the Member States must consider eradication as the defined aim.

Hence, most EU regulations apply only to *M. bovis*, and sometimes *M. tuberculosis*, screening. Based on sequential Commission Decisions, 14 member states of the EU are classified as free of bovine TB at the time of writing. Initially, Denmark, Germany, Luxembourg, the Netherlands, Austria, Finland and Sweden were classified as bovine-TB free (Commission Decision, 1999). In 2003, Belgium and France were added to the list.
(Commission Decision, 2003), and by 2009, the Czech Republic, Slovakia, Slovenia and Poland were also listed as bovine-TB free (Commission Decision, 2009a). Outside but close to the EU, Norway and Switzerland also have the status ‘free of bovine TB’ (de la Rua-Domenech, 2006). Member states can also apply to the EU to classify part of their regions as free of TB, such as the UK obtained for Scotland (Commission Decision, 2009b). The main criterion for keeping the ‘officially free of TB’ status for a country or a region is to maintain the prevalence of bovine TB in cattle herds below 0.1% (OIE, 2010a) per year for six consecutive years (Council Directive, 1964).

The Balai Directive (EU Council Directive 92/65/EEC; Council Directive, 1992) requires that all ruminants traded between institutions must come from officially bovine-TB-free herds, as mentioned in Trade Control and Expert System (TRACES) health certificates. Moreover, to be ‘Balai-approved’, an institution must be free of ‘bovine TB’ (as listed in Annex A of the Directive), for at least 3 years, or free of ‘TB’ (this term includes all mycobacteria of the TB complex) for primates, felidae and ruminants, if the Member State has a control monitoring programme regarding these particular species. During a transfer between two approved bodies, TB testing is not required by the Balai Directive, but many member states are allowed to add TB testing as an additional requirement when importing ungulates, whatever institutions they come from (e.g. requested by UK, Sweden). Nevertheless, zoo mammals are still being exchanged in national and international transfers within Europe without appropriate individual testing.

TB prevalence in both domestic species and free-ranging wildlife that surrounds zoo premises and animal enclosures are also a major factor that can modify the compulsory TB requirements of a zoo. Any TB monitoring/action programme on neighbouring wildlife or livestock should impact zoo TB policy and must take into consideration the relationship between zoo animals and exogenous species (e.g. badgers Meles sp, deer within nearby forests or TB in livestock on a nearby farm).

Even if they do not provide compulsory content, valuable guidelines and recommendations are also produced by the professional zoo community, such as the European Association of Zoos and Aquaria position statement on the Animal Health Strategy for the EU (EAZA, 2009), led by the European Association of Zoo and Wildlife Veterinarians (EAZWV), or the European Endangered Species Programme (EEP) or Taxon Advisory Group (TAG) recommendations on pre-transfer health requirements for managed species.

**US states programmes and zoo concerns**

The Animal and Plant Health Inspection Service (APHIS) Uniform Methods and Rules (UM&R) have produced the minimum standards for the maintenance of TB-free accredited herds of cattle and bison, and the maintenance of state or zone status in the US Department of Agriculture’s (USDA) TB eradication programme (USDA, 2004). These minimum standards do not preclude the adoption of more stringent standards by any state or zone. Each individual state has various regulations with regard to the testing requirements for exotic ruminants before importation and several have no specific requirements at all. The current status of each state can be checked at the APHIS website. Within the zoo community in North America, the Association of Zoos and Aquariums (AZA) and American Association of Zoo Veterinarians (AAZV) recommend TB testing for zoo ungulates, but no standard protocols are in place. Regulations are similar to the EU in that *M. bovis* and *M. tuberculosis* are reportable disease. In a confirmed diagnosis of *M. bovis* the state veterinarian, USDA Veterinary Services and Animal Care (for facilities licensed under the Animal Welfare Act), public health officials and other relevant regulatory agencies should be promptly notified. If *M. tuberculosis* is diagnosed, local public-health officials are notified first.

All livestock herds, within a 10 mile (16 km) radius, will be tested within 6 months of any
diagnosis of bovine TB in livestock or free-ranging wildlife (USDA, 2004).

OVERVIEW OF CURRENT DIAGNOSTIC METHODS

Diagnostic methods are seldom homogenous, are rarely validated for zoo species and can hardly ever be transposed from one species to another. As population size and the nature of wild species would often prevent decent validation for these tests (M. Miller, 2008), zoo veterinarians must be aware of the actual diagnostic tools available, their limits and the current progression of scientific knowledge in TB diagnostic means.

