X. TUBERCULOSIS IN ZOO SPECIES: DIAGNOSTIC UPDATE AND MANAGEMENT ISSUES.

EAZWV Tuberculosis Working Group

1. Introduction

One person out of three is currently infected by tuberculosis over the world. More than 2 millions of people die from it every year. Recent raising of Multi Drug Resistance or even Extreme Drug resistance, co-infection with HIV and incidence of non tuberculous mycobacteria (NTM) makes the battle against tuberculosis more difficult: the disease is now placed in the top 3 list by WHO, together with AIDS and Malaria.

Most of the mycobacteria from the 'tuberculosis complex' have the ability to infect wild species, in whom the pathogenesis, receptivity and immune responses vary widely. Genetic key factors (e.g. Interferon Gamma receptor or vitamin D genotypes) are obviously acting towards these differences (SCHLUGER, 2005). Course of disease and occurence of latent infection vs. open disease are variable among species, from extremely sensitive old world monkeys to apparently resistant equids.

Table 1: Tuberculosis complex mycobacteria and their reported hosts.

Mycobacteria of the Tuberculosis complex	Major historical known host or burden	Reported Wild and Zoo Host
M.tuberculosis	Human, Non Human Primates (NHP)	Elephant, NHP, Beisa Oryx, Addax, Goats, Birds, Lowland Tapir, Giraffes, Springboks, mongoose, Rhinoceros
M.bovis	Cattle (+buffalo, Bison)	All ruminants, Badgers, Possums, Meerkats, Big Cats, Canids, Rodents, NHP, Wild boars Elephants, Camelids, Rhnoceros, Onager, Horse, Birds
M.africanum	Human	Cattle, Swine, NHP
M.microti	Vole, Camelids	NW Monkeys, Big Cats
M.pinnipedii	Pinnipeds	Camel, Tapir, Big Cats
M.caprae	Goat, Sheep, Swine	Swine, Cattle Wild Boars, Red & WT deer, Camel, Bison
M.canetii	Human	?
« Dassie bacillus »	Hyraxes	Meerkats

2. European regulation

National programs

Domestic species of cattle in zoos and safari parks should normally be subjected to routine tuberculin testing as often as the indicated testing interval for the area in which the zoo is located.

Zoo species are generally exempted from statutory TB testing and, in any case, there is no recognized, approved screening test for TB in species other than bovines and deer.

Several texts are defining sanitary policy for tuberculosis within country members. The EU policy mainly focuses on the eradication of bovine tuberculosis and is based on two fundamental principles:

1/ The Member States are primarily responsible for the eradication of bovine tuberculosis and may receive community financial support for the eradication program
2/ Eradication of bovine tuberculosis in the EU must be the final target and the Member States must consider eradication as the defined aim.

Hence, most of the EU regulations apply only to *M.bovis*- sometimes *M.tuberculosis* - screening.

Table 2: European legislation concerning animal tuberculosis.

LEGISLATION RELATED TO THE ERADICATION OF BOVINE TUBERCULOSIS
Council Directive 64/432/EEC of 26 June 1964 on animal health problems affecting intra-Community trade in bovine animals and swine
Council Directive 77/391/EEC of 17 May 1977 introducing Community measures for the eradication of brucellosis, tuberculosis and leucosis in cattle
Council Directive 78/52/EEC of 13 December 1977 establishing the Community criteria for national plans for the accelerated eradication of brucellosis, tuberculosis and enzootic leukosis in cattle
Commission Decision 2003/467/EC of 23 June 2003 establishing the official tuberculosis, brucellosis, and enzootic-leukosis-free status of certain Member States as regards bovine herds
Commission Decision 2003/849/EC of 28 November 2003 approving the programmes for the eradication and monitoring of animal diseases and for the prevention of zoonoses presented by the Member States for the year 2004
Council Decision 90/424/EEC of 26 June 1990 on expenditure in the veterinary field
Council Decision 90/638/EEC of 27 November 1990 laying down Community criteria for the eradication and monitoring of certain animal diseases

Based on the Commission Decision 1999/467/EC of 15 July 1999 seven states of the European Union were classified as free of bovine tuberculosis: Denmark, Germany, Luxembourg, the Netherlands, Austria, Finland, and Sweden. In 2004 Belgium, the Czech Republic, and France were added to this group and on 4th March 2005 an eleventh country, Slovakia was added. Switzerland also has the status “free of bovine tuberculosis”.

BALAI application within Europe

The BALAI directive 92/65 requires that all ruminants traded between institutions must come from officially free herds, as it's mentioned in TRACES health certificates.

Moreover, to be BALAI approved, an institution must be free of "bovine tuberculosis", as listed into the Annex A of the directive, for at least three years, or "tuberculosis" (this term includes all mycobacteria of the TB complex) for primates, felidae and ruminants, if the member state has a control monitoring progam. During a transfer between two approved bodies, Tb testing is not required by the BALAI directive, but many member states (UK, Sweden,…) added TB test as an additional requirement when importing ungulate, whatever institutions it is coming from.

Given these requirements, a lot of zoo mammals are still being exchanged in national and international transfers without any serious individual testing.

3. Quick overview of current diagnostic methods

Standard screening tools like skin testing and sputum smears have limited application in wildlife species, especially when prevalence is low. Inversely, investigations of cell-mediated immunity through an vitro assay of gamma interferon have numerous advantages (good sensitivity), as long as technical limits are known and can be improved. Furthermore, new tools based on the investigation of humoral immunity seem very promising for the detection of antibody directed against certain immunogenic mycobacterial antigens in a wide range of species. All these methods are currently evaluated in field studies, despite difficulties to ensure rigorous validation.

Thus, diagnostic methods are hardly homogenic and never validated for any zoo species. Bearing in mind that validation will not easily be possible, zoo actors must be aware of actual tests available.

Limits of unspecific diagnosis

Clinical signs of TB are rarely seen prior to death in zoo animals (LYASCHENKO & AL, 2006, MONTALI & AL, 2001). If cough and dyspnea are noticed, this is always in a very late and irreversible stage of the pulmonary form of disease. The most frequent sign noticed among all mammals is chronic weight loss.

Imagery techniques can help, especially laparoscopy followed by biopsy of suspect granulomas or tissue. A CT scan is useful to detect lesions in non-palpable lymph nodes. On the other hand, though X-rays are a regular step in human diagnostics, it is not practically useful in zoo veterinary medicine: the size of animals may prevent X-R use, references images are often missing, skills required to interpret pictures are often out of "average zoo vet" range and, on top of that, a lot of species do not show any calcified lesions.

Limits of direct examination

Culture stands as the "gold standard" method, but requires at least 2 to 6 weeks delay before a test result can be obtained, even with recent fast techniques (VARGAS & al, 2005) which 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 microscopic exam (Ziehl Neelsen and other stains) is around 10^4 bacilli / ml, which is a rather important and infectious value. In a culture, it is possible to detect bacilli loads superior to 10^1-10^2 / ml. As a comparison, less than 10 colony forming units (CFU) of *M.tuberculosis* per ml is enough to infect a cynomolgus macaque (LIN & al, 2006).

