Immunodiagnosis of tuberculosis in captive African elephants (*Loxodonta africana*) in the Victoria Falls and Livingstone area

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List of Abbreviations

MOTT	Atypical mycobacteria or mycobacteria other than TB
BCG	Bacillus Calmette–Guérin
PO SID	By mouth once a day
CMI	Cell-mediated immunity
CBC	Complete blood count
CFP-10	Culture filtrate protein 10
DPP	Dual Path Platform
ESAT-6	Early secretory antigenic target-6
ETH	Ethambutol
IGRA	IFN-γ release assay
IFN-γ	Interferon gamma
INH	Isoniazid
LTBI	Latent tuberculosis infection
µg/ml	Microgram /millilitre
mg/kg	Milligram / kilogram
pg/ml	Picograms/millilitre
MAPIA	Multiantigen print immunoassay
MTBC	*Mycobacterium tuberculosis* complex
M.Tb	*Mycobacterium tuberculosis*
NO	Nitric oxide
NTM	Non-tuberculous mycobacteria
PPD	Purified protein derivative
PZA	Pyrazinamide
RLU	Relative light units
RIF	Rifampin
TB	Tuberculosis
USDA	United States Department of Agriculture
$	United States dollar
WBC	Whole-blood cultures
Xpert MTB/RIF	Xpert Mycobacterium tuberculosis/ rifampin
rEpIFN γ	Recombinant elephant specific interferon gamma
RD	Region of difference

Thesis Summary

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Rationale: Tuberculosis (TB) in elephants is a chronic respiratory disease, most often caused by *Mycobacterium tuberculosis*, and infected animals show little or no clinical sign until the disease is in its advanced stages. There are currently no validated diagnostic assays for TB in elephants and those currently used appear to have suboptimal sensitivity or specificity. This urges for the establishment of an affordable and sensitive diagnostic algorithm able to detect TB in elephants at an early stage.

Aim of the study: To determine the immune response profiles of fifty elephants using the DPP™VET TB Assay , ElephantTB Stat Pak®, PPD ELISA and Elephant specific IFN-γ assay assay and for their usefulness to diagnose tuberculosis in captive African elephants.

Study design: Fifty captive African elephants *(Loxodonta africana)* in six privately owned facilities in Victoria Falls (Northwestern Zimbabwe) and Livingstone (South Eastern Zambia) were sampled during the period September 2014. Follow up testing was done on 17 animals previously test positive for the Elephant specific IFN-γ assay in November 2015. Heparinised blood samples and serum from all elephants were tested using the Elephant specific IFN-γ assay, (stimulation was done with bovine and avian tuberculin as well recombinant antigens ESAT-6; CFP10 and positive and negative controls) and serological tests respectively. (ElephantTB Stat Pak® / DPP™VET TB Assay and PPD ELISA).

Results: The four different tests used in fifty elephants in this study during the period September-November 2014 indicated the following prevalences of immune reactivity to *Mycobacterium tuberculosis* complex Elephant specific IFN-γ assay: Elephant TB Stat Pak®: 36%; DPP™ VET TB Assay: 2%; 12% and PPD ELISA: 6%.

Chapter 1
Literature Review

1.1 Introduction

Tuberculosis has shown to be a major health problem for elephant populations in captivity across the world (Landolfi *et al.* 2014). Elephants have been trained and used by humans for thousands of years (Csuti 2006). Transmission of *Mycobacterium tuberculosis* between humans and elephants is due to prolonged close contact (Michalak *et al.* 1998*).* Various authors (Une & Mori, 2007; Angkawanish *et al*. 2010; Murphree *et al*. 2011) have reported evidence of *Mycobacterium tuberculosis* transmission from humans to elephants or even other animal species. In addition, reports of tuberculosis in elephants appeared over the last two decades (Michalak *et al*. 1998, Mikota *et al*. 2001, Oh *et al*. 2002, Payeur *et al*. 2002, Lewerin *et al*. 2005, Une & Mori, 2007; Angkawanish *et al*. 2010 & Murphree *et al*. 2011). Hence, for effective tuberculosis control an innovative and efficient diagnostic approach that can identify infection at all stages from early infection until progressed disease is urgently needed.

Identification of bacteria through culture from trunk washes and necropsy tissue samples is currently considered as the gold standard for confirmation of tuberculosis in elephants (Mikota *et al*. 2001). This method unfortunately has limited sensitivity. Commercial diagnostic assays that are able to detect antibodies specific for the *Mycobacterium tuberculosis* complex, unfortunately have their own shortcomings. *Mycobacterium tuberculosis is* an intracellular bacteria, which triggers an early cell mediated immune response immediately after infection this has been considered to contribute to control (Greenwald *et al*. 2009). The tuberculin skin test, which is used for the screening of tuberculosis in cattle, has been unsuccessful in thick-skinned species like elephants and rhinoceroses. In humans, the IFN-γ assay has shown great promise and is currently replacing the Mantoux test (Schiller *et al*. 2010). The-IFN-γ assay is being used successfully in cattle (Vordermeier *et al*. 2001, 2006), domestic cats (Rhodes *et al*. 2008), lions (Maas *et al*. 2012), rhinoceroses (Morar *et al.* 2007), and elephants (Angkawanish *et al*. 2010).

Tuberculosis in captive elephants has been recognized as a re-emerging zoonotic disease (Mikota *et al*. 2000, Montali *et al*. 2001 & Mikota 2008). *Mycobacterium tuberculosis,* the bacterium which causes tuberculosis in humans has been known to also affect captive elephants especially those with prolonged close contact with humans. There are at least fifty domesticated elephants at four establishments in the Victoria Falls and two establishments in the Livingstone area, which are primarily set up for tourism purposes and there is a clear need to determine if this population had been exposed to the *Mycobacterium tuberculosis* complex infection.

The findings of this study will help in understanding *Mycobacterium tuberculosis* infection in elephants and possible transmission patterns between elephants and humans. The tuberculosis infection status in captive elephants in the Victoria Falls and Livingstone area is currently unknown.

Aim of the study

To determine the immune response profiles of fifty elephants using the Elephant specific IFN-γ assay, DPP™VET TB Assay , Elephant TB Stat Pak® and PPD ELISA for their usefulness to diagnose tuberculosis in captive African elephants.

1.2 History of tuberculosis in elephants

Evidence of a disease similar to tuberculosis in elephants has been documented as far back as 2000 years ago according to Sanskrit documents. A study on mastodont foot bones and ribs by Rothschild in 2006 showed that 52% of 113 had bone lesions which were typical for tuberculosis infection. The first reported case of tuberculosis in elephants concerned an Asian elephant *(Elephas maximus)* that died in 1875 in a zoo in London (Garrod 1875). Subsequently sporadic case reports of tuberculosis in Asian elephants were published by various authors (Damman, 1909; Thieringer, 1911; Narayanan, 1925 & Baldrey, 1930). Tuberculosis in an African elephant (*Loxodonta africana*) was first reported only in 1962 (Gorovitz 1962).

The first successful anti-tuberculosis drug study in an Asian circus elephant was published late in the 20th century (Gutter, 1981 & Devine, *et al* 1983). According to a retrospective study by Mikota, Sargent, & Ranglack in 8 out of 379 zoo elephants, tuberculosis was

confirmed in the period 1908 and 1994. This study did not include privately owned elephants (Mikota 1994). In 1997 of cross-transmission of tuberculosis between humans and elephants was documented at an Illinois facility (Michalak *et al*. 1997).

In 1996 tuberculosis became a major public health concern after two circus elephants from a privately owned travelling herd, died three days apart in the United States. The USDA immediately set up the National Tuberculosis Working group for Zoo and Wildlife species, which came up with the first guidelines on the control of tuberculosis in elephants in 1998. The guidelines recognised trunk wash culture as the best method for the diagnosis of tuberculosis in elephants (Shimshony 2008). The European elephant Technical Advisory Group (Elephant TAG) has recently recommended the Elephant specific IFN-γ assay (Angkawanish *et al*. 2010) as an important pre-movement screening tool in their guidelines on tuberculosis control published in March 2018.

1.3 Aetiology

Mycobacterium tuberculosis and other mycobacterial species have been isolated from a variety of captive zoo species, including non-human primates, elephants and other exotic ungulates, carnivores, marine mammals, and psittacine birds (Montali *et al*. 2001). Elephants are susceptible to *Mycobacterium bovis* and *Mycobacterium tuberculosis* infection (Thoen & Chiodini 1993), though most cases of tuberculosis in elephants are caused by *Mycobacterium tuberculosis* (Mikota *et al.* 2001, Montali *et al.* 2001 & Paudel *et al.* 2014).

Tuberculosis is defined as a disease caused by infection with any *Mycobacterium tuberculosis* complex organisms. The complex includes *Mycobacterium tuberculosis*, *Mycobacterium bovis*, vaccine strain *Mycobacterium bovis* (*Bacille Calmette-Guérin*), *Mycobacterium africanum, Mycobacterium microti, Mycobacterium orygis, Mycobacterium canetti, Mycobacterium caprae, Mycobacterium mungi, Mycobacterium suricattae, Dassie bacillus* and *Mycobacterium pinnipedii* (van Ingen *et al*. 2012).

Mycobacteria are rod-shaped acid-fast bacteria, which typically measure 0.5 μm by 3 μm. The structure of the mycobacterial cell wall is pivotal in the survival and growth of the bacteria (Lee *et al*. 2005). It contains a multitude of fatty and mycolic acids, attached to the underlying peptidoglycan-polysaccharide arabinogalactan layer. This provides a unique lipid barrier, which is crucial for the survival of the bacteria from host defence mechanisms. The virulence of mycobacteria is dependent on composition and quantities of the various components of the cell wall (Lee *et al*. 2005). The rigidity of the cell wall is provided by a peptidoglycan polymer outside the cell membrane and this also contributes in creating a permeable barrier. The cell wall also has carbohydrate structural antigen lipoarabinomannan which has facilities which protect the outside of the mycobacteria inside host macrophages (Lee *et al*. 2005 & Joe *et al*. 2007).

1.4 Epidemiology

Vedic literature over 2000 years ago has described infections similar to tuberculosis in elephants (Iyer 1937) however; significant research only began in 1996 after *Mycobacterium tuberculosis* was observed in a herd of circus elephants Maslow & (Mikota 2015). During the period between 1994 and 2013, 57 cases of tuberculosis in elephants were confirmed through culture in the United States, and more animals showed positive results on other commercially available test methods, mostly assessing contact with *Mycobacterium tuberculosis* complex by assessing cellular or humoral immune responsiveness (Maslow & Mikota 2015).

Tuberculosis in elephants has been noted across the world through various screening programs (Maslow & Mikota 2015). In Europe, tuberculosis in elephants was first recorded at the Kolmarden Zoo in Stockholm (Lewerin *et al.* 2005 & Moller *et al*. 2005). Culture-confirmed cases have also been found in India, Nepal, and Thailand amongst animals seropositive in sero-surveys, and all the confirmed cases to date have been due to *Mycobacterium tuberculosis*. The potential of mixing of infected captive and wild elephants in Asia remains a huge concern to the endangered species. (Chandranaik *et al*. 2017) In Australia, tuberculosis was noted in an Asian elephant that had been imported from Thailand. The infection spread to chimpanzees, which were housed 110 metres away (Stephens *et al*. 2013). In Africa the first case of tuberculosis in an ex captive wild African elephant was documented in Kenya (Obanda *et al.* 2013).

The primary source of tuberculosis infection is generally thought to originate from elephant handlers who have prolonged close contact with the elephants however, elephant-to elephant spread or even to other animal species has been noted (Maslow & Mikota 2015). Genotypic analysis of mycobacterial isolates by (Mikota *et al*. 2001) from herds having multiple infected animals are mostly identical although sometimes genetically distinct strains may be present (Payeur *et al*. 2002 & Michalak *et al*.1998). Elephants infected at a Stockholm zoo were all infected with an identical strain (Lewerin *et al.* 2005).

1.5 Transmission of tuberculosis in humans

Mycobacterium tuberculosis is mostly spread by small airborne droplets, generated during coughing by an infected person. Tiny infected droplets can remain in the air for several minutes to hours after expectoration depending on environmental conditions (Lee *et al.* 2005). Prolonged close contact with infected humans has been attributed to be the primary source of infection in captive elephants (Miller & Olea-Popelka 2013).

Effective transmission is dependent on the amount of bacilli in the inhaled droplets, the virulence of the bacilli, ventilation, and frequency of aerosolisation (American Thoracic Society and Centers for Disease Control and Prevention 2000).

1.6 Pathogenesis of tuberculosis in humans

Infection occurs when infectious droplets are inhaled and tubercle bacilli deposit in the mucus of the upper respiratory tract (Frieden *et al.* 2003). Some of the bacilli may end up in the lungs leading to infection of the lower respiratory tract.