Limits of an unspecific diagnosis

Clinical signs of TB are rarely seen before death in zoo animals (Montali et al., 2001; Lyashchenko et al., 2006). If a cough and dyspnoea are noticed, this is always in a very late and irreversible stage of the pulmonary form of disease. The most frequent sign noticed among mammals is chronic weight loss.

Different imaging techniques can play a role in TB diagnostics, but they are only informative on localization and aspect of the lesions. Although radiography is a regular step in human diagnostic procedures, its performance may be restricted by the limitations of animal species in zoo veterinary medicine: the size of the thoracic cage of some animals may prevent the use of X-rays (e.g. ungulates over 250 kg), reference images are often missing and lesions may be too subtle for radiographic detection. Moreover, in some species, calcified lesions are very rare (e.g. felids and non-human primates).

High-frequency ultrasonography, computer tomography (CT), magnetic resonance imagery (MRI) and even positron emission tomography (PET) scans could also be useful to detect granulomas or lesions in non-palpable lymph nodes, when they are in the thorax or because animal conformation prevents easy palpations, such as in pinnipeds (Jurczynski et al., 2007; Lacave et al., 2009). For radiographs, the size of animal patients, anaesthesia considerations and availability of the devices, except for ultrasounds, often prevent their use in zoo species.

For species such as birds, laparoscopy remains one of the most useful tools (Pavlas et al., 1993; Speer et al., 1999), followed by biopsy of suspect granulomas or abnormal tissue for further direct examinations [stains, polymerase chain reaction (PCR) and culture].

Limits of direct examination

Culture stands as the ‘gold standard’ method (M. Miller, 2008) but requires from 2 weeks to 3 months for culture to occur (Liebana et al., 1995), depending on whether the mycobacteria belong to the slow-growing group (e.g. all mycobacteria from MTC) or rapidly growing group (e.g. Mycobacterium abscessus or Mycobacterium fortuitum). Various modifications and improvement have been added to culture methods (i.e. use of liquid or semi-liquid culture media) in order to reduce the delay to below 30 days (White-law & Strum, 2009). Some mycobacteria species hardly grow on these alternative media (e.g. M. pinnipedii, M. L. Boschiroli, pers. comm.) and some of these culture techniques are still not validated in veterinary medicine. Hence, faster testing still relies on microscopic examination and mycobacterium DNA amplification methods.

The threshold number of bacilli needed to obtain a positive result by microscopic examination of Acid Fast Bacilli stained with the Ziehl Neelsen method (ZN) is around 5000 bacilli ml$^{-1}$ and is dependent on the reader. Other bacteria also share the Acid Fast resistant property, and also react to ZN dyes, such as Nocardia sp. Detection limit could be enhanced using fluorescence microscopy (FM), although access to this technique is not as widespread as ZN. The usual staining techniques (ZN and modified ZN) allow the detection of bacilli loads superior to 103–104 colony-forming units (CFU) per ml: as a comparison, fewer than ten CFU of M. tuberculosis per ml are enough to infect a
Cynomolgus macaque *Macaca fascicularis* (Lin *et al*., 2006). This means that a sample may indicate an infection source while its microscopic results remain negative.

Some biological samples may contain very few bacilli (Martin-Hernando *et al*., 2007); for example, because of highly calcified lesions (Capuano *et al*., 2003) or intermittent shedding in biological fluids, such as bronchial secretions or milk. As an example, 15–20% of active pulmonary forms are not even confirmed by any culture in humans (Frieden *et al*., 2003). Therefore, while positive culture provides evidence of disease, negative culture results may not rule out infection in exposed or suspect animals.

The detection of DNA material directly from raw biological samples is sometimes performed by some laboratories. The biological media issued from zoo animals could be broncho-alveolar lavage (Flynn *et al*., 2003), gastric lavage, saliva from oral swab and stools or elephant trunk wash (Mikota *et al*., 2000). Amplification methods (e.g. PCR) are now well developed and can help to specify the mycobacteria with the use of selected probes. In the recent years, sensitive and specific PCR probes became available, sometimes associated with rapid processing (e.g. Genoquick®) able to provide species identification of mycobacteria or antibioresistance profile within a few hours. However, some of these biological samples are likely to host many other bacteria or biological compounds as inhibitors that are impairing PCR efficacy. Even with the simplification of molecular techniques, they still cannot be used as a broad and primary screening tool as described in Table 2. It should be noted that those DNA-based techniques show the best sensitivity and specificity values when applied on pure culture samples, even those that are early stage (Fitzgerald *et al*., 2000).