Many biological samples contain very few bacilli (highly calcified lesions, intermittent shedding in biological fluids like bronchial secretions or milk). As an example, 15 to 20% of active pulmonary forms are not even confirmed by any culture in human being (FRIEDEN & al, 2003). Therefore, only positive culture findings provide evidence of disease, whereas negative culture results may not rule out infection in exposed/suspected animals.

Detection of DNA material in biologic samples have been used already in various zoo species: elephant trunk wash (MIKOTA & AL), broncho-alveolar lavage (FLYN & al, 2003), gastric lavage,.. Amplification methods (PCR) are now well developed and help specify the mycobacteria with the use of selected probes. However, some of these biological samples are likely to host many other bacteria that are impairing PCR efficacy. Within the last years, very sensitive and specific PCR became available, but it still can't be used as a broad screening tool as described in table 3.

Table 3: Example of PCR sensitivity in human sputum with 5% prevalence. From (VEZIRIS, pers. com.). With those given sensitivity and specificity, a positive PCR result is meaning an even probability (49%)of TB infected or free animal, mainly because of the low prevalence. The lower the prevalence is, the lower the PPV turns out.

PCR **Se=72%** **Sp= 96%**	Disease (Active TB)	No Disease (Latent or no TB)	Positive predictive value PPV=3.6/(3.6+3.8) **=49%**
Prevalence 5%	5	95	
PCR +	3.6	3.8	Negative predictive value NPV=91.2/(91.2+1.4) **=98%**
PCR -	1.4	91.2	

When there is enough DNA (rich sample or culture), molecular typing should be performed. Spoligotyping or other methods (VNTR,..) allow 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 tuberculosis strains were spoligotyped and their fingerprint can be compared to other circulating strains. Whenever a tuberculosis mycobacterium is discovered, this kind of typing should always be performed, or at least samples must be kept frozen (-25°C or -80°C) until further typing is available.

Cell mediated immunity (CMI) exploration

Memory of T cells regarding previous contact with *Mycobacterium* can be assessed by presenting selected antigen(s) to them, either *in vivo* (skin test or "Mantoux" test) or *in vitro* (Lymphoblastic Transformation Test –LTT- or Interferon Gamma tests).

Skin testing is far from being validated for zoo species, as there are great variations between tegument and dermal structure within species. The test relies on local inflammatory cell recruitment, which can be low or absent for many reasons: tegument cellular organization, immunosuppressive status, superficial temperature, … Skin test sensibility is often poor: e.g 70 to 90% in human, 50 to 90% in zoo hooftstock (COUSINS AND FLORISSON, 2005). Specificity depends on antigen (tuberculin) injected intradermally but could also be low because of species-specific features (e.g. orangutans) or co-infection by other non tuberculous mycobacteria (e.g. *avium* complex), leading to false positive results.

Materials and methods of application of the skin test vary between countries and vets: standardized quality of tuberculin is still missing within EEC, reading methods also vary between vets with a lot of “distant” appreciations of TST local reaction. Whenever possible, a close exam, palpation and caliper measurement of tegument is strongly recommended in order to avoid false negative results.

In vitro tests of the cellular immunity rely on (re)stimulation of T lymphocytes memory. Thus, first compulsory step is to keep cells alive until they reach the lab. Blood sample should reach lab for stimulation no later then 8-10h after collection and should be kept at ambient temperature until there. Particular care must be paid to homogenic and full mixing of blood and anticoagluant (heparine) at collection.

Sometimes part of experimental study, LTT is not a current option to a zoo vet because of its limited reproducibility and the use of radio-elements. Gamma interferon (IFNg) tests stand as a good option as they are already available and marketed for cattle, non human primates, deer and humans. These tests are performed in two steps, for a total run of 48h minimum. The first step is to incubate T cells with selected mycobacterial antigen (usually bovine and avium ppd), leading them to produce IFNg when they were previously in contact with the same antigen.. The second step is to reveal the amount of IFNg produced through an ELISA or an ELISPOT (more sensible). This step can be delayed as soon as stimulation wells have been frozen.

Commercial kits are available, designed for cattle (BOVIGAM®), primates (PRIMAGAM®), deer (CERVIGAM®, but production is discontinued at this time) and human (QUANTIFERON GOLD®). Recent studies show that these tests can be used in some exotic species : for example a cattle-designed test will detect INFg of a large range of exotic Bovidae, but also some animals standing outside of this family (e.g. Girafidae). On the other hand, other artiodacyl species just don’t do well with the usual positive control antigens, or are not detected by ELISA (RIQUELME, 2009). This also counts for the primate-referred test: although a list of “validated” species is provided on its leaflet, field studies showed contrasted results (LÉCU, 2008, RIQUELME, 2009).

In order to overcome the problem of specificity in ELISA detection of INFg, in-house modified tests can be created. One solution is the detection of mRNA coding for INFg, which seems to have broad nucleotid sequences, shared by a lot of different mammal species. This can only be achieved by experienced laboratories. Another solution is to design a specific interferon antibody, as is currently being developed in elephants and rhinoceros.

Whatever kind of cellular exploration is chosen, the duration of the cell-mediated immunity is rather unknown. All studies concerning longest periods of time (VERVENNE & AL, 2004) and work on BCG vaccination protection length suggest that stimulation tends to go back to a baseline level, which remains unknown beyond a year.

Humoral immunity exploration

Serodiagniosis of tuberculosis suffered from “bad reputation” because early trials were assessing antibody against broad mycobacterial antigens, showing a very low specificity in these tests. During the course of an infection, Th1 activity (CMI pathway) is thought to be initially greater than Th2 (humoral route) in order to control and confine infection. An inversion of this Th1/Th2 balance control is often associated with a relapse or with active disease (DOHERTY & ROOK, 2006). Thus, seeking for antibodies maybe of little help to screen for latent infected animals, but is becoming more relevant to detect and monitor more active, “sick” and shedding individuals. It has been clearly noticed also in human (ABEBE & al, 2007) that some antibody rise occurs during the shift from latent to active disease, so that a prognostic value may be added to certain serological results

Some mycobacterial antigens elicit humoral response in a wide range of species. Most relevant antigens have been selected to design ELISA and Rapid Lateral Flow Technology test, with good results in a lot of various species (elephants, primates, tapirs, camels, deer,…). However, the panels of immunostimulant antigen will remain directly linked to the type of mycobacterium involved, host species, timeframe of disease and also individual condition. Some commercial tests validated for Asian and African elephants (ElephantTB STATPAK®, DPP® VetTB) or primates (PrimaTB STATPAK®) also look promising in non target species (GREENWALD ET AL. 2009; LYASHCHENKO ET AL. 2008).

Sensibility (Se) and specificity (Sp) of these serological assays are encouraging. However, Se and Sp values are 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, maybe jeopardizing the real Se and Sp. Moreover, serological profiles of animals in long-term quiescent infection status are still remaining poorly known.