Macrophages in the alveolar spaces (Korf *et al.*2006), form part of the innate immune system and if activated may destruct invading mycobacteria preventing infection (van Crevel, *et al.* 2002).

Several defense mechanisms are involved in the uptake of mycobacteria by macrophages (van Crevel *et al*. 2002).The mycobacterial lipoarabinomannan is a key ligand for a macrophage receptor. (Nicod 2007). Likewise, the complement protein C3 binds to the mycobacterial cell wall and enhances recognition by macrophages. Phagocytosis of bacilli by macrophages leads to a series of events, which determine latency, or development of active tuberculosis (Frieden *et al*. 2003). These two outcomes are essentially determined by the strength of the host immune system or virulence of the mycobacteria (van Crevel *et al*. 2002 & Goyot-Revol *et al*. 2006).

Upon ingestion of bacilli by macrophages proteolytic enzymes may contribute to destruction of the invading bacteria that protect themselves by inhibition of the phagosome lysosome fusion within the macrophage. Mycobacteria continue to multiply slowly after being ingested by host macrophages (Frieden *et al*. 2003) with bacterial cell division occurring every 25 to 32 hours. (American Thoracic Society and Centers for Disease Control and Prevention, 2000; Porth, 2002 van Crevel *et al.* 2002, & Nicod, 2007). Molecules released by the mycobacteria and cytokines produced by the macrophages attract and activate T lymphocytes and other cells. (van Crevel *et al.* 2002).

As a result of the cell mediated immunity, thus induced, granulomas are formed around the macrophages contains the mycobacteria which create an environment that limits the spread and multiplication of the bacteria (Rosenkrands *et al.* 2002; Frieden *et al*. 2003 & Nicod, 2007). The granuloma microenvironment restricts growth and eventually results in latency resulting in the lesion undergoing fibrosis and calcification, but bacilli are able to survive (Dheda 2005). Solid necrosis at the centre takes 2 or 3 weeks to resemble soft cheese, often referred to caseous necrosis, and is characterized by low oxygen levels, low pH, and limited nutrients (Dheda 2005).

1.7 Clinical signs in elephants

Ante mortem signs in elephants are difficult to see however, chronic weight loss, anorexia, dyspnea, coughing, and exercise intolerance has been noted, though these signs can be confused with other similar diseases (McGaughey, 1961, Seneviratna *et al.* 1966, Pinto *et al*. 1973, Gutter, 1981 & Saunders, 1983). Anorexia, which results in significant loss of fat and muscle, is a very common feature in terminally infected elephants (Paton *et al*. 2004).

However, chronic weight loss can be misleading as elephants can lose weight due to malnutrition and other chronic diseases in old elephants (Furley 1997). In Ceylon, ancient Ayurvedic elephant physicians more than 3 000 years ago associated ventral oedema with an incurable lung disease (Pinto *et al.*1973). Ventral oedema has been observed in some infected elephants though; it may be confused with other conditions such as concurrent congestive heart failure and anaemia, (Seneviratna *et al*. 1966 & Pinto *et al*. 1973).

1.8 Pathology

Pathology in elephants is usually observed in the lungs and thoracic lymph nodes, variable involvement of other extra-thoracic sites has also been noted (Seneviratna *et al*. 1966, Pinto *et al.* 1973, Gutter, 1981, Saunders, 1983, Michalak *et al.* 1998, Montali, 1999 & Mikota *et al.* 2000). Lesions tend to vary with the stages of the disease. Elephants with less severe forms of the disease usually have firm granulomatous lesions with or without caseous foci in the pulmonary tissue and thoracic lymph nodes. Cases with extensive involvement of both lungs usually result in death from caseous lesions, which often result in abscesses from which *Mycobacterium tuberculosis,* and other opportunistic bacteria such as *Pseudomonas aeruginosa* have been isolated. Mucopurulent bronchial plugs have often been observed in advanced cases of tuberculosis in elephants. Bronchial and other thoracic lymph nodes are enlarged as well (Mikota *et al.* 2000).

Tuberculosis lesions in the spleen, adrenals, kidneys, liver and mesenteric lymph nodes have been noted in some advanced cases. This could indicate that shedding of the bacteria can occur through other routes besides the respiratory system (Mikota *et al*. 2000).

1.9 Immune response in tuberculosis infection in animals

1.9.1 Cell mediated immunity

Knowledge of the immunology of tuberculosis in elephants is inadequate, making it a challenge to design efficient procedures for testing, treatment, management, and control. The immune response to tuberculosis infection is both cell mediated and humoral (Mikota 2008). Cell-mediated immunity is activated earlier than humoral immunity and IFN-γ a cytokine produced by TH1 and natural killer (Flynn *et al*. 1993). IFN- γ causes the

activation of macrophages that are meant to kill obligate intracellular microbes (Delves *et al.* 2006 & Paudel *et al.* 2015).

1.9.2 Humoral immunity

Humoral immunity in elephants with tuberculosis is mediated by specific antibodies, as well as complement proteins, and antimicrobial peptides belonging to innate immunity. It involves individual antibody variation in antigen recognition (Lyashchenko *et al.* 1998, 2000). This heterogeneity determined by both pathogen and host factors, has been suggested to be the result of differential expression of stage-specific *Mycobacterium tuberculosis* genes leading to the changing profiles of the predominant immune recognition of corresponding proteins in the course of disease in animals including elephants (Davidow *et al.* 2005 & Lyashchenko *et al.*1998).

1.10 Diagnosis of tuberculosis in elephants

The options for the diagnosis of tuberculosis in elephants at the various stages of the disease are very limited making it rather a challenge to manage and control the disease. (Mikota *et al.* 2001, Gavier-Widen *et al.* 2002, Lewerin *et al*. 2005, Moller *et al*. 2005).

1.10.1 Trunk wash culture

Bacterial identification from a trunk wash is currently the only acceptable confirmatory ante mortem diagnosis of tuberculosis in elephants. Despite this trunk wash culture has various limitations which include poor sensitivity, slow turnaround time for sample processing and bacterial growth due to which it usually takes 8-12 weeks to get a culture result, inconsistent specimen quality and sample collection logistics. (Isaza & Ketz 1999 & Mikota.*et al.* 2000). The method involves the collection of test samples from an elephant trunk for bacterial culture and identification. This is done by instilling sterile saline into the trunk, elevating the trunk and getting the elephant to exhale in a sterile plastic bag, repeated three times with a one day interval The veterinarian or the technician as well as the elephant should be well trained in order to collect a good quality sample (Griffin & Buchan, 1994 & Larsen *et al.* 2000). A positive trunk culture confirms presence of the infection but a negative culture does not rule out its presence. Samples are prone to

contamination by fungi and bacteria as elephants use their trunks for different functions (Moller *et al.* 2005).

1.10.2 ElephantTB Stat Pak®

The ElephantTB Stat Pak®, test is a commercially available rapid test that uses lateral flow technology in detecting antibodies specific for a number of recombinant antigens ESAT-6, CFP10, and MPB83 shared between *Mycobacterium tuberculosis* and *Mycobacterium bovis* in elephant serum or plasma. The ElephantTB Stat Pak®, may be used as a screening test together with other cellular and humoral diagnostic methods in the diagnosis of tuberculosis in elephants (Lyashchenko *et al*. 2006 & Greenwald *et al.* 2009).

1.10.3 Dual Path Platform (DPP™VET TB Assay)

The Dual Path Platform DPP™VET TB Assay is a commercially available test that detects IgG antibodies; specific for a number of recombinant antigens (MPB83 and CFP10/ESAT-6) shared by *Mycobacterium tuberculosis* or *Mycobacterium bovis,* in elephant serum, plasma. The Chembio, designed diagnostic tool is a new generation single use rapid test kit that uses immuno-chromatography in detecting IgG antibodies in the diagnosis of tuberculosis in elephants. The Dual Path Platform DPP™VET TB Assay may be used as a screening test together with other cellular and humoral diagnostic methods in the diagnosis of tuberculosis in elephants (Greenwald *et al.* 2009).

1.10.4 PPD ELISA

The PPD ELISA is a, multiple-antigen enzyme-linked immunosorbent assay (ELISA) based serological test, which uses PPD (Purified protein derivative) tuberculin in the detection of antibodies specific to *Mycobacterium tuberculosis* complex in infected elephants (Sayin & Erganis 2013). The test is used to screen for tuberculosis in elephants (Larsen *et al*. 2000).

1.10.5 The Elephant Specific Interferon Gamma Release Assay (IGRA)

The IGRA has recently become available as in an in-vitro whole blood test to diagnose infection with *Mycobacterium tuberculosis* complex by assessment of the of *Mycobacterium tuberculosis* specific T-cells sensitised by prior exposure to the bacteria. The assay detects the elephant IFN-γ, produced in infected elephant whole-blood cultures

after stimulation with (ESAT6) the 6 kDa early secretory antigenic target / 10 kDa culture filtrate antigen (CFP10) Purified protein derivative-bovine antigen (PPD-B), derived from *Mycobacterium bovis* and Purified protein derivative-Avian antigen (PPD-A) derived from *Mycobacterium.avium,* in concentration ranges elicited in whole blood cultures by *Mycobacterium tuberculosis* specific T-cells.IFN- γ release assays are designed on the basis that T-lymphocytes will release-IFN-γ when exposed to specific tuberculosis antigens.

Apart from the human Interferon-gamma release assays (IGRA) the first commercial IFN-γ release assay, BOVIGAM, was produced by Prionics AG (Zurich, Switzerland) for the diagnosis of tuberculosis in bovine species. Experimental IGRA assays have meanwhile been developed for cattle, badger, deer, (Vordermeier *et al*. 2001,2006), domestic cats (Rhodes *et al*. 2008), lions (Maas *et al*. 2012) rhinoceroses (Morar *et al*. 2007), and elephants (Angkawanish 2013). Interferon-gamma release assays have shown great promise in the diagnosis of latent tuberculosis infection in both humans especially in exotic species.

1.11 Diagnosis of tuberculosis in humans

The diagnosis of tuberculosis in humans using signs and symptoms is often misleading due to other respiratory diseases, which usually exhibit similar a clinical picture to that of tuberculosis. Direct and indirect diagnostic test methods are available which are used to confirm tuberculosis diagnosis.

Bacterial culture from a diagnostic specimen usually sputum is a direct standard method for the diagnosis of tuberculosis in humans. Mycobacterium takes 3 to 6 weeks to grow on solid media, which makes this test method less convenient. Liquid chromatography can also be used to differentiate cell wall mycolic acid and is able to confirm presence of disease in 4-14days (Center Of Disease Control And Prevention, 2009). The sputum smear test is another example of direct quick screening diagnostic method. The test involves the use of sputum smeared on a microscope slide, stained with Ziehl-Neelsen stain and fixed with alcohol. The test takes at least 24 hours to give a result (Center Of Disease Control and Prevention 2009).

Indirect tuberculosis test methods include the skin test, xyray imaging, the QuantiFERON-TB-Gold test, and the Xpert MTB/RIF assay. The tuberculin skin testing is commonly used to screen for latent *Mycobacterium tuberculosis* in humans (Goldrick 2004).The skin test is not specific to the *Mycobacterium tuberculosis* complex. Immunocompromised individuals can produce false negative results whereas patients with previous exposure to other mycobacteria which may not have been *Mycobacterium tuberculosis* can show false positive results (Anderson *et al.* 2006) in addition vaccination with *Bacillus Calmette–Guérin* (BCG) for tuberculosis may cause responses that cannot be differentiated from those occurring after infection.

Chest x-rays are an important screening tool in the diagnosis of tuberculosis in humans especially in cases of respiratory signs or known unprotected contact with an (active) TB patient. Pulmonary tuberculosis shows as infiltrates with cavitation in the upper and middle lobes of the lungs (Thrupp *et al.* 2004).

The QuantiFERON-TB-Gold Test is an interferon-gamma (IFN-γ) release assay based on T-cell mediated IFN-γ release after stimulation with specific *Mycobacteria tuberculosis* antigens in humans (Hermensen *et al*. 2014).The test is able to differentiate infection between *Mycobacteria tuberculosis* and non-tuberculous mycobacteria due to its high specificity (Kobashi *et al.* 2009 & Detjen *et al.* 2007).

The Xpert MTB/RIF assay is able to rapidly detect *Mycobacterium tuberculosis* complex and resistance to rifampicin in less than two hours. The assay aids in choosing the correct treatment protocol and makes the management of tuberculosis cases much quicker and easier (Center of Disease Control and Prevention 2009).

1.12 Diagnosis of tuberculosis in other animals

Tuberculosis in livestock has significant impact on international trade of livestock and animal products and cannot be diagnosed based on clinical signs because they are only observed in terminal stages of the disease. The intradermal tuberculin skin test is the most used field diagnostic test for live animals. The test relies on measuring the immune response of the animal to injections of tuberculin. Enviromental, and host factors can however, affect the performance of the tuberculin skin test (Stear 2005).