When there is sufficient DNA (rich sample or culture), molecular typing should be performed. Spoligotyping or other methods [e.g. variable number tandem repeat (VNTR)] allow researchers to identify strains of mycobacteria and these techniques can be a very useful tool to track the epidemiological circuit of the TB. In recent years, several TB strains from zoos were spoligotyped and their fingerprint can be compared with other circulating strains, either from captive or from feral animals. Whenever a TB mycobacterium is discovered, this kind of typing should always be performed, or at least samples must be kept frozen (−25 or −80 °C) until further typing analyses are available. The standard use of all molecular techniques is application on pure culture material. However, PCR techniques, and sometimes spoligotype, could also be applied to raw material (lavages, tissues), which decreases the delay before the results are available, although this application may compromise sensitivity and specificity.

### CMI-based diagnostics

During the CMI process, mycobacterial antigens are presented by macrophages to Th

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<table>
<thead>
<tr>
<th>TB TEST (E.G. PCR)</th>
<th>DISEASE (ACTIVE TB)</th>
<th>NO DISEASE (LATENT OR NO TB)</th>
<th>POSITIVE PREDICTIVE VALUE</th>
</tr>
</thead>
<tbody>
<tr>
<td>SE = 72% SP = 96%</td>
<td>5</td>
<td>95</td>
<td>PPV = 3·6/(3·6+3·8) = 49%</td>
</tr>
<tr>
<td>Prevalence 5%</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PCR +</td>
<td>3·6</td>
<td>3·8</td>
<td></td>
</tr>
<tr>
<td>PCR –</td>
<td>1·4</td>
<td>91·2</td>
<td></td>
</tr>
</tbody>
</table>

**Table 2.** Example of polymerase chain reaction (PCR) sensitivity in human sputum, with 5% tuberculosis (TB) prevalence situation: from N. Veziris, pers. comm. With the given sensitivity (SE) and specificity (SP), a positive TB test result means an even probability (49%) of a TB-infected or a TB-free animal. The lower the prevalence, the lower the positive predictive value.
lymphocytes, which become activated and then produce various cytokines, inducing their own replication and the activation/mitosis of other ‘helped’ cells. Memory of Th cells regarding previous contact with Mycobacterium can be assessed (Chapel et al., 2006) by presenting selected antigen(s) to them, either in vivo [tuberculin skin test (TST) also called the ‘Mantoux’ test] or in vitro [lymphocyte proliferation assay (LPA) or IFN-γ tests].

TST is far from being validated for zoo species, as there is considerable variation between tegument and dermal structure within species, compounded by a lack of understanding of immunophisiology across various taxa. The test relies on local inflammatory cell recruitment (Ciftci et al., 2005; Olsen et al., 2010), which can be low or absent for many reasons, such as tegument cellular organization, immunosuppressive status or superficial temperature. TST sensitivity is often poor; for example, 70–90% in humans and 50–90% in zoo hoofstock (Cousins & Florisson, 2005). Specificity is also dependent on species-specific features (e.g. orangutans Pongo sp prone to an unspecific TST-positive reaction) or individual conditions, such as malnutrition, medical treatments or unrelated inflammatory status, that have previously been shown to affect the results of tuberculin testing, potentially resulting in false negatives (Kiwanuka, 2005).

The co-infection by other NTM (e.g. avium complex) may also lead to false-positive results: specificity is influenced by the kind of antigen (tuberculin) injected intradermally; for example, mammalian old tuberculin (MOT) is a crude culture filtrate preparation that contains antigens common to many mycobacterial species; therefore, cross reactions owing to NTM sensitization sharing these antigens could be expected. Other tuberculin have more selected contents [protein-purified derivate (PPD)], either for M. bovis screening (bovine PPD) or for Mycobacterium avium complex (MAC) screening (avian PPD).

The materials and methods of application of the skin test vary between countries and veterinarians. For bovine tuberculin production, a standardized quality of tuberculin is still missing within the European Economic Community, despite a common regulatory definition from resolution 64/432/EEC (Council Directive, 1964). In the United States, tuberculin is standardized.