Repeated testing seems to be the only way to adapt with these actual limitations, as titers is thought to arise when relapse is about to happen. As zoonotic risk increases a lot within this shifting time, detection of early serological change may be relevant to trigger a deeper screening (direct exam) and preventive measures towards staff and public.

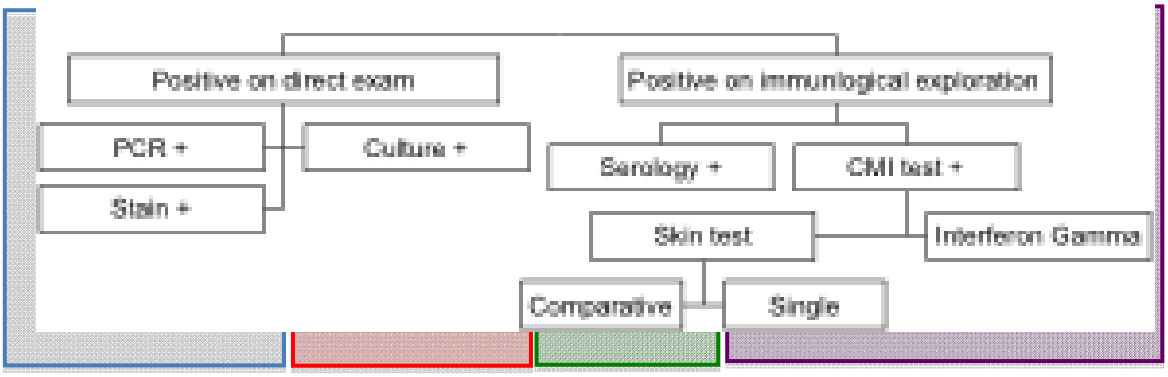
4. Current recommendations

Within some species, current captive population shows more than 10% of infected herds. To draw a parallel, for a country to keep the TB-free status regarding bovine tuberculosis, the OIE requires that the percentage of herds confirmed infected with *M. bovis* has not exceeded 0.1% per year for 3 consecutive years.

All the zoo community should make an effort, even if available tools are still – and will likely remain - unvalidated. Crossing the three exploration paths (direct, CMI and serology) is a good way to partially get rid of some of their respective limitations.

The aim of detecting TB in zoo species has two levels: the first one is to protect staff (keepers, vet) and public from zoonotic contamination. Few actual tools (serology, PCR) are aimed at this predictive purpose, focusing on the active (=excretion) phase of the disease. The second level is the census of latently infected individuals and their monitoring. For this purpose, CMI testing can then help a lot, as long as their sensibility / specificity limits are taken into account.

Table 4: Testing categories available. A positive test from one of these boxes should always initiate a test from another box.



Any repeated positive outcome in the immunological investigationary tests should trigger exploration of the direct exam category, in order to determine the shedding status of the animal. If direct exam is positive, measures should be taken to avoid contamination of staff, surrounding animals and premises.

"Euthanasia or treatment" is a choice that should be considered first with veterinary officials, occupational physician, all related actors and then according to global captive population status and dynamics (EEP, TAG, …). Depending on TB extension analysis, treatment may be an alternative to euthanasia, to prevent infected animals from shedding mycobacteria. Treating an animal for TB implies following strict rules of drug administration, pharmacokinetic check, observance and excretion follow-up, that are very hard to deal with. Zoos who engage in treatment must stick to these rules and require permission for treatment from the official authorities. It must be borne in mind that failure in the treatment could lead to emergence of resistant strain as it was already noticed (LYASHCHENKO ET AL, 2006).

The effect of a successful treatment is to bring the animal back to a latent stage, which means that reactivation will always be possible in any stages 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.

An updated database –working group leaded- is under progress, in order to gather and share information on tests, categorized by zoo species. The feedback of test results is of primary concern, as well as follow-up of suspect populations and fingerprinting of circulating strains. Zoo vets, TAG & EEP advisor must arouse this interest within their relative animal & human population. It's not acceptable anymore to exchange animals without any medical history including TB.

Table 5: Current TB risk and monitoring levels, and methods of diagnostic available for relevant selected species in zoo. Refer to Annex 3 for more specific recommendations when existing.

Species (G): see TB guidelines in Annex 3	**Risk - Recommended monitoring level**	**Recommended methods**
Bovidae	Depends of status of surrounding animals, and above all, contact with feral animals. Maintenance monitoring = testing on occasions: exchange, anesthesia – if purpose doesn't interfere with TST (infection, use of AI drugs,…)	• Skin test: 0.1ml of bovine PPD, 1mg/ml or at least 2000 UI/test injected intradermally into a shaved cervical area. Reading at 24, 48 and 72h includes palpation and measuring the skin thickness with an appropriate caliper. Caudal tail fold less appropriate to wild species. • Bovigam® : blood should be tested within 8h after sampling. Refer to literature for adjusting TST and elicit booster effect. • Serological test
Primates **(G)**	Old world monkeys are highly susceptible to infection, new world monkeys are fairly resistant but able to develop TB. OIE recommends quarantine on recently imported	• Skin test with 0.1ml bovine ppd (at least 2000 IU/test, 1000 to 10000 times greater than human) into eyelid or abdomen skin. Reading at 24, 48 and 72h. Old mammalian tuberculin may be used to increase sensibility but it decreases specificity

	primates with 2 to 3 skin test at a 2-week intervals.	• Primagam® : whole blood or white cells should be stimulated 8h max. after sampling. Refer to literature for validated or successful species. • RapidTest (PrimaTB STATPAK®) on serum
Carnivores	Not a standard test to perform except when there is a risk of exposure (Tb cases in zoo or fed with suspect carcass)	• Comparative cervical test(CCT) maybe used • Serological assays looks promising (ELISA and RapidTest) • In-house interferon test could be used (badgers), see references.
Sealions **(G)**	Some South American sealions (*O.byronia*) infected with *M.pinnipedii*. Likely to come from imported animals. Transmission to other species of pinnipeds (seals, Californian sealion) and terrestrial mammals occurred. Test should be required for any trade or as soon as animals are trained enough.	• Skin test with 0.1ml bovine ppd in cervical area, up the shoulder. • RapidTest (ElephantTB STATPAK®) : prefer serum to whole blood • ELISA on serum • Stains, PCR and culture of any nasal discharge
Camelids	As bovids	• Skint test as indicated for bovids but one has to be aware that the percentage of false positive and false negative is much higher than in bovids • Serologic tests (ELISA, ElephantTB STAT PAK)
Tapirs (G)	Malayan tapirs are most at risk with a not yet defined proportion of captive population infected.	• TST or Comparative skin test can be performed with 5000 IU=0.1ml bovine ppd tuberculine and 2000 IU avian ppd tuberculine injected in the inguinal area. Non specific reactions are not uncommon • Serological test showed good results
Elephant (G)	Infected Asian elephants are still detected.	• Serological tests show the best sensibility and specificity : ELISA, ElephantTB STATPAK® and DPP®VetTB
Deers	Clusters of infected feral deer remain in Europe.	• Comparative Cervical Test with avian and bovine ppd (*M.avium* infection is not rare in cervids) • Serologic tests (ELISA, CerivdTB STAT PAK®, DPP®VetTB)
Rhinoceros	M.tuberculosis infection from infected human reported in black rhino M.bovis infected white and black rhino reported in zoos.	• Caudal Fold skin test • Gastric lavage with culture and PCR • Serologic tests (ELISA, CerivdTB STAT PAK®, DPP®VetTB)