The BOVIGAM^{TM,} is another routinely used gamma interferon realease assay in many countries for the detection of *Mycobacterium bovis* infected cattle, buffalo and goats. Sensitivity has varied between 81.8% and 100% for culture-confirmed bovine TB and specificity between 94% and 100%. The gamma interferon assay detects *Mycobacterium bovis* infection earlier than the skin test (Wood and Jones 2001).

1.13 Treatment of tuberculosis in elephants

Very little has been published with regards to treatment of tuberculosis in elephants (Mikota *et al.* 2000). The current treatment protocols being used in elephants have been derived from regimens used in humans. Tuberculosis therapy involves the use of a four drug regimen for a period of 6-12 months (American Thoracic Society 1994).

The primary objective for tuberculosis treatment in elephants is to stop the infected animal from shedding *Mycobacterium tuberculosis* organisms to other elephants, humans and other species. It is also imperative that the sick animal does not get ill due to the treatment thus the amount of drugs administered should be adapted when causing adverse effects. The main drugs used in tuberculosis treatment, thus in elephants are isoniazid, rifampin, ethambutol, and pyrazinamide.

Tuberculosis drug administration in elephants is done either orally or rectally. Long term oral administration can be a challenge as elephants are discerning eaters. However, the oral route results in higher blood levels than the rectal route. Oral administration over food fed ad lib is no longer recommended. Elephants can be trained to accept a bite block for oral administration with an equine dosing syringe (Pandey & Khuller 2005). Rectal drug administration techniques including suppositories have also been developed. A precise weight determination is essential for accurate dosing of the drugs (Mikota *et al.* 2000).

Table 1.1 Antitubercular drug characteristics and usage

DRUG & DOSAGE	MECHANISM OF ACTION	ROUTE	DIRECTIONS OF USE
Isoniazid 2-5 mg/kg	Inhibits synthesis of mycolic acids in the bacterial cell wall. Rapid killing of actively dividing MTB organisms	Oral or rectal	Isoniazid should be used in all MTB treatment protocols unless elephant is intolerant of it Supplementation with vitamin B6 (pyridoxine) at a daily dose of 1 mg/kg is recommended
Rifampin 10 mg/kg	Inhibits DNA dependent RNA polymerase activity in the bacteria. Kills latent or inactive Mtb organisms and resolves cavitary lesions	Oral only	If the elephant is not trained for consistent rifampin oral ingestion, substitute enrofloxacin or levofloxacin.
Ethambutol 15 mg/kg	Diffuses into actively growing Mycobacteria and inhibits cell wall biosythesis. This leads to increased cell wall permeability and bacterial death	Oral only	Must be used in conjunction with other drugs Symbiotic with other drugs
Pyrazinamide 20 mg/kg	After metabolism into pyrazinoic acid, it interferes with Mycobacteria's ability to synthesize the fatty acids it requires for growth and replication	Oral or rectal	Must be used in conjunction with other drugs Used in conjunction with Isoniazid to prevent resistance
Levofloxacin Oral at 5 mg/kg OR Rectal at 15- 25 mg/kg*	fluoroquinolones inhibit bacterial topoisomerase and DNA gyrase, enzymes needed for bacterial DNA replication, transcription, repair Treats latent populations of MTB organisms and to prevent relap	Oral or rectal	Can be used with or as a substitute for RIF of RIF is not accepted by elephant.

(2017 Recommendations for the diagnosis, treatment, and management of tuberculosis *Mycobacterium tuberculosis* in elephants in human care 2017)

Anti-tuberculosis drug doses for individual elephants should be determined by measuring drug blood-levels. Elephants should be weighed before and throughout treatment (Mikota *et al.* 2000). The treatment period for tuberculosis in elephants is still experimental but it is generally done for a year or longer. (Shimshony 2008). Complete blood count and serum chemistry panels are recommended monitoring tools for elephants receiving anti-tuberculosis drugs (Mikota *et al*. 2000).

Elephant treatment costs are generally substantial estimated between $50-60,000 for a single elephant. Drug costs based on the recommended dosages for an adult Asian elephant were roughly $5/day for isoniazid, $65/day for rifampin, $190/day for pyrazinamide, and $155/day for ethambutol. Given that multiple drugs and quantities are used for different elephants, the costs usually vary from one animal to the other though the exercise is quite expensive. Getting an adequate supply of the different drugs in bulk is quite a challenge, which often results in regular interruption of treatment facilitating the emergency of antimicrobial resistance (Maslow *et al.* 2005).

Adverse effects associated with elephant anti-tubercular therapy has been observed. Combining the various drugs is more likely to increase the likelihood of these effects. Some of the effects may be transient, spontaneous or might persist consistently. (Wiedner & Schmitt 2007).

Chapter 2
Materials and Methods

2.1 Introduction

Elephant TB, caused by *Mycobacterium tuberculosis* is considered worldwide as an important infectious disease which poses a serious zoonotic threat (Une & Mori, 2007; Angkawanish *et al*. 2010 & Murphree *et al*. 2011). The African elephant (*Loxodonta africana*) is classified under CITES appendix I and II as a critically endangered species due to poaching and loss of habitat. The potential threat of elephant TB and other diseases in wild and captive populations stands to dampen conservation efforts to save the species from extinction.

The first confirmed case of tuberculosis in Africa was reported in a 12-year-old wild Kenyan African elephant (Obanda *et al.* 2013). There is no published data of elephant TB in Zimbabwe and Zambia and this study was the first of its kind. There are no official elephant TB screening programs in both Zambia and Zimbabwe.

Current elephant TB diagnosis strongly relies on trunk wash culture; however, this method has limited sensitivity (Mikota *et al*. 2001 & Lyashchenko *et al.* 2006). Innovative and efficient diagnostic methods are therefore urgently needed to complement or even replace the trunk wash culture.

Available assays measuring MTBC specific immune responsiveness were employed in the study, which also allowed comparison of their results. While apparently not useful in early stages, in advanced stages of disease elephants produce antibodies specific for *Mycobacterium tuberculosis* which were assessed using the commercially available serological diagnostic tests (the ElephantTB STAT-PAK, Dual Path Platform [DPP] VetTB, and Multiantigen print immunoassay [MAPIA], ChemBio Diagnostic Systems, Inc., Medford, NY in this study (Greenwood *et al*. 2009 &, Lyashchenko *et al.* 2006).

The Interferon gamma release assay is now considered a gold standard in diagnosis of TB both in humans and cattle, the Elephant specific interferon gamma assay.despite not being validated, has potential in being an alternative test to the trunk wash in early and convenient diagnosis, screening and treatment monitoring of TB in elephants.

2.2 Materials

2.2.1 Study design

The project was a cross-sectional study that involved testing for *Mycobacterium tuberculosis* complex infection of fifty captive African elephants (*Loxodonta africana*) at six privately owned facilities in Victoria Falls, northwestern Zimbabwe and Livingstone, southern Zambia. The elephants were tested between September 2014 and November 2015 using four different immunoassays: the Elephant specific IFN–γ assay, the DPP™VET TB Assay (ChemBio Diagnostic Systems, Inc.), the Elephant TB Stat Pak® (ChemBio Diagnostic Systems, Inc.) and the PPD ELISA.

2.2.2 Study population

Each facility had between 2 and 17 captive elephants. The sex ratio of the sampled elephants was 26 females to 24 males. The ages of the elephants ranged between 3.5 and 60 years with an average of 23 years of which 51 % had reached the breeding age. Eighty three precent of the elephants were born in the wild.

Two facilities, with 15 animals were situated in Zambia. The ages of these African elephants ranged from 5-60 years with an average of 49 years. The male to female ratio was 1:1.4.

Table 2.1 Demographic data of elephants sampled in Zambia in 2014 and 2015

Animal	Facility	Age	Sex
ZAM36	E	29	male
ZAM37*	E	18	male
ZAM38*	E	22	female
ZAM39*	E	16	female
ZAM40*	E	16	female
ZAM41	E	13	female
ZAM42	F	60	male
ZAM43	F	55	male
ZAM44	F	35	male
ZAM45	F	35	male
ZAM46	F	35	female
ZAM47	F	35	female
ZAM48	F	10	female
ZAM49	F	8	female
ZAM50	F	5	male

* Elephants tested both in September 2014 and in November 2015 (14 months interval).

Thirty animals animals selected from four facilities were sampled in Zimbabwe. The ages of the African elephants ranged from 3.5 to 43 years with an average of 22 years. The male to female ratio was 1:1.06.

Table 2.2 Demographic data of elephants sampled in Zimbabwe in 2014 and 2015

Animal	Facility	Age	Sex
ZIM1	A	29	male
ZIM2*	A	31	male
ZIM3*	A	30	male
ZIM4*	A	36	female
ZIM5	A	7	male
ZIM6	A	28	female
ZIM7*	A	3.5	female
ZIM8*	A	27	female
ZIM9*	A	8	female
ZIM10	A	4	female
ZIM11*	A	28	female
ZIM12	A	12	male
ZIM13	B	27	male
ZIM14	B	21	female
ZIM15*	B	20	female
ZIM16	B	24	female
ZIM17	C	40	male
ZIM18	C	33	female
ZIM19	C	16	female
ZIM20	C	18	female
ZIM21*	C	22	male
ZIM22	C	14	male
ZIM23	C	15	female
ZIM24	C	5	male
ZIM25*	D	42	male
ZIM26	D	42	male
ZIM27	D	43	male
ZIM28	D	39	male
ZIM29	D	18	female
ZIM30	D	16	male
ZIM31	D	16	male
ZIM32*	D	16	female
ZIM33	D	15	female
ZIM34*	D	14	female
ZIM35*	D	12	male

* Elephants tested both in September 2014 and in November 2015 (14 months interval).

The six facilities under study in Zimbabwe and Zambia had 57 elephants and employed 78 elephant handlers with an average of 1.36 handler per elephant. The four Zimbabwean facilities had 68% of the elephant population.Eigthy eight percent of this captive African elephant population were sampled during the study.

Table 2.3 Elephant and handler numbers at the facilities in Zimbabwe and Zambia

Facility ID	Country	Total no of elephants	No of elephants sampled	No of handlers
A	Zimbabwe	16	12	14
B	Zimbabwe	4	4	8
C	Zimbabwe	9	8	12
D	Zimbabwe	11	11	18
E	Zambia	6	6	12
F	Zambia	11	9	14
Total		57	50	78

2.2.3 Study site

The study area was located in the Kavango Zambezi Transfronteir (KAZA TFCA) conservation area in the resort border towns of Victoria Falls and Livingstone. On the Zambian side Livingstone The KAZA TFCA is made up 444 000km^2 of vast expanse of mainly protected areas, national parks game reserves, conservancies, forest areas, wildlife management areas and communal areas of 5 southern Africa member states of Zimbabwe, Botswana, Zambia, Namibia and Angola. The transfrontier conservation area is home to one of the largest populations of transboundary African elephants.

2.2.4 Management of elephants and elephant facilities

Four elephant safari facilities were located in the northwest of Zimbabwe near the resort town of Victoria Fall, which is within Victoria Falls National park. The other two facilities had fifteen elephants and were located in the surroundings of the southern Zambian border town of Livingstone within the Mosi-oa-Tunya National Park. The facilities were owned and managed independent of each other.

The six elephant facilities employed a total of seventy-eight elephant handlers. On average, each facility had two handlers per elephant. The handlers had very close contact with the elephants during the course of their duties, which included feeding, cleaning training and daily back safari rides with the tourists. Supplementary feeding was provided daily in the form of game cubes, tree branches, and hay before and after their daily rides. Warthogs and monkeys had access to the facility and came into contact with the captive elephants during their feeding routines.

The local wildlife veterinarian checked the health of the animals monthly and an animal health record was maintained. Private and state wildlife veterinarians were called to investigate and carry out post-mortems each time a captive elephant died. Elephant bulls on the facilities were regularly treated with hormonal treatment that suppressed periodic highly aggressive behaviour associated with high levels of testosterone (musth). It was common practice on the six elephant facilities for owners to trade or loan the elephants amongst each other. The captive elephant population was considered very valuable and old or sick animals would be retired from daily activities and sold to other animal sanctuaries. No individual elephant was assigned to a particular handler though the elephants preferred particular handlers over others.

Each elephant facility operated based on an annual renewable permit from the Parks and Wildlife Management Authority that stipulated the conditions under which the captive elephants were expected to be kept. An annual return of elephant deaths, births or rescues was a prerequisite for the renewal of the permit. Captive elephants that posed a risk to humans or other elephants were euthanized in consultation with the national parks authority.The euthanasia is motivated by unruly behaviour or old age and is independent to the immunodiagnostic results of this study.

2.2.5 Sample collection

Blood was collected from each elephant by the researcher after prior written consent from the facility owners. The exercise was carried out either early in the morning or later in the afternoon. The samples were collected from the auricular vein with a 19-gauge needle into heparin and serum tubes. A leg chain on one of the front legs, a behaviour restraint and azaperone a mild sedative at a dosage rate of 0.02mg/kg were used to help fully and safely restrain the elephants during the exercise.