Although the worldwide source for tuberculin production is M. bovis strain AN5 (Schiller et al., 2010), there is an important discrepancy between PPD quality, leading to a relevant variation in the TST results (Rangel-Frausto et al., 2001; Good et al., 2007). Although named ‘purified’, tuberculin PPD still contains a very large panel of different antigens (Borsuk et al., 2009) in non-predictable proportions. Reading methods may also vary between veterinarians when a ‘distant’ opinion about TST local reactions is applied to avoid repeated immobilizations. Therefore, whenever possible, close examination, palpation and caliper measurement of tegument are strongly recommended in order to avoid false-negative results. Skin test interpretation guidelines exist only for humans (Katial, 2003), non-human primates (Bushmitz et al., 2008), cattle (OIE, 2010a), deer (Clifton-Hadley & Wilesmith, 1991; OIE, 2010b) and tapir (Gomis et al., 2008). Visualization is often used to read intrapalpebral TST performed on primates and, while not official for regulatory purposes, this method is used within zoological facilities and as a means of surveillance. However, close eyelid evaluation and palpation is also recommended in these species, as it could enhance the sensitivity of TST (Panarella & Bimes, 2010). All regulatory testing must be read by palpation.

To increase the specificity of TST as well as in cases of doubtful or inconclusive results, a comparative TST is strongly recommended, with the use of both bovine and avian tuberculin (Cook, 1993; Clifton-Hadley et al., 2001). This comparative test is still the only official test allowed by some national regulations for species such as deer (Sockett, 1993). A time interval between two TSTs is required to overcome the desensitizing effect of tuberculin and is usually set to a minimum of 42 days (de la Rua-Domenech et al., 2006).

In vitro tests of cellular immunity rely on (re)stimulation of T lymphocyte memory.
Thus, the first mandatory step is to keep cells alive until they reach the laboratory. Blood samples should arrive at the laboratory for stimulation no later than 8–10 hours after collection and should be kept at ambient room temperature (below 22°C), avoiding temperatures that are too cold (below 4°C) or too high (more than 25°C) (Waters et al., 2007) in order to keep lymphocytes alive until they arrive at the laboratory to be incubated. Particular attention must be paid to immediate, homogenic and full mixing of blood and anticoagulant (heparin) at collection.

These CMI in vitro tests are performed in two steps, for a total run of 48 hours minimum. The first step is to incubate T cells with a selected mycobacterial antigen (usually bovine and avium PPD; sometimes purified antigen, such as ESAT-6 or CFP10), leading the lymphocytes to produce cytokine and to proliferate if they had previously been exposed to the same mycobacterial antigen(s). T cells are usually issued from blood centrifugation and separation techniques, but other relevant fluids (pleuretic or abdominal effusion fluids) could also be considered (Ariga & Harada, 2008) as long as their cellular content is elevated. The second step is to reveal either the LPA rate through radio-isotopic techniques or the amount of relevant cytokines (e.g. IFN-γ) produced through an enzyme-linked immunosorbent assay (ELISA) or an enzyme-linked immunosorbent spot (ELISPOT, which is more sensitive). Once the cells have been stimulated to produce IFN-γ, then the ELISA or ELISPOT can be delayed if the test wells are frozen.

LPA is less available to current practice than IFN-γ tests, owing to the use of radio isotopes in their process, while commercial kits for the IFN-γ test are available, designed for cattle (BOVIGAM®: Wood & Jones, 2001), primates (PRIMAGAM®), deer (CERVIGAM® production discontinued at this time) and humans (QUANTIFERON GOLD®, T-SPOT-TB®). Recent studies show that the use of these tests can be extended to some exotic species (Grobler et al., 2002; Riquelme, 2009); for example, a cattle-designed test will detect IFN-γ of a large range of exotic Bovidae, but also some animals outside this family (e.g. Giraffidae). However, artiodactyls may respond poorly with the standard positive control antigens (unspecific mitogen) or their IFN may not be detected by the ELISA (Riquelme, 2009). It is likely that similar problems occur with the primate-referred test; although a list of ‘validated’ species is provided on its leaflet, field studies showed contrasting results between different species (Lécu & Riquelme, 2008; Riquelme, 2009; S. Hoby & C. Wenker, pers. comm.); for example, the IFN-γ of certain species may trigger a greater optical signal through the ELISA included QUANTIFERON® rather than the one of the PRIMAGAM®.