Annex 1

References

ABEBE, F., HOLM-HANSEN, C., WIKER, H.G., BJUNE, G. 2007. Progress of Serodiagnosis of Mycobacterium tuberculosis infection. Scandinavian Journal of Immunology 66: 176-191

ARTOIS, M., LOUKIADIS, E., GARIN-BASTUJI, B., THOREL, M.F., HARS, J. 2004. Infection des mammifères sauvages par Mycobacterium bovis, risques de transmission aux bovines domestiques. Bulletin Epidémiologique de l'AFFSA 13: 1-3

CHAPEL, H., HAENEY, M., MISBAH, S., SNOWDEN, N. 2006. Essentials of Clinical Immunology, 5th Edition. Blackwell Publishing, Oxford. 368pp.

CLIFTON-HADLEY, R.S.,SUATER-LOUIS, C.M., LUGTON, I.W., JACKSON, R., DURR, P.A., WILESMITH, J.W. 2001. Mycobacterium bovis infections. In Infectious diseases of wild mammals (ed E.S. Williams & I.K.Barker). pp 340-361. Iowa State Unisversity Press.

COOK RA (1993): Review and update on USDA Cervid TB regulations and their effects on AAZPA accredited zoos. Proc.Am.Assoc.Zoo.Vet., 205-207.

COUSINS, N.V. & FLORISSON N. 2005. A review of tests available for use diagnosis of tuberculosis in non-bovine species. Rev sci tech Off int Epiz. 24 (3): 1039-1059.

CRANFIELD, M.R., THOEN, C.O., KEMPSKE, S. 1990. An outbreak of Mycobacterium bovis infection in hoofstock at the Baltimore Zoo. Proceedings of the Annual Meeting of the American Association of Zoo Veterinarians, Denver, Colorado, October 21-26 1990 : 128-137.

DOHERTY, T.M. & ROOK, G. 2006. Progress and hindrances in tuberculosis vaccine developpement. Lancet 367: 947-949.

FITZGERALD, S.D., KANEENE, J.B., BUTLER, K.L. & al. 2000. Comparison of post mortem techniques for the detection of *Mycobacterium bovis* in white tailed deer (*Odocoileus vigrinianus*). J Vet DIagn Invest 12(4): 322-327.

FLYNN, J.L., CAPUANOA, S.V., CROIXA, D., PAWARA, S., MYERSA, A., ZINOVIKA, A., KLEINB, E. 2003. Non-human primates: a model for tuberculosis research. Proceedings from the 4th World Congress on Tuberculosis. Tuberculosis 83(1-3): 116-118.

FRIEDEN, T.R., STERLING, T.R., MUNSIFF, S.S., WATT, C.J., DYE, C. 2003. Tuberculosis. Lancet 362: 887-899.

GREENWALD, R., LYASHCHENKO, 0., ESFANDIARI, J., MILLER, M. MIKOTA, S., OLSEN, J.H, BALL, R., DUMONCEAUX, G., SCHMITT, D. MOLLER, T., PAYEUR, J.B., HARRIS, B., SOFRANKO, D., WATERS, R. AND LYASHCHENKO, K.P. 2009. Highly Accurate Antibody Assays for Early and Rapid Detection of Tuberculosis in African and Asian Elephants. Clin. Vaccine Immunol.; 16(5) : 605-612.

GROBLER D.G., MICHEL A.L., DE KLERK L.M., BENGIS R.G. 2002. The gamma-interferon test: its usefulness in a bovine tuberculosis survey in African buffaloes (*Syncerus caffer*) in the Kruger National Park. Onderstepoort J Vet Res. 69(3):221-227.

HAAGSMA, J. & EGER, A. 1990. ELISA for diagnosis of Tuberculosis and Chemotherapy in zoo and wildlife animals. Proceedings of the Annual Meeting of the American Association of Zoo Veterinarians, Denver, Colorado, October 21-26 1990: 107-110.

JURCYNSKI, K., LYASHCHENKO, K., GOMIS, D., LÉCU, A., TORSTCHANOFF, S., KLARENBEEK, A., MOSER, I. 2007. Mycobacterium pinnipedii infection is South American sea lions (*Otaria byronia*) in Europe. Proceeding 43rd Internationalen

Symposiums über die Erkankungen des Zoo un Wildtiere, Edinburgh 2007 : 180.

KIERS A, KLARENBEEK A, MENDELTS B, VAN SOOLINGEN D, KOËTER G. 2008. Transmission of *Mycobacterium pinnipedii* to humans in a zoo with marine mammals. Int J Tuberc Lung Dis. 12(12): 1469-73.

LECU, A. & RIQUELME, L. 2008. Evolutions des outils diagnostiques de la tuberculose des éspèces sauvages. Bulletin de l'académie vétérinaire de France, 161 (2) : 151-158.

LEWERIN,S.S., OLSSON,S.L., ELD,K., ROKEN,B., GHEBREMICHAEL,S., KOIVULA,T., KALLENIUS,G., BOLSKE,G., 2005. Outbreak of Mycobacterium tuberculosis infection among captive Asian elephants in a Swedish zoo. Vet Rec. 156:171-175.

LIN, P.L., PAWAR, S., MYERS, A., PEGU, A., FUHRMAN, C., REINHART, T.A., CAPUANO, S.V., KLEIN, E., FLYNN, J.L. 2006. Early Events in Mycobacterium tuberculosis Infection in Cynomolgus Macaques. Infection and Immunity 74 (7): 3790–3803.

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. 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.

LYASHCHENKO, K.P., GREENWALD, R., ESFANDIARI, J., GREENWALD, D., NACY, C.A., GIBSON, S., DIDIER, P.J., WASHINGTON, M., SZCZERBA, P., MOTZEL, S. & AL. 2007. PrimaTB STAT-PAK Assay, a Novel, Rapid Lateral-Flow Test for Tuberculosis in Nonhuman Primates. Clin. Vaccine Immunol 14(9): 1158-1164.

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(1-2): 91-100.

LYASHCHENKO K.P., GREENWALD, R., ESFANDIARI, J., CHAMBERS, M.A., VICENTE, J., GORTAZAR, C., SANTOS, N., CORREIA-NEVES, M., BUDDLE, B.M., JACKSON, R., O'BRIEN, D.J., SCHMITT, S., PALMER M.V., DELAHAY, R.J., WATERS, W.R. 2008. Animal-side serologic assay for rapid detection of Mycobacterium bovis infection in multiple species of free-ranging wildlife. Vet Microbiol.;132(3-4): 283-92.