The collected samples were kept in a container not exceeding a temperature of 25 °C until they were processed at the Victoria Falls Wildlife Trust Laboratory between 30 minutes and two hours after collection. Serum samples were allowed to settle for 24 hours before plasma was harvested and stored at -20 °C until assays were performed using the DPP™VET TB Assay (ChemBio Diagnostic Systems, Inc.), PPD ELISA and Elephant TB Stat Pak® according to the research protocol.

2.2.5.1 Ethical considerations

Approval for the study was secured from the following

1. Facility owners of the six facilities were asked to sign consent forms to authorize the use of their animals for the study.
2. Permission was sought and approved from the research committee in Zimbabwe for the study to take place see attached appendix 1.
3. The study was also approved by the Animal Ethics Committee of the University of Pretoria, South Africa see attached ethics approval under Appendix 2.

2.3 Methods

The study involved the use of four different test methods, namely the Elephant specific IFN- γ assay, Elephant TB Stat Pak®, PPD ELISA, and the DPP™VET TB Assay to test the absence or presence of TB in the fifty elephants under study.

2.3.1 Elephant specific IFN-γ assay

The assay detects IFN- γ in whole-blood cultures (WBC) from African elephants after stimulation with TB specific (PPDA, PPDB ESAT6/CFP10, PC EC &PC HP,) and control antigen (Pokeweed mitogen and cell culture medium).

2.3.1.1 Whole blood stimulation

African elephant blood samples were collected in heparinized tubes, (BD Vacutainer heparin, Becton Dickinson, Breda, the Netherlands) 10 ml, kept at a temperature below 25°C and were processed within 6 hours of collection. Whole blood in two sets of duplicates was diluted in equal volumes with complete medium [RPMI1640 + Glutamax containing 5% FCS, 50 U/ml penicillin, 50 U/ml streptomycin, 5000 U heparin, 5 × 10−5 m

2-mercaptoethanol and l-glutamine (Gibco®, Grand Island, NY, USA)] and incubated in 1 ml Eppendorff tubes at 37°C,with mitogens used as positive-control stimulants including pokeweed mitogen (PWM, Sigma®-Aldrich Chemie, Zwijndrecht, the Netherlands), 5 µg/ml and the antigens avian and bovine tuberculin (PPDA 1000 IU/ml and PPDB 600 IU/ml, ThermoScientific, Lelystad, NL), ESAT6 and CFP10 fusion protein (E6/CFP10, Staten Serum Institute, DK) 10 µg/ml.with stimulating antigens Prionics™ PC-EC and Prionics™ PC-HP(lyophilised).Culture medium (RPMI 1640) was used as the negative control. One set of the duplicates was incubated for 24 hours and the other for 48 hours .After incubation the supernatants were harvested and stored at −20°C until they were tested in the Elephant specific IFN-γ capture ELISA.

2.3.1.2 Elephant specific IFN–γ capture ELISA

ELISA plates (96 well flat –bottom / ST MICROLOM® 600 high binding clear Greiner Bio-One, Alphen a/d Rijin, the Netherlands) were coated with 50 µl of 2 µg/ml purified selected capture antibody (MoAb AE16F10C9) (Angkawanish *et al.* 2013) in Phosphate Buffered Saline (PBS) for 1 hour at room temperature. Plates were blocked with 1.3% casein in PBS (Universal casein diluents SDT®) for 1 hour at room temperature, emptied and washed four times with PBS /0.05% Tween-20. The supernatants of the whole blood cultures were diluted 1:2 with 1.3% casein buffer and added in triplicate. To produce a recombinant elephant specific interferon gamma (rEpIFN γ) standard curve, a twofold dilution series (500 pg/ml-0.5 pg/ml in 1.3%casein buffer) was included in the assay.

After 2 hours incubation at room temperature, the plates were washed and 50 µl/well biotinylated detection antibodies (MoAb AE10F4G11) 1:20 000 (0.025 µg/ml) diluted in 0.65% casein buffer were added for one hour at room temperature. Plates were washed and streptavidin –peroxidase (SA-HRP80) diluted 1:20 000 in 50 µl /well 0.5% casein buffer was added for 30min at room temperature. Plates were washed, and substrate 3, 3', 5, 5'-tetramethylbenzidine (TMB reagent) (SDT®, extra sensitive, Baesweiler, Germany) was added, and the colour reaction was stopped after 5 minutes using 1% Hydrochloric acid (HCl (1M; 50 µl/well) and the optical density was determined at 450 nm and 650 nm.

The OD reading at 450 nm was the test reading. The OD reading at 650 nm was the reference reading. The reference wavelength reading was to negate the effect of precipitated proteins or cellular debris that may have interfered with the wavelength

reading. This reading was used to correct for optical variation. If there is particulate matter in the path of the light, it will affect absorbance in all channels by scattering. A more standardized reading was obtained from subtracting the OD 650 nm from OD 450 nm.

Before animals were classified into different reactor categories it was first ascertained that requirements for test validity indicating cell viability and absence of non-specific responses were met. Table 2.4 shows a summary of the test results observed as outcomes of the e IFN-γ assays and how they were used as criteria for classification of the elephants, originally in 9 categories, which were reduced to the 3 main categories: Positive, Suspect and Negative, used in further analyses.

A test result was considered positive if an animal showed a net immune reaction to both the bovine tuberculin (ODPPD-B - ODPPD-A) and at least one recombinant TB antigen. The criterium tuberculin PPD was a critical marker in the first phase of the diagnostic algorithm, which, if above the threshold, indicated a positive response and classified the animal at least as a suspect reactor. In combination with responses to ESAT6, CFP10 and/or the peptide cocktails after, either or both, 24 and 48 hours of stimulation the animal was classified positive. Test negative animals were those that did not show any responsiveness to either the bovine tuberculin (ODPPD-B minus ODPPD-A < 0.1) or the recombinant TB antigens (ODESAT-6 and/or ODCFP10 and/or OD Peptide cocktails < OD-nil + 0.1) at both 24 and 48hours.

Table 2.4 Criteria for Elephant classification based on results (criteria) of the e-IFN-γ assay

Classification	Criteria
Test validity	OD–nil < OD_{PPD-B} and/or OD_{ESAT-6} / OD_{CFP10} OD-PWM > 0.43
Positive Elephants that showed a net immune reaction to both the bovine tuberculin and at least one recombinant TB antigen	OD_{PPD-B} minus OD_{PPD-A} > 0.1 and OD_{ESAT-6} and/or OD_{CFP10} and/or OD $_{Peptide\ cocktails}$ > OD-nil + 0.1 either at 24 h or 48 h or both
Suspect Elephants that either showed a net immune response to the bovine tuberculin or one recombinant TB antigen but not both	OD_{PPD-B} minus OD_{PPD-A} > 0.1 and OD_{ESAT-6} and/or OD_{CFP10} and/or OD $_{Peptide\ cocktails}$ < OD-nil + 0.1 at either 24h or 48h or both OD_{PPD-B} minus OD_{PPD-A} < 0.1 and OD_{ESAT-6} and/or OD_{CFP10} and/or OD $_{Peptide\ cocktails}$ > OD-nil + 0.1 at either 24h or 48h or both
Negative Elephants that showed no net immune reaction to either the bovine tuberculin or the recombinant TB antigens	OD_{PPD-B} minus OD_{PPD-A} < 0.1 and OD_{ESAT-6} and/or OD_{CFP10} and/or OD $_{Peptide\ cocktails}$ < OD-nil + 0.1 at both 24 h and 48 h

2.3.2 ElephantTB Stat Pak®

The ElephantTB Stat Pak® is a one-step lateral-flow test that uses selected *Mycobacterium tuberculosis* antigens (ESAT-6, CFP10, and MPB83) and a blue latex signal detection system for rapid detection of antibodies in serum or plasma samples. The test required 30 µl of elephant serum or plasma and 3 drops (~100 µl) s of sample buffer (included in the kit), which were added to the device sequentially. The results were read visually 20 min later and the results recorded.

2.3.2.1 Interpretation of test outcomes

A blue band visible in the test (T) window signalled a reactive and was considered an antibody-positive result. A blue band in the Control (C) window indicated a valid test. No band in the Test (T) window meant the serum was non-reactive.

2.3.3 DPP™VET TB Assay for Elephants

The DPP™VET TB Assay is a new-generation point-of-care test for TB in elephants developed using Chembio innovative DPP technology. The assay has two nitrocellulose strips that are connected in a “T” shape inside the device to allow independent delivery of test sample and antibody detecting reagent. The first strip receives a serum sample and buffer solution via the sample well. The diluted sample migrates toward the second strip, containing two test lines (MPB83 and CFP10/ESAT-6 printed as separate bands) and one control line.

Adding buffer to the conjugate well releases dried colloidal gold particles coupled with protein A/G and facilitates its migration along the second strip to the test area. If antibody is present in the sample, it binds to the immobilized test antigen, and the gold particles then react with this immune complex, thus making the test band visible. In the absence of detectable antibody, no specific immune complex would be formed on the test line, and therefore, no visible band would appear in the test area. The control band would develop, as the gold particles continue migrating along the second strip irrespective of the presence of antibody, ensuring correct performance of the test. The DPP™VET TB Assay was performed using 5 µl of elephant serum, 2 drops of buffer (~65 µl) in the sample well, and 4 drops of buffer in the conjugate well. The results were read after 15 min visually and recorded.

2.3.3.1 Interpretation of test outcomes

1. Three pink/purple lines, one line in the control area, one line in the test (1) area and one line in the test (2) area indicated a reactive result. This was an indication that the sample was reactive for TB.
2. A pink/purple test (2) line and a pink/purple control line was visible. This was an indication that the sample was reactive for TB.
3. A pink/purple test (1) line and a pink/purple control line were visible. This suggested that the sample was reactive for TB or mycobacteriosis. Test lines were considered reactive regardless of intensity.

2.3.4 PPD ELISA

Antigens used were *Mycobacterium bovis strain* AN5 culture filtrate purified protein derivative from the standard USDA bovine tuberculin modified protein 70, purified from *Mycobacterium. bovis* strain AN5; lipoarabinomannan antigen from the virulent Erdman strain of *Mycobacterium. tuberculosis*; lipoarabinomannan antigen from the virulent H37Ra strain of *Mycobacterium. tuberculosis*; and purified protein derivative from *Mycobacterium. avium*. Samples were diluted 1:100 and detected using non-species specific conjugates (Proteins A and G horseradish peroxidase). Seroreactivity was determined by measuring optic density. Duplicate trials were performed for each sample for each antigen. The mean value of the duplicate trials was subtracted from the mean value of blank controls, and an optic density ratio value (OD) was determined using a sample of *Mycobacterium bovis*-positive bovine serum.

2.3.5 Data analysis

Data obtained from the ElephantTB Stat Pak®, DPP™VET TB Assay and the Elephant specific IFN–γ assay were entered into an excel spreadsheet to form a database. The Stata software (Stata Statistical Software: Release 13 College Station, TX, StrataCorp LP) was used to analyse, describe, and summarize the data collected from this study. Criteria were set for cut off values for test validity for the e-IFN –γ assay. An algorithm for classifying animals into test positive and test negative was developed. Prof Michel (UP), Prof Rutten (Utrecht) and Prof Olea-Popelka (Colorado State University) assisted in the analysis of the research findings.

Classification was deducted from results of stimulation with PPD-B compared to stimulation with recombinant antigens during 24 and 48 hours. The McNemar's statistical test was used to compare the test agreement of immune responses elicited by after stimulation ESAT-6 and/or CFP10 and/or peptide cocktails > OD-nil + 0.1 for 24 and 48 hours The Kruskal-Wallis (Non-Parametric ANOVA) test was used to compare the median ages among elephants in different facilities.