In order to overcome the problem of specificity in ELISA detection of IFN-γ, in-house modified tests can be created. One solution is the detection of mRNA coding for IFN-γ, which seems to have broad nucleotid sequences, shared by several different mammal species; moreover, mRNA IFN-γ sequences from several species are now available in gene banks, and these can be used to develop specific in-house ELISA tests (Harrington et al., 2007). This option is currently developed for some zoo or wild species, although the entire procedure can only be performed by experienced laboratories. An alternative solution is to design a specific IFN monoclonal antibody to be used instead of the regular ELISA, as is currently being developed for elephants and rhinoceros (Morar et al., 2007; Rutten, 2007). At the time of writing, these dedicated tests are still not available for routine use.

The duration of the CMI is rather unknown and its behaviour over the course of infection is only theoretical. All studies concerning long periods of time (Vervenne et al., 2004) and work on bacille Calmette-Guérin (BCG) vaccination protection length suggest that stimulation tends to revert to a baseline level, which remains unknown beyond a year.

**Humoral immunity-based diagnostics**

Serodiagnosis of TB suffered from a bad reputation in human TB medicine as early
trials assessed antibodies against single mycobacterial antigens, which resulted in tests that were low in sensitivity and specificity (Pottumarthy et al., 2000). This has changed in the last few decades as humoral tests began to look at multiple mycobacterial antigens, resulting in improved sensitivity and specificity.

During the course of an infection, Th1 activity (the CMI pathway) is thought to be initially greater than Th2 (the humoral route) in order to control and confine infection (Chapel et al., 2006). An inversion of this Th1/Th2 balance control is often associated with a recrudescence or with active disease and extensive pathology (Welsh et al., 2005; Doherty & Rook, 2006). Although seeking antibodies may appear to be of little help when screening for latent infected animals, it is becoming more relevant in the detection and monitoring of active, ‘sick’ and shedding individuals. It has also been clearly noted in humans (Abebe et al., 2007), cattle (Welsh et al., 2005) and in wildlife (Chambers, 2009) that some antibody elevations occur during the shift from latent to active disease, so that a prognostic value may be added to certain serological results.

A panel of mycobacterial antigens are able to elicit persistent humoral responses in humans (Lyashchenko et al., 2000) and in a wide range of species (Haagsma & Eger, 1990; M. Miller, 2008). Mainly assessed in non-human primates (Khan et al., 2008), most relevant antigens have been screened through different techniques (Cranfield et al., 1990; Lyashchenko et al., 2000), and selected to design ELISAs and rapid lateral flow technology tests, with good results in the species for which they have been validated, such as elephants (Lyashchenko et al., 2006; Greenwald et al., 2009) and primates (Lyashchenko et al., 2007). However, the panels of immunostimulant antigen will remain directly linked to the type of mycobacterium involved, host species, time frame of disease and also individual condition. Some commercial tests validated for Asian elephants Elephas maximus and African elephants Loxodonta africana (ElephantTB STAT-PAK®, DPP® VetTB) or primates (PrimaTB STATPAK®) also look promising in non-target species, such as deer (Lyashchenko et al., 2008; Greenwald et al., 2009), Black rhinoceros Diceros bicornis (Duncan et al., 2009; Espie et al., 2009), tapirs and camels (Moser et al., 2008) or South American sea lions Otaria byronia (Jurcynski et al., 2007).

The sensitivity and specificity of these serological assays are encouraging. However, these values are often issued from descriptive studies performed on either very sensitive species (i.e. prone to start disease soon after infection) or under very clear enzootic conditions, perhaps jeopardizing the real sensitivity and specificity values (Chambers, 2009). Serological profiles of animals in long-term quiescent infection status are still poorly known, and these are likely variable along a lifetime and then able to decline below detection thresholds (Lerche et al., 2008) of current serological tests during certain periods of latent infection.

Repeated testing seems to be the only way to adapt with the actual limitations, as antibody titre is thought to arise when relapse is about to occur. As zoonotic risk increases considerably within this shifting time, the detection of early serological change may be relevant to trigger a deeper screening (direct examination) and preventive measures for staff, the public and animals in the vicinity.

**Booster or no booster?**

In both humoral- and cell-mediated immunity, the phenomenon of anamnestic rise could be considered to enhance the sensitivity of tests: through a TST, the injection of mycobacterial antigens (tuberculin) may initiate a boost of the immune response of host and then increase antibody production and re-launch cellular-mediated activity (i.e. IFN production) if the subject was previously exposed to mycobacterial infection. This ‘booster effect’ has been noticed in several studies in both the humoral and the cellular response of cattle (Palmer et al., 2006) and in a humoral response of elephants (W. Shaftenaar, pers. comm.). In human medicine, this
phenomenon is even used in a ‘two-step TB Skin Test’ (Menzies et al., 1994; Katial, 2003), with two skin tests performed at a 3 week interval. If the second test is negative, then the subject is considered uninfected; if the second test is positive, then the subject may be considered to have a ‘boosted’ reaction to an infection that occurred a long time ago.