MIKOTA SK,LARSEN RS, MONTALI RJ. 2000. Tuberculosis in Elephants in North America. Zoo Biology **19**, 393-403.

MILLER, M. 2008. Current diagnostic methods for tuberculosis in Zoo Animals. In Fowler, M.E & MILLER, E.R. 2008. Zoo and Wildlife Medicine, current therapy, volume 6. Saunders Elsevier Eds. Pp 10-19.

MONTALI, R.J., MIKOTA, S.K., CHENG, L.I. 2001. Mycobacterium tuberculosis in zoo and wildlife species. Rev sci tech Off int Epiz. 20(1): 291-303.

MORAR, D., TIJHAARB, E., NEGREA, A., HENDRIKS, J., VAN HAARLEM, D., GODFROID, J., MICHEL, A.L., RUTTEN V.P.M.G. 2007. Cloning, sequencing and expression of white rhinoceros (Ceratotherium simum) interferon-gamma (IFN-γ) and the production of rhinoceros IFN-γ specific antibodies. Veterinary Immunology and Immunopathology. 115 (1-2): 146-154.

MOSTOWY, S., INWALD, J., GORDON, S., MARTIN, C., WARREN, R., KREMER, K., COUSINS, D., BEHR, M.A. 2005. Revisiting the Evolution of Mycobacterium bovis. J Bacteriol. 187(18): 6386–6395.

OIE. 2007.: Terrestrial Animal health Code. Chapter 3.2.2. Bovine Tuberculosis.

RIQUELME, L. 2009. La tuberculose chez les espèces sauvages captives : possibilité d'utilisation du test interferon gamma pour son diagnostic ante-mortem. Thèse Med. Vet.

Alfort; n°35, 239p.

ROBBE-AUSTERMAN, S., KRULL, A.C., STABEL, J.R. 2006. Time delay, temperature effects and assessment of positive controls on whole blood for the gamma interferon ELISA to detect paratuberculosis. J.Vet.Med. B 53: 213-217.

SCHLUGER, N.W. 2005. The pathogenesis of tuberculosis: the first one hundred (and twenty-three) years. Am J Respir Cell Mol Biol. 32(4): 251-256.

SOCKETT DC (1993): *Mycobacterium bovis* infection in U.S. Deer and Elk Farms. Proc.Am.Assoc.Zoo.Vet., 198-201.

THOEN CO, STEELE JH, GILSDORF MJ (2006): *Mycobacterium bovis* infection in animals and humans. 2nd Edition. Blackwell Publishing. 329pp.

VARGAS D, GARCÍA L, GILMAN RH, EVANS C, TICONA E, ÑAVINCOPA M, LUO RF, CAVIEDES L, HONG C, ESCOMBE R, MOORE DAJ. 2005. Diagnosis of sputum-scarce HIV-associated pulmonary tuberculosis in Lima, Peru. Lancet **365,** 150–52

VERVENNE RAW, JONES SL, VAN SOOLINGEN D, VAN DER LAAN T, ANDERSEN P, HEIDT PJ, THOMAS AW, LANGERMANS JAM. 2004. TB diagnosis in non-human primates: comparison of two interferon-g assays and the skin test for identification of *Mycobacterium tuberculosis* infection. Veterinary Immunology and Immunopathology **100**, 61–71

WOOD, P. R. & JONES, S. L. 2001. BOVIGAM® : an in vitro cellular diagnostic test for bovine tuberculosis. Tuberculosis 81(1-2): 147-155.

Annex 2

List of National Laboratory regarding Mycobacteria diagnostic.

VLA Weybridge
New Haw, Addlestone, Surrey KT15 3NB
UNITED KINGDOM
Tel: (44.1932) 34.11.11 Fax: (44.1932) 34.70.46
Glyn Hewinson
Email: r.g.hewinson@vla.defra.gsi.gov.uk

AFSSA Alfort
Unité Zoonoses Bactériennes
23 avenue du Général de Gaulle, 94706 Maisons-Alfort Cedex
FRANCE
Tel: (33 (0)1) 49.77.13.00 Fax: (33 (0)1) 49.77.13.44
María Laura Boschiroli-Cara
Email: ml.boschiroli@afssa.fr

Veterinary Research Institute
Hudcova 70, 62132 Brno
CZECH (Rep.)
Tel: (420.5) 33.33.16.01 Fax: (420.5) 33.33.12.29
I. Pavlik
Email: pavlik@vri.cz

VISAVET
Laboratorio de vigilancia veterinaria, Facultad de Veterinaria, Universidad Complutense de Madrid
Avda. Puerta de Hierro, s/n. Ciudad Universitaria
28040. Madrid
SPAIN
J.M. Vizcaino
Email: jmvizcaino@visavet.ucm.es
Community reference laboratory for bovine tuberculosis from July 2008 until July 2013.

Friedrich-Loeffler-Institute
Federal Research Institute for Animal Health
Suedufer 10
D-17493 Greifswald - Insel Riems
GERMANY
Phone +49 38351 7-0
Fax +49 38351 7-219
Dr Irmgard Moser
Email : irmgard.moser@fli.bund.de

Central Veterinary Institute - Lelystad
Edelhertweg 15
8203 AA Lelystad
THE NETHERLANDS
Phone: +31 320 238159
Fax: +31 320 238050
Douwe Bakker

Email : d.bakker@wur.nl

National Veterinary Institute (SVA)
Department of Bacteriology
Section of Mycoplasma and Mycobacteria Diagnosis
S-Uppsala
SWEDEN
Göran BÖLSKE
E-mail: goran.bolske@sva.se

Institut National des Recherches Veterinaires (INRV)
Laboratory of Tuberculosis
B-Brussel
BELGIUM
Jacques Godfroid
E-mail: jagod@var.fgov.be

Laboratorio Nacional de Investigaçao Veterinaria
Ministerio de Agricultura, do Desenvolvimento Rural e das Pescas
Estrada de Benfica 701
1500 Lisboa
PORTUGAL
Tel. 351 1 7115200
Fax. 351 1 7115383
Alicia Amado
Email : alice.amado@lniv.min-agricultura.pt

Department of Bacteriology
National Veterinary and Food Research Institute
Hämeentie 57
Helsinki
FINLAND
Tel. 358 9 393 18 26
Fax. 358 9 393 18 11
Sinikka Pelkonnen
Email : sinikka.pelkonen@eela.fi

Sections of Bacteriology, Virology and Serology, and Pathology
National Veterinary Institute
8156 Dep
N-0033, Oslo
NORWAY
Tel. 47 22 96 46 75
Fax. 47 22 60 09 81
Oeivind Oedegaard
Email : oivind.odegaard@vetinst.no