Chapter 3
Results

3.1 Results of the diagnostic tests

3.1.1 Elephant specific Interferon gamma (e-IFN-γ) assay

The elephant specific interferon gamma release assay (e-IFN-γ assay) was used to measure cell-mediated immune responses as an indication of prior exposure to *Mycobacterium tuberculosis* complex in fifty African elephants in 2014 followed by re-measurement in a subset of 17 elephants in 2015. *(Results attached under table 3.1)*

Table 3.1 Summary of the elephant results obtained during the sample collection in 2014 and 2015

Animal ID	e-IFN-γ 2014	e-IFN-γ 2015	DPP VetTB	STAT-Pak	PPD ELISA
1	Negative		Negative	Negative	Negative
2	**Positive**	Suspect	Negative	Negative	Suspect
3	**Positive**	Suspect	Negative	Negative	Negative
4	Suspect	Invalid	Negative	Negative	Negative
5	Suspect		Negative	Negative	Negative
6	Negative		Negative	Negative	Negative
7	Suspect	Suspect	Negative	Negative	Negative
8	Suspect	Suspect	Negative	Negative	Negative
9	**Positive**	Suspect	Negative	Negative	Negative
10	Suspect		Negative	Negative	Negative
11	Suspect	**Positive**	Negative	Negative	Suspect
12	Suspect		Negative	Negative	Negative
13	Negative		Negative	Negative	Suspect
14	Suspect		Negative	Negative	Negative
15	Negative	Negative	Negative	Negative	Negative
16	**Positive**		Negative	Negative	Negative
17	Suspect		Negative	**Positive**	Negative
18	Negative		Negative	Negative	Negative
19	Suspect		Negative	**Positive**	Negative
20	Suspect		Negative	Negative	Negative
21	Suspect	Negative	Negative	Negative	**Positive**
22	Negative		Negative	Negative	Suspect
23	Negative		Negative	Negative	Negative
24	Negative		Negative	Negative	Negative
25	**Positive**	**Positive**	Negative	Negative	Negative
26	Negative		Negative	**Positive**	Suspect
27	Negative		Negative	**Positive**	Negative
28	Suspect		Negative	Negative	Suspect
29	Suspect		Negative	Negative	Negative
30	Suspect		Negative	**Positive**	Suspect
31	**Positive**		Negative	Negative	Negative
32	**Positive**	Suspect	Negative	Negative	Negative
33	Negative		Negative	Negative	Negative
34	Suspect	Suspect	Negative	**Positive**	Negative
35	**Positive**	Suspect	Negative	Negative	Negative
36	Suspect		Negative	Negative	**Positive**
37	Suspect	Negative	Negative	Negative	Negative
38	**Positive**	Suspect	Negative	Negative	Negative
39	Negative	Negative	Negative	Negative	Negative
40	**Positive**	**Positive**	**Positive**	Negative	Suspect
41	Negative		Negative	Negative	Negative
42	**Positive**		Negative	Negative	Negative
43	**Positive**		Negative	Negative	**Positive**
44	**Positive**		Negative	Negative	Negative
45	**Positive**		Negative	Negative	Negative
46	**Positive**		Negative	Negative	Negative
47	Suspect		Negative	Negative	Negative
48	Suspect		Negative	Negative	Negative
49	**Positive**		Negative	Negative	Negative
50	**Positive**		Negative	Negative	Negative

3.1.2 Interferon gamma responses to antigen stimulation

The mean value of OD values obtained from all whole blood samples stimulated with pokeweed mitogen 1.536 with a standard deviation of 0.69. Based on this analysis the cut-off for viability was determined as follows: Mean OD_{PWM} minus 2*stdev = 0.43. Test results for blood samples not meeting validity requirements and hence classified as invalid either due to OD_{nil} values above limit or due to OD_{PWM} values below the cut-off (see criteria table 2.4) were observed at a proportion of 4.7% (9/192) of all blood samples collected. In most cases only one of the blood samples incubated for either 24 hours or 48 hours was affected.

Following the reactor classification in three categories the response profiles obtained from the OD_{PPD-B} - $OD_{PPD-A,}$ ESAT 6 and CFP10 values in each reactor category (positive reactors , suspect reactors and PPD no reactor) were analysed statistically.

ESAT 6 values for positive reactors had a median value of 0.71 and an interquartile range of between 0.533 and 1.725. The highest ESAT 6 values were 2.273 and lowest was 0.140 for positive reactors. The median value for ESAT 6 suspect reactors was 0.268 and an interquartile range of between 0.097and 0.625. The median for non-reactors was 0.071 and a interquartile range of between 0.066and 0.112.The highest and lowest ESAT-6 readings were 0.188 and 0.0198

CFP10 values for positive reactors had a median value of 0.412 and an interquartile range of between 0.25 and 0.886. The highest CFP10 values were 1.77 and lowest was 0.068 for positive reactors. The median value for CFP10 non-reactors was 0.068 and an interquartile range of between 0.056 and 0.0915. The median for suspect reactors was 0.111 and an interquartile range of between 0.605 and 0.2215 The highest and lowest CFP10 readings were 0.026 and 0.642.

PPD reactors which, in connection with ESAT6 or CFP10 or peptide cocktail reactivity, were classified as e-IFN-γ positive animals had a median of OD_{PPD-B} - OD_{PPD-A} value of 0.37 and an interquartile range of between 0.2 and 0.4. The highest observation for OD_{PPD-B} - OD_{PPD-A} values was 0.69 and the lowest was 0.1 for positive reactors. The median OD_{PPD-B} - OD_{PPD-A} values for PPD non reactors was -0.02 and an interquartile range of between -0.034 and 0.1, with the lowest being -0.4.and the highest being 0.1. The median

for suspect reactors was 0.071 and an interquartile range of between -0.017 and 0.035. The highest and lowest observations were 0.10 and -0.25, respectively. the distributions are illustrated in fig 3.1.

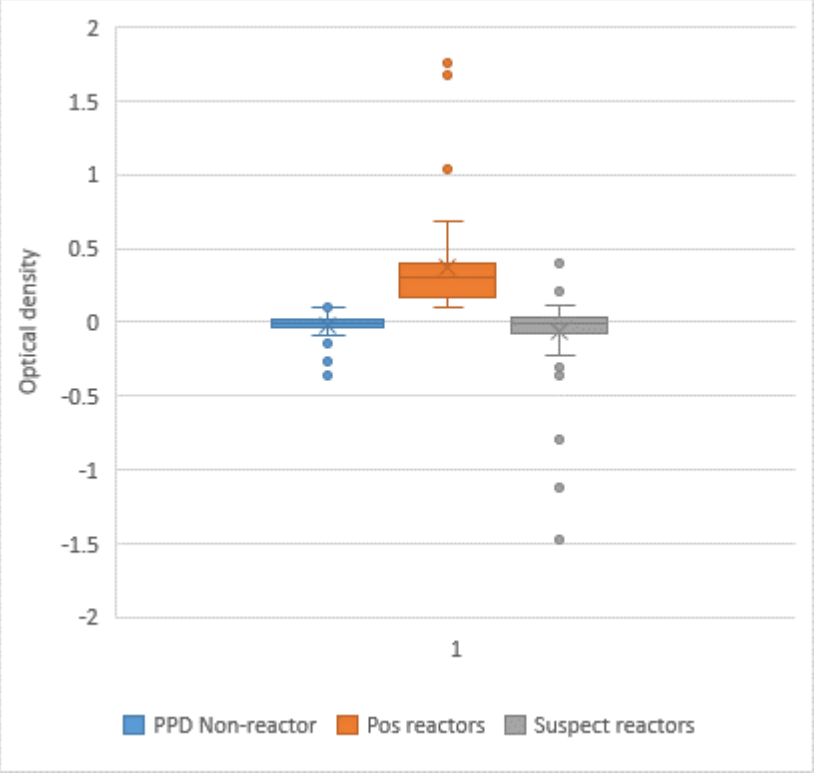

Figure 3.1 Box & Whisker plot to demonstrate the separation of OD_{PPD-B} - OD_{PPD-A} values based on the reactor classification in Table 3.2

In general using the criteria in table 3.2 the e-IFN-γ assay classified 34% (18/50) of the animals tested in 2014 as positive for prior exposure to *Mycobacterium tuberculosis* complex infection, 22 (44%) as suspect and 11 animals (22%) as negative.

Facility F (n=11) had the highest positive reactor rate of 7/9 elephants sampled, two were classified as suspect and none were classified as negative. Facility C (n=8) had no animals classified as test positive, four animals were classified in the suspect category; four animals were non-reactive. Facility A (n=12) had the largest number of animals in the test suspect category (5/12 animals). Thirty-two percent animals were classified as non-reactors with facilities C (n=4) 8% and D (n=4)8%.

Table 3.2 Reactor classification of fifty elephants per facility based on the e-IFN-γ assay tested in 2014

Property	e-IFNγ +	e-IFNγ sus	e-IFNγ neg	Total
A	4	5	3	12
B	1	1	2	4
C	0	4	4	8
D	4	3	4	11
E	2	2	2	6
F	7	1	1	9
Total	18	16	16	50

Table 3.2 and Fig 3.2 show reactor classification of fifty elephants per facility based on the elephant specific interferon gamma assay .as tested in 2014. Eighteen animals were identified as positive. Sixteen animals were identified as test negative. Facility F had the highest number of test positive animals. Facility C had no animals calssified as test positive. Facilities C and D had the highest number of animals classified at test negative animals. Facility A had the most number of animals classified as suspect reactors.

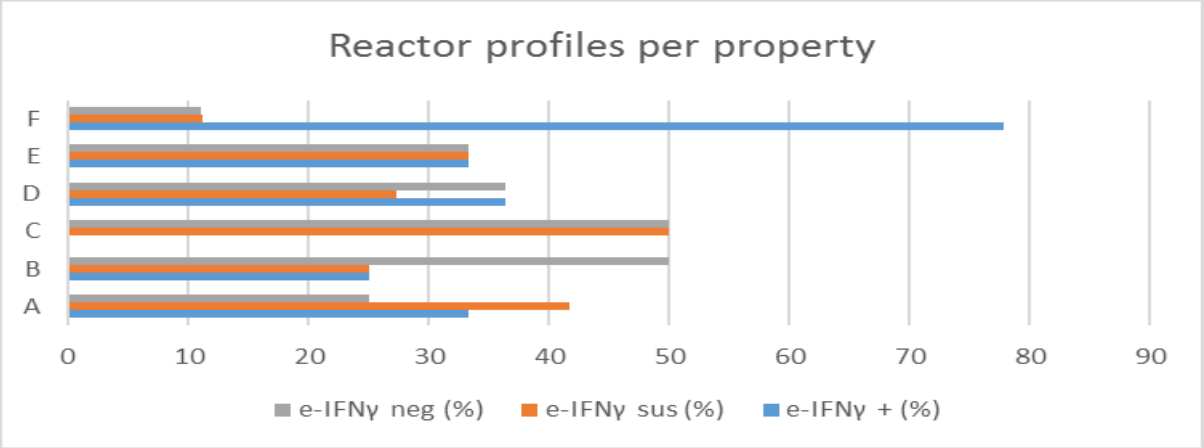

Figure 3.2 Reactor classification of fifty elephants per facility based on the e-IFN-γ assay tested in 2014 as derived from Table 3.3

As follow up, sampling and testing of a subset of seventeen of the fifty elephants were repeated in November 2015 (interval 14 months). The e-IFN-γ assay classified two animals as consistently test positive. Consistent test results were observed (Table 3.7) in 41% of the animals classified as either positive, suspect or negative. Six animals (35%) changed status from positive in 2014 to suspect in 2015. One animal (12 %) changed status from suspect to negative. Two animals (11 %) changed their test status from suspect to positive. The two animals that classified as negative, kept that status upon repeated testing. Seventy five pecent of the animals remained as reactors (positive or suspect), whereas two animals changed in classification from reactor to negative. Two animals remained non-reactors at both test intervals. None of the animals that were classified as positive changed status into negative. Nine animals yielded negative results in all the immunoassays used during the study. One animal gave an invalid result

Table 3.3 Classification of seventeen elephants from four facilities that were tested both in 2014 and 2015 using the e-IFN-γ assay

Test status 2014	Test status 2015	Number of elephants
Positive	Positive	2 (12%)
Positive	Suspect	6 (35%)
Positive	Negative	0 (0%)
Suspect	Suspect	3 (17%)
Suspect	Negative	2 (12%)
Suspect	Positive	1 (6%)
Negative	Negative	2 (12%)
Negative	Suspect	0 (0%)
Negative	Positive	0 (0%)
Invalid result		1 (6%)
Total tested twice		17 (100%)

The McNemar's test which was used to compare test agreement between the tuberculin induced immune response and the recombinant TB antigens showed that there was a significant difference ($p<0.0001$) (serious disagreement) between PPD B minus PPDA and ESAT-6 test classification after 24 hours of stimulation. There were 46 animals that showed a test result after calculation/interpretation to ESAT-6 stimulation but had no

response to OD_{PPD-B} – OD_{PPD-A}. There was one animal that showed a positive immune response to PPD-B but no response to ESAT-6 (Appendix 3).

To determine the agreement between tuberculin PPD and the recombinant antigens ESAT-6 and CFP-10 as markers the Kappa coefficient was used and found to be 0.3, indicating a “fair agreement” agreement between these two tests (Appendix 3).

Classification as deducted from results of stimulation with PPD-B was compared to stimulation with recombinant antigens after 48-hour stimulation. The McNemar’s test was used to compare the immune responses that were elicited by tuberculin PPD (PPD-Bovine tuberculin minus PPD-Avian tuberculin > 0.1 at 48 hours) and ESAT- 6 (ESAT-6 and/or CFP10 > 2 times OD-nil at 48 hours). There were 37 aliquots that showed immune responses to ESAT-6 compared to 2 aliquots that showed an immune response to tuberculin PPD. The McNemar’s test showed that there was a significant difference ($p<0.0001$) (serious disagreement) between these two markers at this test condition. The immune responses elicited by tuberculin PPD and ESAT-6 can therefore are not be used independently of each other if whole blood stimulation for 48 hours is used to classify an animal’s reactor category. The Kappa coefficient of 0.13 indicated a **“**slight agreement” agreement between these two markers at 48-hour stimulation (Appendix 3).