However, in zoo animals, the possibility of producing a false positive because of a PPD injection (e.g. direct immune stimulation of PPD antigens in the absence of any infected background) should remain a real concern. Studies on tuberculin injection effects on the immune response of uninfected animals are under progress (A. Lécu, unpubl. data). Thus, the use of a booster effect should be carefully applied as an ancillary tool and must be interpreted with caution.

CURRENT SCREENING RECOMMENDATIONS

Within some species, current captive populations show more than 10% of infected herds (e.g. elephants; Mikota et al., 2000). To draw a parallel, for a country to keep the TB-free status regarding bovine TB, the OIE (2010a,b) requires that the percentage of herds confirmed infected with *M. bovis* has not exceeded 0·1% per year for three consecutive years.

The entire zoo community should make an effort, even if available tools are still – and will likely remain – unvalidated. Using the three diagnostic modalities (i.e. direct, CMI and serological techniques) available for TB screening in combination for each individual case reduces the limitations of each type of test, reducing the likelihood of false negatives and false positives.

Zoological managers have the duty to ensure the protection of the visitors and staff, as well as welfare of their animal collection. Detecting TB in zoo species has two important aims. The first is to protect staff (keepers, veterinarians) and visitors from zoonotic contamination. Serology and PCR are aimed at this predictive purpose, focusing on the active (i.e. excretion) phase of the disease. The second aim is the detection of latently infected individuals and their monitoring. For this purpose, CMI tests are used. The application of screening methods focuses the attention of all zoo stakeholders and encourages priority monitoring of animals with doubtful results, which is the first essential step to preventing the silent extension of TB within such a multispecific animal population.

In the United States, a *Tuberculosis Surveillance Plan for Non-Domestic Hoofstock* has been developed by the National Tuberculosis Working Group for Zoo and Wildlife Species (2001). This group identified the following guidelines to prevent the transmission of TB between institutions. First, identify the TB status of the sending institution. Avoid animal exchanges with facilities that do not have a TB surveillance plan in place. Maintain farm animals separate from non-domestic ungulates in the collection. The transfer of domestic livestock from zoos to farms should be avoided when possible to minimize the potential risk of transmission from the zoo to the livestock industry, as is the case between wildlife and domestic stock (Artois et al., 2004; Wilson et al., 2009). An institutional surveillance plan should be developed and instituted. Pre-employment and annual tuberculin testing are recommended for employees. All animals that are shipped should be tuberculin tested on the premises of the sending institution as part of pre-shipment clinical examination. Tuberculin tests should be read by palpation. Test results should be interpreted taking into consideration the history of the individual animal enclosure, the previous movement history of the animal and the TB risk status of the institution. All animals should have a permanent identification (transponder or tattoo). Ungulates should be quarantined upon arrival according to the guidelines established by AZA (Quarantine Procedures Recommended for AZA Accredited Institutions: R. E. Miller, 1995) and AAZV (Preventive Medicine Recommendations: Junge, 1995). Animals not screened for TB before shipment should be tested during quarantine at the receiving institution.

Immunological *in vitro* tests are often the easiest to perform because they only require a
blood sample but any repeated positive outcome in this immunological investigation should trigger a secondary confirmation test that uses direct techniques, in order to determine the shedding status of the animal (Fig. 1). If direct examination is positive, measures should be taken to avoid contamination of staff, surrounding animals and premises. However, it should be considered that in a known outbreak of TB (i.e. prevalence is higher), the positive predictive value of testing is increasing and then a positive result may be sufficient when used in conjunction with current history to confirm TB-infected status.

Management considerations for positive or suspected animals

The choice for euthanasia or treatment should be considered carefully in concordance with veterinary, animal management, occupational physician, government officials and the respective studbooks of the species involved [e.g. EEP, Species Survival Plan (SSP), TAG].