Institute of Veterinary Research of Thesaloniki
80, 26th October Street
Thessaloniki
GREECE
Tel. 30 31 55 20 28
Fax. 30 31 55 40 22
Zoi Dimareli-Malli

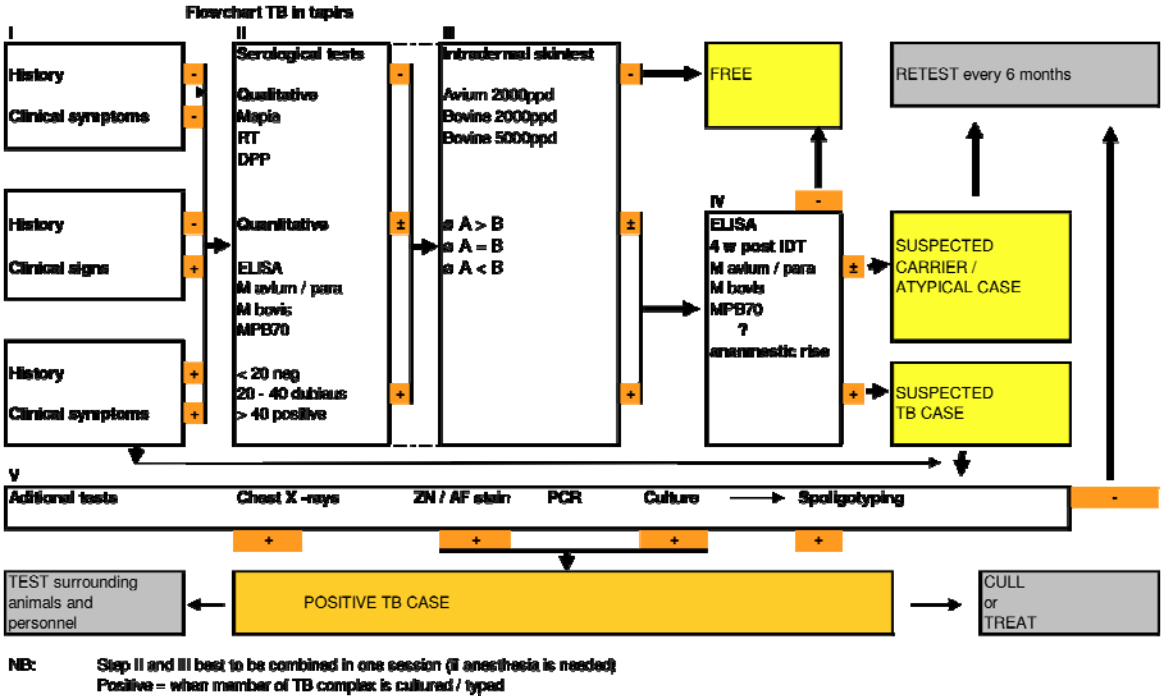
Email : vetresi@the.forthnet.gr

EAZWV Working Group members

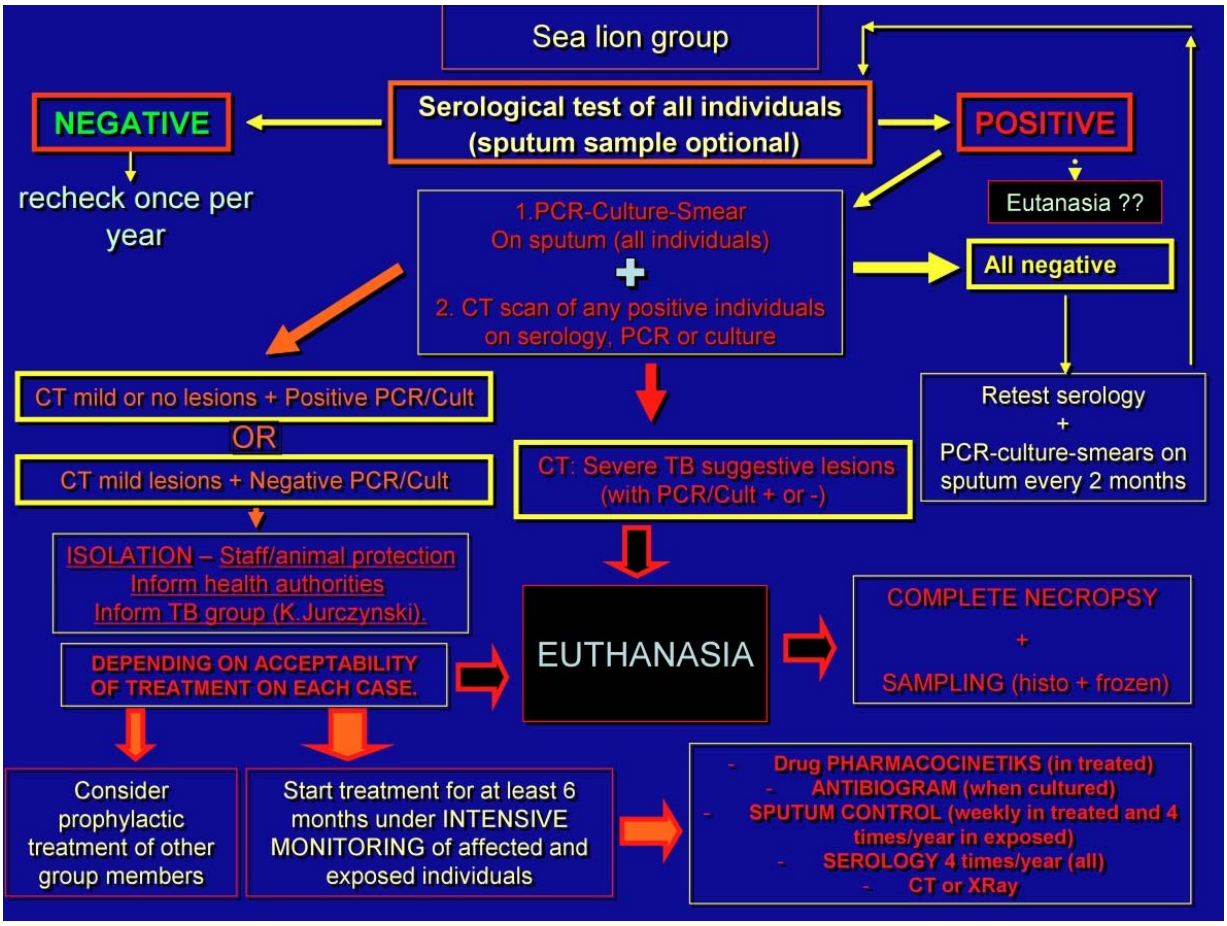
Annex 3

Tuberculosis guidelines for some species

Malayan Tapirs *(Tapirus indicus),* from M.Hoyer 2009.