3.1.3 Elephant Stat Pak®

The one-step lateral-flow test ElephantTB Stat Pak® was used to test fifty elephants for tuberculosis in 2014. It detected antibodies specific for antigens shared by *Mycobacterium tuberculosis* and *Mycobacterium bovis* antigens in six (12%) of the animals, these were from facilities C (n=2) and D (n=4) as shown in Table 3.4. No antibody reactions (evident by a visible test line in the test device) were detected in the remaining 44 elephants (88%).

Table 3.4 ElephantTB Stat Pak® test results of fifty elephants from six facilities

Facility	Country	Positive	Negative
A	Zimbabwe	0	12
B	Zimbabwe	0	4
C	Zimbabwe	2 (ZIM 17&19)	6
D	Zimbabwe	4 (ZIM 26,27,30,34)	7
E	Zambia	0	6
F	Zambia	0	9
Total		6 (12%)	44 (88%)

3.1.4 DPP™VET TB Assay for Elephants

The DPP™VET TB Assay for elephants was used to test fifty elephants in 2014. In the assay 2% of the elephant population were classified as positive, namely one elephant from facility E (n=1) ZAM40. Ninety eight percent of the population were non-reactive in the assay. The positive response was obtained from an animal, which was positive in the e-IFN-γ assay.

3.1.5 PPD ELISA

A PPD ELISA was used to test for tuberculosis in fifty elephants of six different facilities in 2014. The PPD ELISA classified eight animals (16%) as suspect reactors, based on OD readings. 78% of the elephant population was classified as test negative. A total of 3 elephants were classified as test positive according to the criteria described. Facility A (n=3) and D (n=3) had the highest number of test suspect animals. All animals from facilities B were classified as non-reactive as shown in Table 3.5.

Table 3.5 PPD ELISA test results of fifty elephants collected from six facilities

Facility	Country	Positive	Suspect	Negative
A	Zimbabwe	0	3 (ZIM2,11,13)	9
B	Zimbabwe	0	0	4
C	Zimbabwe	1 (ZIM21)	1 (ZIM22)	6
D	Zimbabwe	0	3 (ZIM26,28,30)	8
E	Zambia	1 (ZAM36)	1 (ZAM40)	4
F	Zambia	1 (ZAM43)	0	8
Total		3 (6%)	8 (16%)	39 (78%)

3.2 Comparative analyses of test results of the e-IFN-γ assay and serological assays

A combination of the Logistic Regression analysis and the correlation coefficient was used to assess the test agreement between the DPP® VetTB, STAT- PAK®, PPD ELISA and e-IFN-γ in the detection of *Mycobacterium tuberculosis* complex infection.

Eighteen elephants were classified as positive based on the e-IFN-γ assay (34%, 95% CI: 11.6–36.4%), six based on the STAT- PAK® assay (12%, 95% CI: 5.6–30.4%), one in the DPP® VetTB (2%, 95% CI: 0–14.4%) and three in the PPD ELISA (6%, 95% CI: 0–18.4%).The e-IFN-γ assay detected one DPP® VetTB positive animal (ZAM 40) and one PPD ELISA (ZAM 43). For nine animals, all four immunoassays yielded a negative result.

3.2.1 Correlation coefficients for the four immunoassays used to test for Mycobacterium tuberculosis complex infection in fifty elephants

The results show that the e-IFN-γ results had no correlation with the DPP® VetTB (each with correlation coefficient, R, of 0) with a slight correlation with STAT- PAK® test results (correlation coefficient, R, of 0.2209) which was statistically significant. The correlation between DPP® VetTB results and STAT- PAK® results was statistically significant (correlation coefficient, R, of 0.0904). A slight correlation between DPP™VET TB Assay results and that of PPD ELISA was observed (correlation coefficient, R, of 0.3105). ElephantTB Stat Pak® and PPD ELISA results had no significant correlation (correlation coefficient, R, of 0.0273) as shown in table 3.6.

Table 3.6 Correlation coefficients for the four immunoassays used to test for *Mycobacterium tuberculosis* complex infection in fifty elephants

	e-IFN-γ	**DPP® VetTB**	**STAT- PAK®**	**PPD ELISA**
e-IFN-γ	1			
DPP® VetTB	0	1		
STAT- PAK®	0.229	0.0904	1	
PPD ELISA	0	0.3105	0.0273	1

The results in table 3.10 show the comparison of the test results between the cell-mediated test (e-IFN-γ) and amongst humoral tests (DPP® VetTB, STAT- PAK®, and PPD ELISA). Overall, statistical analysis of the test results of the tests DPP® VetTB, STAT-PAK®, PPD ELISA and e-IFN-γ indicated that these tests did not agree on the results of tuberculosis in the study population of African elephants.

3.3. Age distribution of elephants by facility

Descriptively, the mean age of the animals in the facility F was 30.9 years, older than that of elephants in the other five facilities. However, the Kruskal-Wallis (Non-Parametric ANOVA) test to compare the median ages (used in view of the small sample size) among elephants in different facilities indicated no significant difference between the median ages among elephants in different facilities (p=0.53).

Chapter 4
Discussion

4.1 Elephant specific Interferon gamma (e-IFN-γ) assay

The INF-γ assay platform measures the detectable levels of INF-γ after stimulation with various MTBC specific antigens and has been used successfully in many other species including cattle, badger, deer, (Vordermeier *et al*. 2001,2006), domestic cats (Rhodes *et al*. 2008), lions (Maas *et al*. 2012) rhinoceroses (Morar *et al*. 2007) and humans.

During *Mycobacterium tuberculosis* infection cell mediated immunity involves the production of pro-inflammatory cytokines IFN-γ and tumour necrosis factor (TNF) -α (Keane *et al*. 2001). Early secreted antigen 6 (ESAT-6) and culture filtrate protein 10 (CFP-10) are important antigenic proteins responsible for *Mycobacterium tuberculosis* virulence. ESAT-6 inhibits antigen presenting cell function by reducing IL-12 production by macrophages (Samten *et al.* 2009) by lysing epithelial cells and macrophages (Hsu *et al.* 2003 & Gao *et al.* 2004).

In cattle the Bovigam (Prionics AG, Zurich, Switzerland) has a varied sensitivity of between 81.8% and 100% for culture-confirmed bovine tuberculosis and specificity between 94% and 100% (Woods & Jones 2001).

In 2014, from the fifty elephants the e-IFN-γ assay detected immune responses following stimulation with either tuberculins PPDB and PPDA (as specificity control) or the recombinant MTBC antigens CFP 10 and ESAT 6 or both, indicative of *Mycobacterium tuberculosis* complex infection in 34% of the animals tested (18/50). The e-IFN-γ assay classified 22 (44%) as suspect and 11 animals (22%) as negative.

The assay done in 2014 gave an indication of the disease status of the fifty captive elephants that were sampled during the study, based on multiple stimulants (biomarkers) and test conditions (24 and 48 hour incubation periods, duplicate sample aliquots) used in a test algorithm for maximum reliability of test results. Additional data supporting the reactor classification based on the algorithm defined, were provided through repeat sampling and e-IFN-γ testing of 17 of the 50 study elephants in 2015. These data showed

that 41% (7/17) retained the same test status and none of the animals changed in test status from positive to negative after 14 months (table 3.7). The distribution of the test positive animals in the six different facilities that were sampled in 2014 showed that facility C had no test positive and facility F had 7 test positive out of a total of 9 elephants. For maximum test performance the immune responses elicited by tuberculin $OD_{PPD-B} – OD_{PPD-A}$ and CFP10/ESAT6 and the peptide cocktails should be used in combination if whole blood stimulation for 24 and 48 hours is used to classify an animal's reactor category.

In summary, this study found that the e -INF-γ might be a very useful test for the diagnosis of TB in elephants in early stages of the infection when clinical signs are absent.

4.2 Effect of elephant age on the e- IFN-γ reactor rate

Most of the wild elephants sampled during the study were brought in as orphans from culling operations carried out in Zimbabwe during the 1990s. Eighty- three percent of the elephants were born in the wild.The perception would have been older animals that had a prolonged period in captivity would have been at a greater risk of being e-IFN-γ assay reactors than younger animals. However, the study did not show any particular trend.

The zoonotic potential of *Mycobacterim tuberculosis* transmission between captive elephants and humans who work closely with elephants was first described in the late 1990s. An elevated risk of infection was found to be among those who had prolonged close contact with elephants, those who live inside an elephant barn, who do elephant treatments, necropsies or who participate in cleaning of the elephant barns (Davis, 2001 & Oh. *et al*. 2002).

4.3 Sero-diagnostic tests

Elephants infected with *Mycobacterium tuberculosis* have been shown to develop robust antibody responses, and serological tests like the ElephantTB Stat Pak® and the DPP™VET TB Assay for elephants have shown to be of value in the detecting the presence of infection in the advanced stages of the disease (Larsen *et al*. 2000, Lyashchenko *et al*. 2000,Greenwald *et al*. 2009). The serological tests used in the study

are frequently used in routine elephant TB screening and treatment monitoring. However, in the present study they did detect potential contact with MTBC in 10 elephants only and when applied in combination.

Overall, statistical analysis of the results of DPP® VetTB, STAT- PAK®, PPD ELISA and e-IFN-γ indicated that these tests did not agree on the results of tuberculosis in domesticated African elephants.For the comparison between the e-IFNy assay and the serological tests, disagreements were expected. Cell-mediated immunity during *Mycobacterium tuberculosis* infection is activated earlier than humoral immunity (Flynn *et al*. 1993), but also fades away earlier which may be responsible for disagreement between the cell-mediated and humoral-based test methods. It is generally accepted that the immune system's response to tuberculosis is mainly cell-mediated rather than humoral, so that serology cannot be expected to serve as more than a supplementary test in advanced cases. The e-IFN-γ classified the18 out of 50 study animals sampled in 2014 as having *Mycobacterium tuberculosis* complex infection.

Following the test results from the elephants sampled in 2014, the owners of the six facilities encouraged their employees to voluntarily get screened for tuberculosis at their nearest health facility. Of the total group of 50 handlers, 22 were voluntarily subjected to testing and four (18.2 %) were confirmed positive for tuberculosis and advised to undergo tuberculosis therapy. The exact prevalence of tuberculosis among the handlers remains unknown as the other 28 handlers did not heed the call to get tested.

Tuberculosis screening in captive elephants and elephant handlers is not a statutory requirement in both Zambia and Zimbabwe. The positive results findings from this study could help policy makers and elephant facility owners to appreciate risk and adopt a routine screening protocol to limit and manage the possible zoonotic spread of *Mycobacterium tuberculosis* between elephants and handlers.

It was interesting to note that two of the test positive handlers (sputum test) worked at the same facility in Zimbabwe. The other two TB test positive (chest x-ray) handlers worked at the same facility in Zambia. There were test positive elephants at the same facilities where test positive handlers were found. We could not manage to link the infection in the handlers and the elephant population. It is less likely that the elephants in the study could

have been exposed to infection though the regular contact with tourists during the elephant safari rides.

Mycobacterium tuberculosis is the most common cause of tuberculosis in humans and elephants although infection in birds and other mammals has been documented especially with a history of prolonged contact with humans (Saunders, 1983, Mikota *et al*. 1994 & Michalak *et al.* 1998). The assumption in this instance will be that the source of tuberculosis in the African elephants could be coming from the handlers, though further investigations is required.

The zoonotic potential of *Mycobacterium tuberculosis* transmission between captive elephants and humans who work closely with elephants was first described in the late 1990s. An elevated risk of infection was found to be among those who had prolonged close contact with elephants, those who live inside an elephant barn, who do elephant treatments, necropsies or who participate in cleaning of the elephant barns (Davis, 2001 & Oh. *et al.* 2002).Elephant and human isolates were observed to be identical. An elephant handler with active tuberculosis infection was suspected to be the source of infection to one of the Asian elephant that had died of confirmed *Mycobacterium tuberculosis. (*Oh *et al.* 2002*).*

4.4 Limitations

1. The positive e-INF-γ test status of 18 elephants could not be ascertained during the study given that we could not confirm infection by bacterial culture or PCR for MTBC
2. Owners of some of the facilities did not give consent to carry out follow up sampling on the suspected positive animals chiefly due to the innate stigma and fear associated with a diagnosis of *Mycobacterium tuberculosis* complex infection in their elephants and loss of business thereof.