Treating an animal for TB implies following strict rules of drug administration, pharmacokinetic controls, observance and excretion follow-up, which are very difficult to deal with. Zoos that engage in treatment must strictly comply with these rules and seek permission for treatment from the official authorities. When considering treatment options, a collection must be aware of the potential sequela resulting from a failure in treatment and the production of resistant strains of mycobacteria (Lyashchenko et al., 2006). It is important to consider that the result of a successful TB treatment is rarely eradication, but bringing the animal back to a latent stage, which means that reactivation will always be a possibility at any stage of its life when treatment is discontinued. Thus, treated animals should be monitored closely for the rest of their life. It could be recommended that treated animals remain in the premises where they have been treated or are transferred to an institution with at least the same level of monitoring abilities.

It is recommended that any animal with a culture positive for *M. tuberculosis* or *M. bovis* should be euthanized owing to the potential for zoonotic transmission, transmission to conspecifics and the documented development of MDR-TB. Treatment might be encouraged in the case of sero-positive animals or other ancillary diagnostics, but only if the clinical evidence supports the potential for latent TB without any excretion.

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**Fig. 1.** Any positive results from boxes 1, 2-1, 2-2 or 3 should trigger the use of an ancillary test from another box to complete the testing process: CMI, cell mediated immunity; ELISA, enzyme-linked immunosorbent assay; IFNg, interferon-γ; LPA, lymphocyte proliferation assay; MAPIA, multi-antigen print immunoassay; PCR, polymerase chain reaction; TST, tuberculin skin test; ZN, Ziehl–Neelsen stain.
NTM
There are currently over 140 species of NTM, which are a widely diverse group of organisms with a broad spectrum of virulence and potential for causing disease in humans and animals. The NTM share an important characteristic in that they are all found in some niche in the environment. Zoonotic risk is, therefore, not as great with NTMs as animals and humans may have an equal opportunity for environmental exposure. The notable exception is in a post-mortem examination of an animal that has disseminated NTM disease, allowing potential exposure of the pathologist to unusually high levels of mycobacteria. Diagnostically, this proves challenging when mycobacterial disease is suspected and an NTM is cultured.

MAC consists of two major groups of slow-growing acid-fast bacilli: M. avium and Mycobacterium avium intracellulare. The route of infection is typically via the gastrointestinal tract and it is typically shed via faeces and will contaminate soil. MAC has produced disease in numerous species of birds and mammals, and is an important pathogen for respiratory disease in humans (Griffith, 2010). Captive marsupials seem to be pre-disposed to infection more than their wild counterparts and disease is typically from MAC. Clinical disease often manifests itself as osteomyelitis (Richardson & Read, 1986; Bush et al., 1995). Marsupials appear to be highly susceptible to specific mycobacterial infections that may be linked to deficiencies in their cellular immunity. Histopathological inspection of affected tissues indicates that, unlike most eutherians, marsupials are unable to wall off infection sites, resulting in the formation of satellite lesions and generalized disease (Buddle & Young, 2000).

Johne’s disease is an infectious disease of ruminants caused by Mycobacterium avium paratuberculosis (MAP). The organism primarily affects the small intestines, resulting in poor digestion, diarrhoea and chronic weight loss. Johne’s disease has been diagnosed in captive non-domestic hoofstock and in free-ranging wildlife. The Johne’s Testing Center at the University of Wisconsin reports isolating the organism from 9.1% of US zoos submitting faecal samples in a 2 year period (1998–2000). Diagnosis in non-domestic hoofstock is complicated by the fact that serologic assays have not been validated in these species and, therefore, culture is still the standard.

There are NTM species, such as Mycobacterium kansasi and Mycobacterium szulgai, that are almost always associated with significant disease when isolated from respiratory specimens in humans (Griffith, 2010) and have been recognized recently as significant pathogens in zoological collections. Mycobacterium kansasi is particularly problematic in that it closely resembles M. bovis infection in ruminants. Disease from this organism has been reported in Bontebok Damaliscus pygargus (Terrell et al., 2009) and llama (Johnson et al., 1993), and has been seen in Cape buffalo Syncerus caffer and Addax Addax nasomaculatus (R. Ball, unpubl. data). Western blot analysis was useful in the case of the Addax to differentiate it from M. bovis. Mycobacterium szulgai was recently implicated as the cause of osteoarthritis and pneumonia in African elephants (C. Lacasse et al., 2007), but could also create severe liver lesions in amphibians (Chai et al., 2006) and pneumonia in crocodiles (Roh et al., 2010).