South American Sea Lions *(Otaria byronia)* issued from the 1st Sealion TB Meeting, Duisburg, Sept. 2009. Contact



Elephants *(Elephas maximus)*

W.Shaftenaar, vet advisor of Elephant EEP
26 June 2009

These recommendations for the control of tuberculosis in captive Asian en African elephants are aimed to prevent the spread of Mycobacterium spp. that can cause tuberculosis in mammals. The recommendations are based on the document "TB testing in captive elephants
in the EEP, 23 July 2008", (see annex to this document and are reflecting the current possibilities for testing within Europe. The document will be updated when new relevant developments become available.
The interpretation of the available diagnostic tests is under constant evaluation and the panel of experts involved in TB-testing in elephants in recent years will be consulted when questions arise.

Glossery
Antibody test (serum or plasma): ELISA. At present, the Central Veterinary Institute Lelystad is the only institute in Europe running this ELISA on a routine base.
Antigens used: M.bovis, MPB70 and M.paratuberculosis.
Address:
Central Veterinary Institute,
DSU
Edelhertweg 15,
8219 PH Lelystad,
the Netherlands

Elephant TB STAT-PAK Assay: Also known as ERT. Test to be performed by a qualified zoo veterinarian or veterinary institute.
The test is available through the following website: www.zootest.com
Culture of suspected material to be sent to: National Veterinary Laboratory
Tuberculin to be obtained from: National Veterinary Institute
Trunk wash for culture and/or PCR See definition in the annex

How to act when an elephant has to be transported from or to a zoo that participates in the elephant EEP?

Before the transport of an elephant to or from an institute that participates in the elephant-EEP, the veterinary advisor to the elephant TAG must be notified. Together with the institute that is sending the animal as well as the receiving institute, the veterinary advisor will propose the measures to be taken in order to reduce the risk of transmission of tuberculosis. In case of disagreement, the studbook coordinator will be informed and the EAZA veterinary committee will be consulted if the parties involved do not resolve these issues. The advice of the veterinary advisor is always subject to the (inter-)national veterinary legislation applicable in the countries involved at the time of shipment. The veterinary advisor will base his advice on the pre-transport screening and specific test results as described below.

Pre-transport screening

A report about the history of the animal should be made, including the following data:
- Species / ID / date of birth / location of birth
- Locations where the animal has been kept during its entire life, including dates of entry in any new location

- Has the animal previously been suspected of tuberculosis or treated for this disease?
- Has there been any known direct or indirect contact with confirmed or suspected TB-cases in herd mates or other mammalian species, including humans?
- List of data when blood was sampled, tested and stored at below -20°C
- Test results of any TB-test performed in the animal in the past
- Does the animal show clinical signs that are suggestive for tuberculosis?

Specific tests

The animal(s) that will be transported must be tested 4-6 weeks prior to transport for the presence of specific antibodies using the following tests: ELISA's and Elephant TB STAT-PAK Assay (ERT). Serum or heparin plasma of the elephant(s) must be shipped on dry ice to the Central Veterinary Institute (CVI) in Lelystad to be tested in the ELISA's that are routinely used for elephants. The Elephant TB STAT-PAK Assay will also be performed at the CVI-Lelystad. Costs for these tests will be covered by the sender of the samples.

On the same day when the blood sample is taken, a comparative skin test will be done: 2000 IU of ppd avian tuberculin* will be injected intracutaneously in a thin part of the skin on the base of the backside of the ear. At the same time 3000-5000 IU of ppd bovine tuberculin* will be injected intracutaneously on the contra-lateral ear at a similar location. If only one ear can be used, both injections should be given at a minimum distance of 10 cm from each other.
** Standardization of the quality of the tuberculin is of outmost importance. In case of any doubt, the tuberculin can be provided by the veterinary advisor for the elephant TAG.*

The results of all tests and the anamnesis data will be evaluated by the zoo vet and the veterinary advisor of the elephant-TAG. The latter will write his advice to the EEP studbook-coordinator.

What to do when an elephant is suspected of tuberculosis?

An elephant can become suspected of tuberculosis for different reasons. The anamnesis may contain certain data that raise suspicion or one or more tests may give a positive result. When this is the case, the veterinary advisor will consult international experts in the field of tuberculosis and may propose additional tests, which may include: repeated antibody tests (ELISA, ERT), additional intradermal tuberculin test, trunk wash and other tests available at that particular moment). Attempts will also be made to send a serum sample to the USA to perform a Multi Antigen Print Immuno Assay. It is the responsibility of the zoo veterinarian to consult the local veterinary authorities about the situation.

Willem Schaftenaar, DVM
Veterinary advisor to the elephant TAG
Rotterdam zoo
PO box 532
3000 AM Rotterdam, the Netherlands
Tel: +31 10 4431 485
Fax: +31 10 4431 414
E-mail: W.Schaftenaar@Rotterdamzoo.nl

TB testing in captive elephants in the EEP
23 July 2008

TB testing in elephants is a concern for all elephant keeping institutions. In Europe, no validated tests for elephants, approved by EU-authorities or any other European country, are available at this time. However, veterinary authorities often request elephants to be tested on TB when they are moved to other countries. Zoos that receive elephants must be well aware

of the risk of the import of TB in their collection. This makes it very important to build up a well documented history of elephant herds, including regular testing and monitoring other animals and personnel.

In the EAZWV-recommendations for approval of zoos according to the EU Directive 92/65, the trunk wash procedure for regular testing on tuberculosis (TB) has been discussed. In the final recommendations, a more general text was chosen (see point C.5.h.): “Specific guidelines for the systematic testing of specific animal species may be developed and recommended by the Infectious Diseases Working Group of EAZWV”. This means, that at this time, no official guidelines for TB-testing of elephants are described under the BALAI-directive.

However, this does not mean that zoos keeping elephants should not prepare themselves to establish a routine testing protocol. The risk of introducing TB in a zoo through elephants is quite realistic as we can see from cases in the past decade both in the USA as well as in Europe. This document does not touch the issue of introduction of TB through other animal species and human contact, but should leave no doubts that a complete TB-surveillance is the only way to reduce the risks of TB in a zoo.

Every national or regional government may have its own interpretation about how to deal with suspected TB-cases. Much depends on the relationship between the zoo veterinarian and the local veterinary authorities. Generally it helps if the zoo has a clear policy regarding its health surveillance system. There is one approach that should **not** be practiced: the “sleeping dog policy”. Zoos that make all efforts to be certified according to the EU Directive 92/65 should have the intrinsic desire to stay free of infectious diseases like TB. This document describes methods that can be used to collect information about the TB-status of individual elephants and an elephant herd. One should be aware that the European situation can not be compared to the situation in the USA, where one federal government determines guidelines for the entire country. Legislation in the EU and other non-EU-member states is far from uniform. Quality of PPD’s even within the EU differs significantly between the member states. This makes it impossible to write a guideline with an official status. This document can only help to convince institutions that keep elephants to use any means available to minimize the risk of contracting and spreading tuberculosis in their animals and personnel. Finally it may help decision making in case of a planned elephant transfer; the reliability of a “TB- history report” depends a priori of the amount of data collected over the years, not only of a single test taken “on the day of transport”.