4.5 Conclusion

In summary, the work done improves understanding of the ability of the various test methods to detect early *Mycobacterium tuberculosis* complex infection in captive elephants in the absence of clinical signs. The e-INF-γ assay though not validated, showed consistency in results obtained from blood stimulated for 24 and 48hours, respectively, duplicate samples from the same animals, stimulation with different TB antigens clustering of reactor animals or on-reactors according to facilities and the consistency of the reactor status when re-tested after 14 months.

The exact tuberculosis status of the fifty African elephants remains unknown but it is highly probable that certain facilities are infected with the *Mycobacterium tuberculosis* complex given the results shown by the e-INF-γ assay and the serological tests. Tuberculosis in elephants has never been reported nor studied in Zimbabwe though *Mycobacterium mungi* has been observed in other species like lions (*Panthera leo)* and the banded mongoose *Mungos mungo (*Unpublished reports, 2014).

Although the study was not designed to establish the route of transmission of the infection between the handlers and the visiting public,it is likely that (one or more of) the elephant handlers could have been the primary source of infection to the elephants given their prolonged close contact and published literature that supports this claim. Although, it cannot be excluded in case of short-term contact with infected individuals, from elephant to humans and elephants to elephants. The risk for tuberculosis transmission from an animal with a case of active tuberculosis, higher for daily handlers than for persons with only brief contact (Michalak *et al.* 1998), is unacceptable for animal and human welfare and may cause damage to the tourist industry. Four elephant handlers that were found to be positive for tuberculosis also add weight to this claim.

Mycobacterium tuberculosis was isolated from four elephants and four elephant handlers at an exotic animal farm in Illinois between 1994 and 1996. One handler from the twenty-two handlers that were screened had smear-negative, culture-positive active tuberculosis test result. DNA fingerprint comparison by IS6110 and TBN12 typing showed that the isolates from the four elephants and the handler with active tuberculosis were the same strain (Michalak *et al.* 1998). Possible animal-to-animal transmission was reported when

there was outbreak in a Swedish zoo between 2001 and 2003 in which elephants, buffaloes, rhinoceroses, and giraffes were involved. Five elephants and one giraffe were found to have been infected by four different strains of *Mycobacterium tuberculosis* (Lewerin *et al*. 2005).

Anti-tuberculosis therapy in elephants remains an option should animals be identified to have active disease through trunk wash culture. The effectiveness of tuberculosis treatment in elephants remains very doubtful and very expensive given the lengthy treatment periods and amount of drugs the animals have to take. (Mikota & Miller 2005).

4.6 Recommendations

Based on the preliminary data gathered in this study a standardised *Mycobacterium tuberculoisis* complex prevention programs should be a prerequisite for every elephant facility.

The elephant specific IFN- γ assay has shown promise in indicating the TB reactor status of the elephants in comparison with the serodiagnostic tests. For added benefit towards the validation of the test, it would be worthwhile that the e-IFN-γ assay be used in more elephants both wild and captive, and preferentially longitudinal, to help fully optimize the test. Using this assay in combination with serological assays might add to the sensitivity.

Animal health and human health authorities together (One health approach) in the two countries should consider the enforcement of protocols that reduce the zoonotic risk of transmission of *Myocobacterium tuberculosis* complex to and from animals in the captive elephant industry (Murphee *et al*. 2009). Such initiatives should include the regular tuberculosis screening and treatment of elephant handlers. Veterinary authorities should ensure an annual ante-mortem tuberculosis-screening programme for all elephants in captivity preferentially through e-IFNy testing combined with serological assays and trunk wash culture in case of positive or suspect resulys. Records of elephant tuberculosis screening results should be shared with public health authorities. Post-mortem examination of all elephants that die in captivity or euthanized should be checked by an experienced veterinary pathologists and screened for tuberculosis infection especially

suspicious elephants with unexplained weight loss, cough, rhinorrhoea or sudden death (Centers for Disease Control and Prevention 1994).

The threat of *Mycobacterium tuberculosis* complex infection getting into the wild population through close contact with the infected captive population during grazing and mating is a cause for concern. The African elephant population on the CITES classification except in Zimbabwe, Namibia and Botswana is listed under Appendix 1 (Animals threatened with extinction) The survival elephant species into the next millennium remains uncertain due to rampant poaching, habitat loss and human elephant conflict (Elephant stakeholder taskforce 2017). It is therefore imperative that authorities undertake this ethical conservation mandate and limit the possible risk of transmission to wild populations by early tuberculosis detection and control initiatives.

Chapter 5
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Appendix 1:
Animal Health Research Committee letter of approval

All communications should be addressed to
"Director Division of Veterinary Technical Services"

Telephone: 263-4-705885/6/7
Telefax: 263-4-794980/707952
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ZIMBABWE

Reference:

DIVISION OF VETERINARY TECHNICAL SERVICES
(Incorporating Veterinary Public Health, Diagnostics & Research)
MINISTRY OF AGRICULTURE, MECHANISATION AND IRRIGATION DEVELOPMENT
P O Box CY 551, Causeway
Harare

Dear Sir/Madam

Ref: To whom it may Concern

The Animal Health Research Committee of Zimbabwe has granted authority to Dr T G Hanyire to carry out his research on "**Pilot project to study prevalence of tuberculosis in domesticated African Elephant (*Loxodonta Africana*) and their handlers in the Victoria Falls and Livingstone areas.**" His research has been found to be in line with the requirements of the National animal welfare ethics.

Your consideration on his study will be greatly appreciated

Best Regards

28/05/2014

Dr P V Makaya

Chairperson Animal Health Research Committee

DIRECTOR
DEPARTMENT OF VETERINARY
TECHNICAL SERVICES
LIVESTOCK & VET. SERVICES DIVISION
28 MAY 2014
P.O. BOX CY 551, CAUSEWAY
HARARE ZIMBABWE

Appendix 2:
Animal Ethics committee approval letter

UNIVERSITEIT VAN PRETORIA
UNIVERSITY OF PRETORIA
YUNIBESITHI YA PRETORIA

Faculty of Veterinary Science
Animal Ethics Committee

Ref: V080-17

31 July 2017

Prof. A Michel
Department of Veterinary Tropical Diseases
Faculty of Veterinary Science
Onderstepoort
(anita.michel@up.ac.za)

Dear Prof. Michel

PROJECT V080-17
Pilot project to study the prevalence of TB in domesticated African elephant (TG Hanyire)

The application was evaluated by the Animal Ethics Committee of the University of Pretoria at the July 2017 meeting.

We have no ethical concern regarding the study done in Zimbabwe

If you have any questions, please feel free to contact the committee.

Yours sincerely

Prof V Naidoo
CHAIRMAN: UP-Animal Ethics Committee
Copy Dr Hanyire (Researcher)
Mrs. R Serfontein (DVTD – Post graduate coordinator)

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Fakulteit Veeartsenykunde
Lefapha la Diseanse tša Bongakadiruiwa

Appendix 3:
Elephant specific IFN-γ results for 2014 and 2015

Date	Incubation	Elephant name	Test interpretation per test date	Classified interpretation per test date	Final interpretation (parallel) per animal	PPD-B	PPD-A	B-A (corrected for M)	PWM	Nil control (med)	ESAT6-M	ESAT6-DI	CFP10	PC-EC	PC-HP	DPP	STAT-PAK	PPD ELISA	Facility	Age (yrs)	Sex
Sep-14	1-24H	Jake	NEG	NEG	NEG	0.098	0.119	-0.021	0.74	0.072	0.104	0.079	0.051	0.048	0.042	Neg	Neg	Neg	A	29	M
Sep-14	1-24H	Jake				0.103	0.102	0.001	0.736	0.056	0.077	0.065	0.038	0.035	0.028				A		
Sep-14	1-48H	Jake	Invalid			0.495	0.961	-0.466	2.729	1.342	0.173	0.181	0.088	0.05	0.047				A		
Sep-14	2-24H	Jock	POS	POS	POS	0.322	0.202	0.12	0.907	0.064	0.179	0.219	0.193	0.043	0.027	Neg	Neg	SUSP	A	31	M
Sep-14	2-24H	Jock				0.266	0.2	0.066	0.685	0.068	0.134	0.152	0.163	0.038	0.025				A		
Sep-14	2-48H	Jock				0.864	0.653	0.211	2.351	0.021	0.871	0.992	0.118	0.029	0.042				A		
Nov-15	2- 24H	Jock	SUSP ESAT 24 &48 HRS	SUSP		1.6245	1.5805	0.044	1.806	0.2785	0.814	0.8605				Neg	Neg		A		
Nov-15	2-48H	Jock	Invalid			0.594	0.689	-0.095	0.0165	0.0145	0.026	0.3235							A		
Sep-14	3-24H	Jumbo	POS	POS	POS	0.332	0.272	0.06	0.645	0.058	0.916	1.078	0.224	0.034	0.027	Neg	Neg	Neg	A	30	M
Sep-14	3-24H	Jumbo				0.299	0.253	0.046	0.642	0.053	1.099	0.995	0.193	0.035	0.029				A		
Sep-14	3-48H	Jumbo				1.679	1.328	0.351	2.596	0.018	2.393	2.341	1.191	0.026	0.028				A		
Nov-15	3-24H	Jumbo	SUSP ESAT 24 &48 HRS	SUSP		0.146	0.155	-0.009	1.7085	0.1275	0.12	0.6525				Neg	Neg		A		
Nov-15	3-48H	Jumbo				1.5945	1.5355	0.059	1.892	0.0135	1.355	1.8985							A		
Sep-14	4-24H	Coco	POS-48H	SUSP	SUSP	0.094	0.099	-0.005	0.761	0.068	0.051	0.063	0.041	0.041	0.032	Neg	Neg	SUSP	A	36	F
Sep-14	4-24H	Coco				0.093	0.117	-0.024	0.726	0.077	0.072	0.07	0.056	0.048	0.051				A		
Sep-14	4-48H	Coco				0.617	0.176	0.441	2.49	0.028	0.697	1.118	0.289	0.051	0.023				A		
Nov-15	4-24H	Coco	INVALID	SUSP		0.416	0.683	-0.267	1.3225	0.4585	0.142	0.31				Neg	Neg		A		
Nov-15	4-48H	Coco	SUSP ESAT 48 hrs			0.18	0.2445	-0.0645	2.2745	0.065	0.101	0.3155							A		
Sep-14	5-24H	Pfumo	SUSP-ESAT24H	SUSP	SUSP	0.046	0.062	-0.016	1.028	0.036	0.224	0.477	0.098	0.039	0.038	Neg	Neg	Neg	A	7	M
Sep-14	5-24H	Pfumo				0.05	0.052	-0.002	0.869	0.031	0.234	0.535	0.09	0.04	0.038				A		
Sep-14	5-48H	Pfumo				0.134	0.176	-0.042	2.543	0.071	0.091	0.157	0.046	0.071	0.089				A		
Sep-14	6-24H	Janet	INVALID	NEG	NEG	0.043	0.04	0.003	0.286	0.025	0.024	0.026	0.021	0.018	0.024	Neg	Pos	Neg	A	28	F
Sep-14	6-24H	Janet	INVALID			0.041	0.035	0.006	0.244	0.025	0.018	0.022	0.016	0.021	0.019				A		
Sep-14	6-48H	Janet	NEG			0.042	0.046	-0.004	2.309	0.013	0.019	0.024	0.013	0.012	0.014				A		
Sep-14	7A-24H	Ntombi	SUSP-ESAT24& 48H	SUSP	SUSP	0.047	0.06	-0.013	0.615	0.028	0.646	0.69	0.124	0.025	0.024	Neg	Neg	SUSP	A	3.5	F
Sep-14	7A-24H	Ntombi				0.036	0.048	-0.012	0.768	0.021	0.524	0.675	0.116	0.024	0.022				A		
Sep-14	7A-48H	Ntombi				0.086	0.116	-0.03	1.498	0.043	1.432	1.572	0.54	0.043	0.066				A		
Nov-15	7-24H	Ntombi	SUSP ESAT 24& 48HRS	SUSP		1.71	1.7405	-0.0305	2.045	0.0895	2.408	2.5285							A		
Nov-15	7-48H	Ntombi				1.587	1.683	-0.096	1.9205	0.0095	0.964	1.6865							A		
Sep-14	8A-24H	Tendai	SUSP-ESAT24/48H	SUSP	SUSP	0.044	0.052	-0.008	0.797	0.029	0.334	0.517	0.1	0.022	0.026	Neg	Neg	Neg	A	27	F
Sep-14	8A-24H	Tendai				0.043	0.057	-0.014	0.75	0.036	0.321	0.525	0.11	0.025	0.032				A		
Sep-14	8-48H	Tendai				0.172	0.438	-0.266	1.977	0.032	0.704	1.086	0.26	0.024	0.065				A		
Sep-14	8-48H	Tendai				0.165	0.435	-0.27	2.953	0.028	0.695	1.145	0.229	0.031	0.079				A		
Nov-15	8-24H	Tendai	SUSP PPD 24 HRS	SUSP		1.9655	1.8575	0.108	1.946	1.694	1.7465	1.7815							A		
Nov-15	8-48H	Tendai				0.284	0.34	-0.056	1.8585	0.0725	0.044	0.0845							A		
Sep-14	9A 24H	Ntembi	POS	POS	POS	0.06	0.117	-0.057	0.049	0.062	1.284	0.834	1.112	0.275	0.036	Neg	Neg	SUSP	A	8	F
Sep-14	9A 24H	Ntembi				0.053	0.093	-0.04	0.045	0.06	1.125	0.802	0.957	0.219	0.031				A		
Sep-14	9A-48H	Ntembi				0.263	0.063	0.2	1.699	0.034	1.491	1.404	0.586	0.037	0.19				A		
Sep-14	9A-48H	Ntembi				0.295	0.073	0.222	2.585	0.029	2.004	1.902	0.58	0.051	0.197				A		
Nov-15	9 24H	Ntembi	POS	POS		1.2285	0.902	0.3265	1.173	0.0355	0.45	0.462				Neg	Neg		A		