Mycobacterium marinum is a water-borne atypical Mycobacterium species that commonly infects fish and amphibians. The infection is typically limited to the skin, mostly involving limbs, but spread to deeper structures (joints) has been reported (Petrini, 2006). At least 150 fish and frog species (Talaat et al., 1997), aquatic mammals (e.g. dolphins) and snakes are known to acquire natural M. marinum infection. Individuals who fish or work with aquariums are at an increased risk of exposure (A. Lacasse et al., 2009).

One complication of NTMs is the possibility of producing false positives in some TB testing, especially TST, as they share several molecular components with the TB complex mycobacteria. To counter this, some humoral screening tools now use more specific recombinant antigens, and IFN-γ tests now also rely on specific
proteins’ stimulation to differentiate the immune reaction between NTMs and the MTC.

As mentioned previously, orangutans consistently produce false positives on TST and other diagnostics must be utilized to determine the status of mycobacterial infection with regard to the MTB complex. Orangutans are well-known reactors to intradermal skin testing of essentially any tuberculin (Calle et al., 1989; Wells et al., 1990) without any evidence of mycobacterial disease. One consideration for this sensitization is that there is less exposure to the soil-borne organism in the natural history of such arboreal animals and that in managed situations they become sensitized. In the case of the Cape buffalo with M. kansasii mentioned above, TST was positive to bovine tuberculin on one occasion.

CONCLUSION

MTC has existed for more than 40 000 years, and obviously benefits from human and animal dispersion (Wirth et al., 2008). It is now classified as a ‘re-emerging’ disease, with a strong burden in developing countries. Zoo collections should not consider themselves out of reach from the TB threat. In the absence of serious surveillance, MTC mycobacteria are able to spread silently within a zoo as latency forms, multiplying potential sources of excretion for the remaining zoo inhabitants, including visitors and staff. The diagnostic techniques available are not as reliable as stand-alone tools but when used in combination they facilitate a reduction in the risk of keeping or importing infected animals. Long incubation periods and latency of any mycobacterial disease require that surveillance plans are established on a long-term basis in order to be effective.

An important aspect to remember in all mycobacterial infections and diseases is that the host is often in a compromised state; then additional efforts should be directed towards discovering what is allowing opportunistic bacteria to cause disease.

It should be noted that the variety of species in zoos, the apparent naïve status towards mycobacteria for some of them and the diversity of response to mycobacterial infection are powerful sources of knowledge for TB science. A large proportion of published literature on the ‘newest’ TB diagnostic tools research involves wild species, both free ranging and captive, demonstrating that the zoo community is not only following scientific improvements on TB diagnostics but also actively participating in developing them.

PRODUCTS MENTIONED IN THE TEXT

BOVIGAM®: in vitro test for the diagnosis of bovine TB, manufactured by Prionics AG, CH-8952 Schlieren-Zurich, Switzerland.

CERVIGAM®: whole-blood assay for the cytokine (production discontinued), manufactured by Prionics AG, CH-8952 Schlieren-Zurich, Switzerland.

DPP VetTB®: Dual Path Platform, rapid serological assay detecting antibody against mycobacterial antigens MPB83 and ESAT6/CFP10 on two separated lines, manufactured by Chembio, Medford, NY 11763, USA (not available at the time of writing).

ELEPHANT-STATPAK®: rapid serological assay detecting specific antigens to MTC in elephants, manufactured by Chembio, Medford, NY 11763, USA.

GENOQUICK-MTB®: molecular genetic assay for the rapid direct detection of the MTC, manufactured by Hain Lifescience GmbH, Hardwiesenstraße 1, 72147 Nehren, Germany.

PRIMAGAM®: in vitro test for the diagnosis of TB in non-human primates, manufactured by Prionics AG, CH-8952 Schlieren-Zurich, Switzerland.

PRIMATB-STATPAK®: rapid serological assay detecting specific antigens to MTC in elephants, manufactured by Chembio, Medford, NY 11763, USA.

QUANTIFERON GOLD®: whole-blood test for diagnosing MTC in humans, manufactured by Cellestis GmbH, D-64293 Darmstadt, Germany.

T-SPOT TB: whole-blood test for diagnosing MTC in humans, manufactured by Oxford Immunotec, Abindon, Oxfordshire, OX14 4RY, United Kingdom.

TUBERCULIN OT®: prepared from culture filtrates of Mycobacterium tuberculosis (strains Pn, C, and Dt), which are heat-inactivated, manufactured by Symbiotics Corp., 11011 Via Frontera, San Diego, CA 92127, USA.

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