What are the diagnostic possibilities on live elephants?

As there are no tests that provide 100% accuracy, it is of outmost importance that each serum or plasma sample obtained from elephants should always be banked at below -20°C. This can be of great value for the interpretation of future test results and the evaluation of the TB-status in an individual elephant as well as the herd status. To test elephants for carrying TB-organisms, a very limited range of tests can be used, of which none has been validated for elephants:

1. *Immunologic tests*

None of these tests are 100% conclusive, but positive results should be followed by further investigations :

a. (Comparative) intradermal tuberculin test. In most countries, this test is only validated for cattle, but it is used in a large range of other species. In elephants it may have some diagnostic value, but results should be interpreted with great care. Bovine and avian tuberculins are injected intradermally in an area where the skin is thin, like the ear base area. The skin reaction is measured after 3, 4 and 5 days (swelling,

temperature, sensibility). This test may provide information about the cellular immune response of the animal as a reaction on previous contact of the animal with *Mycobacterium sp*. A positive response on bovine tuberculin should be followed by further investigations.

b. Humoral antibodies:
ELISA: some laboratories run an ELISA for the detection of antibodies. There is no official ELISA recognized in Europe for use in exotic mammals. The Central Veterinary Institute in Lelystad (the Netherlands) is the only official institute that runs these ELISA's on a routine base for zoos. The antigens used at this CVI are M.bovis, MPB70 and M.paratuberculosis. Positive results should be followed by further investigations.
The ***Elephant TB STAT-PAK Assay*** is officially recognized in the USA and can be obtained in Europe too (www.zootest.com) However, the test has no official status in Europe at this moment.

c. Gamma interferon test: not yet available for elephants. This test is currently under development at the Department of Infectious diseases and Immunology of the Veterinary Faculty in Utrecht, the Netherlands. A positive test result should be followed by further investigations.

2. Culture of Mycobacterium sp.

For several years the method of choice for sample collection has been the trunk wash*. If positive, this is a conclusive test: you know that you have an animal that is excreting the *Mycobacterium sp*. Action should be undertaken immediately, regardless the consequences it may have for the status of the zoo. No zoo can allow its personnel to work with an animal which is a serious risk for its employees without taking protective measures nor to expose other animals to this disease. If the culture is negative, the animal may still be a carrier of *Mycobacterium sp*. and may excrete the pathogens in low quantities. It may take several months before you get the result back from the lab; in the Netherlands the Central Veterinary Institute in Lelystad declares a culture to be negative for *M. bovis-complex* only after **4** months. A two phase TB diagnostic method combining the culture method and the PCR technique may shorten this period. However the PCR is especially useful, when the samples are ZN positive, otherwise many cycles are needed to get a signal and this will result in a certain numbers of false positive reactions in negative samples.

NB: In recent years the sensitivity of the trunk wash method has been proven to be extremely low. If you are dealing with a TB-positive animal, the risk of spreading the Mycobacteria by using this method is a great concern! In the view of the authors this method is not recommended unless performed by qualified persons that are aware of the risks and low sensitivity of the test.

* Trunk wash is an active manipulation at the elephant trunk which can be performed in free and protected contact systems in non immobilized elephants after they are conditioned for this procedure. The principle is that a sterile saline solution (approx. 100 ml) is injected in each nostril of the trunk. The nostrils must be kept closed by an elephant keeper and the trunk has to be lifted actively by the elephant or passively by the keeper so that the solution is running up to the basis of the trunk. The mixture of the solution and trunk mucus is collected in sterile plastic bags by active blowing of the elephant through its trunk or by lowering the level of the nostrils. The sample must be sealed and stored at 4°C for immediate culture or stored at –20°C if culture is to be performed at a later stage. Trunk wash under non-contact situation requires a full anesthesia of the elephant and a portable fluid pump and sucking system which allows the operation under sterile condition. The external pump and sucking system will be connected to a sterile PVC tube (1 cm diameter) with a length of approx. 2 meter. The amount of sterile solution and the collection bag are like described before.

Every zoo should work on a TB-free status, also in elephants. This can be accomplished by regular testing, especially when new animals are being brought in frequently. We propose to test:

a. Every elephants at least opportunistically whenever blood can be collected, by ELISA's (Central Veterinary Laboratory-Lelystad, using antigens derived from M.bovis, MPB70 and M. paratuberculosis) and the Elephant TB STAT-PAK Assay. Training of the elephants should include blood collection;
b. New animals by ELISA's (Central Veterinary Laboratory-Lelystad, using antigens derived from M.bovis, MPB70 and M. paratuberculosis) and Elephant TB STAT-PAK Assay) and skin test;
c. All suspect animals and contact animals by all immunological tests available. It is advisable to repeat the antibody tests 6 weeks after the intradermal tests. Potential carriers of TB may possibly react by an increase in the production of antibodies as measured by ELISA's or (if the first sample was negative) by the Elephant TB STAT-PAK Assay. One has to be aware that this phenomenon may not occur in elephants, though it has been seen in a few individual cases. As in other tests described here, it may contribute to obtain more information about an unknown TB/health status.

What to do when an elephant has died or will be euthanized?

An intense search for lesions of tuberculosis is encouraged in all elephant necropsies. This should include all elephants that die or are euthanized for other reasons even though TB is not suspected. Be advised that elephant TB is likely to be caused by *Mycobacterium tuberculosis* and *M. bovis* which are contagious to humans. Therefore be prepared with proper protective apparel, and contain any suspicious organs or lesions as soon as possible.

Ideally, elephants should be tuberculin tested 3 days prior to euthanasia and bled for serology (ELISA and Elephant TB STAT-PAK) at the time of euthanasia. Serum from elephants that die naturally should be harvested from post mortem blood for serological assays.

All elephants undergoing necropsies should have a careful examination of the tonsillar regions and submandibular lymph nodes for tuberculous appearing lesions. Take any nodes that appear caseous or granulomatous for culture (freeze or ultrafreeze), and fixation (in buffered 10% formalin). In addition, search thoracic organs carefully for early stages of TB as follows: after removal of the lungs and trachea, locate the bronchial nodes at the junction of the bronchi from the trachea. Use clean or sterile instruments to section the nodes. Freeze half of the lymph node and submit for TB culture (**even if no lesions are evident**). Submit sections in formalin for histopathology. Carefully palpate the lobes of both lungs from the apices to the caudal borders to detect any firm (nodular size) lesions. Due to the missing pleural elephants intrathoracal handling is required for the lung removal during necropsy. Please, wear a mouth and nose protector for this procedure. Take sections of any suspicious lesions. Open the trachea and look for nodules or plaques and process as above. Regional thoracic and tracheal lymph nodes should also be examined and processed accordingly. Split the trunk from the tip to its insertion and take samples of any plaques, nodules or suspicious areas for TB diagnosis as above. Look for and collect possible extra-thoracic TB lesions, particularly if there is evidence of advanced pulmonary TB.