Nov-15	9-48H	Ntembi	SUSP	SUSP		2.259	2.174	0.085	2.028	2.3475	2.433	2.5825							A		
Sep-14	10A-24H	Naledi	SUSPP-ESAT24/48H	SUSP	SUSP	0.113	0.082	0.031	1.579	0.043	0.942	1.193	0.464	0.039	0.04	Neg	Neg	Neg	A	4	F
Sep-14	10A-24H	Naledi				0.116	0.081	0.035	1.51	0.044	0.923	1.117	0.397	0.043	0.043				A		
Sep-14	11A-24H	Emily	SUSPP-ESAT24 HRS	SUSP	POS	0.107	0.112	-0.005	0.798	0.039	0.598	0.921	0.311	0.03	0.022	Neg	Neg	SUSP	A	28	F
Sep-14	11A-24H	Emily				0.111	0.094	0.017	0.737	0.04	0.51	0.811	0.316	0.03	0.024	Neg	Neg		A		
Sep-14	11-48H	Emily				0.535	0.461	0.074	1.793	0.959	1.573	1.77	0.828	0.089	0.024				A		
Sep-14	11-48H	Emily				0.477	0.599	-0.122	2.802	1.325	2.199	2.665	1.112	0.137	0.046				A		
Nov-15	11-24H	Emily	POS	POS		1.5245	1.226	0.2985	3.3765	1.098	0.617	1.2415							A		
Nov-15	11-24H	Emily				1.1625	0.793	0.3695	2.345	0.649	0.465	0.7755							A		
Nov-15	11-48H	Emily				0.061	1.531	-1.47	1.521	0.0135	0.926	0.1455							A		
Sep-14	12A-24H	Ezibulo	NEG	NEG	NEG	0.066	0.061	0.005	1.133	0.037	0.072	0.084	0.054	0.029	0.027	Neg	Neg	Neg	A	12	M
Sep-14	12A24H	Ezibulo				0.059	0.062	-0.003	0.859	0.047	0.074	0.089	0.058	0.039	0.041	Neg	Neg		A		
Sep-14	12-48h	Ezibulo				0.063	0.129	-0.066	2.415	0.014	0.195	0.284	0.055	0.016	0.016				A		
Sep-14	13A24H	Mbanje	POS	POS	POS	0.024	0.083	-0.059	1.228	0.026	0.148	0.252	0.072	0.059	0.039	Neg	Neg	Neg	B	27	M
Sep-14	13A24H	Mbanje				0.047	0.086	-0.039	1.176	0.028	0.144	0.227	0.057	0.042	0.036				B		
Sep-14	13A48H	Mbanje				0.603	0.281	0.322	1.668	0.025	0.411	0.735	0.194	0.076	0.035				B		
Sep-14	14A24H	Dhampi	NEG	NEG	NEG	0.037	0.042	-0.005	0.907	0.029	0.042	0.06	0.03	0.032	0.029	Neg	Neg	Neg	B	21	F
Sep-14	14A24H	Dhampi				0.038	0.043	-0.005	0.867	0.029	0.043	0.054	0.028	0.028	0.026	Neg	Neg		B		
Sep-14	14-48H	Dhampi				0.053	0.107	-0.054	1.751	0.011	0.047	0.092	0.022	0.009	0.029				B		
Sep-14	15A24H	Lundi	NEG	NEG	NEG	0.047	0.069	-0.022	0.782	0.031	0.04	0.05	0.032	0.032	0.029	Neg	Neg	Neg	B	20	F
Sep-14	15A24H	Lundi				0.057	0.063	-0.006	0.816	0.028	0.04	0.046	0.028	0.03	0.029				B		
Sep-14	15-48H	Lundi				0.085	0.083	0.002	2.625	0.013	0.077	0.114	0.045	0.022	0.041				B		
Nov-15	15-48H	Lundi	NEG	NEG		0.283	0.297	-0.014	2.165	0.2105	0.18	0.169							B		
Nov-15	15-24H	Lundi				0.08	0.061	0.019	1.236	0.026	0.047	0.045							B		
Sep-14	16A24H	Nkanyezi	SUSP-ESAT48H	SUSP	SUSP	0.037	0.046	-0.009	0.718	0.033	0.03	0.098	0.034	0.039	0.029	Neg	Neg	Neg	B	24	F
Sep-14	16A24H	Nkanyezi				0.016	0.012	0.004	0.765	0.007	0.016	0.067	0.02	0.047	0.034				B		
Sep-14	16-48h	Nkanyezi				0.198	0.207	-0.009	2.42	0.009	0.54	1.048	0.132	0.013	0.047				B		
Sep-14	17A24H	Dombo	SUSP-ESAT48H	SUSP	SUSP	0.095	0.19	-0.095	0.797	0.044	0.098	0.186	0.077	0.035	0.032	Neg	Pos	Neg	C	40	M
Sep-14	17A24H	Dombo				0.091	0.172	-0.081	0.852	0.042	0.092	0.163	0.069	0.029	0.029				C		
Sep-14	17-48H	Dombo				0.167	0.419	-0.252	2.351	0.015	0.29	0.742	0.189	0.013	0.024				C		
Sep-14	18A24H	Apongozi	INVALID	NEG	NEG	0.052	0.064	-0.012	0.191	0.038	0.035	0.058	0.038	0.041	0.034	Neg	Neg		C	33	F
Sep-14	18A24H	Apongozi	INVALID			0.056	0.065	-0.009	0.173	0.04	0.033	0.055	0.027	0.035	0.027				C		
Sep-14	18A48H	Apongozi	NEG			0.139	0.233	-0.094	1.187	0.023	0.086	0.09	0.05	0.036	0.032				C		
Sep-14	19A24H	Janet	NEG	NEG	NEG	0.045	0.044	0.001	0.476	0.049	0.04	0.023	0.055	0.034	0.03	Neg	Susp	Neg	C	16	F
Sep-14	19A24H	Janet				0.044	0.04	0.004	0.461	0.046	0.041	0.02	0.044	0.038	0.022				C		
Sep-14	19-48H	Janet				0.178	0.154	0.024	2.447	0.036	0.057	0.092	0.037	0.043	0.067				C		
Sep-14	20A24H	Mana	SUSP-ESAT48H	SUSP	SUSP	0.045	0.081	-0.036	0.515	0.094	0.06	0.027	0.047	0.041	0.026	Neg	Neg	Neg	C	18	F
Sep-14	20A24H	Mana				0.043	0.079	-0.036	0.456	0.113	0.07	0.041	0.067	0.051	0.033				C		
Sep-14	20-48H	Mana				0.162	0.155	0.007	2.49	0.045	0.214	0.509	0.219	0.053	0.075				C		
Sep-14	21-24H	Houdini	SUSP-ESAT24H	SUSP	SUSP	0.164	0.167	-0.003	2.102	0.04	0.066	1.14	0.059	0.044	0.056	Neg	Neg	Neg	C	22	M
Sep-14	21-24H	Houdini				0.154	0.168	-0.014	1.936	0.027	0.045	1.177	0.041	0.026	0.033				C		
Sep-14	21-24H	Houdini				0.106	0.128	-0.022	2.507	0.018	0.046	0.867	0.037	0.037	0.047				C		
Sep-14	21A-48H	Houdini				0.144	0.286	-0.142	1.771	0.028	0.031	0.054	0.025	0.076	0.034				C		
Sep-14	21A-48H	Houdini				0.062	0.291	-0.229	2.442	0.032	0.044	0.075	0.05	0.106	0.056				C		
Nov-15	21-24H	Houdini	NEG	NEG		0.0425	0.0565	-0.014	1.279	0.0625	0.0265	0.0955							C		
Nov-15	21A-48H	Houdini				0.064	0.068	-0.004	1.5065	0.065	0.0305	0.0855							C		
Sep-14	22A-24H	Temba	NEG	NEG	NEG	0.142	0.104	0.038	1.428	0.028	0.105	0.143	0.069	0.029	0.044	Neg	Neg	Neg	C	14	M
Sep-14	22A-24H	Temba				0.147	0.111	0.036	1.519	0.025	0.13	0.158	0.08	0.026	0.042				C		
Sep-14	22-48H	Temba				0.186	0.105	0.081	1.701	0.02	0.055	0.073	0.072	0.025	0.105				C		

Sep-14	23A-24H	Kumbula	SUSP-ESAT 24 HRS	SUSP	SUSP	0.272	0.36	-0.088	2.003	0.03	0.199	0.198	0.115	0.032	0.037	Neg	Pos	Neg	C	15	F
Sep-14	23A-24H	Kumbula				0.264	0.355	-0.091	1.915	0.031	0.176	0.203	0.102	0.027	0.038				C		
Sep-14	23A-48H	Kumbula				0.206	0.276	-0.07	1.678	0.037	0.147	0.128	0.079	0.036	0.046				C		
Sep-14	24A-24	Mondi	NEG	NEG	NEG	0.113	0.084	0.029	1.245	0.037	0.069	0.124	0.074	0.03	0.039	Neg	Pos	Neg	C	5	M
Sep-14	24A-24	Mondi				0.131	0.083	0.048	1.242	0.046	0.078	0.139	0.08	0.039	0.049				C		
Sep-14	24-48H	Mondi				0.056	0.043	0.013	1.28	0.039	0.063	0.066	0.048	0.058	0.045				C		
Sep-14	25A-24H	Tusker	POS	POS	POS	0.966	0.396	0.57	1.683	0.049	1.053	0.817	0.544	0.08	0.033	Neg	Neg	SUSP	D	42	M
Sep-14	25A-24H	Tusker				0.911	0.372	0.539	1.669	0.038	1.009	0.77	0.532	0.074	0.029				D		
Sep-14	25-48H	Tusker	SUSP			0.137	0.176	-0.039	2.027	0.041	0.921	0.826	0.393	0.03	0.159				D		
Nov-15	25-24H	Tusker	POS	POS		2.512	1.469	1.043	3.101	0.5565	1.761	0.842				Neg	Neg		D		
Nov-15	25-48H	Tusker	INVALID			0.938	1.2425	-0.3045	0.0565	0.055	0.0925	0.289							D		
Sep-14	26A-24H	Dhoma	NEG	NEG	NEG	0.354	0.393	-0.039	0.715	0.659	0.193	0.427	0.199	0.031	0.028	Neg	Susp	Neg	D	42	M
Sep-14	26A-24H	Dhoma				0.364	0.386	-0.022	0.623	0.622	0.189	0.352	0.202	0.03	0.021				D		
Sep-14	26A-48H	Dhoma				0.178	0.51	-0.332	1.106	0.021	0.121	0.252	0.105	0.044	0.038				D		
Sep-14	27A-24H	Moka	NEG	NEG	NEG	0.19	0.084	0.106	0.914	0.332	0.103	0.193	0.072	0.3	0.061	Neg	Susp	Neg	D	43	M
Sep-14	27A-24H	Moka				0.178	0.078	0.1	0.905	0.332	0.101	0.191	0.07	0.291	0.061				D		
Sep-14	27A-48H	Moka				0.144	0.072	0.072	1.06	0.022	0.031	0.04	0.033	0.099	0.129				D		
Sep-14	28A-24H	Tatu	SUSP-ESAT24H	SUSP	SUSP	0.143	0.112	0.031	0.769	0.065	0.187	0.208	0.155	0.049	0.047	Neg	Neg	Neg	D	39	M
Sep-14	28A-24H	Tatu				0.186	0.124	0.062	0.72	0.071	0.224	0.23	0.17	0.058	0.05				D		
Sep-14	28-48H	Tatu				0.079	0.081	-0.002	0.433	0.031	0.051	0.066	0.029	0.04	0.083				D		
Sep-14	29A-24H	Mainos	NEG	NEG	NEG	0.068	0.062	0.006	0.939	0.038	0.051	0.066	0.053	0.049	0.041	Neg	Susp	Neg	D	18	F
Sep-14	29A-24H	Mainos				0.056	0.048	0.008	0.963	0.023	0.028	0.039	0.034	0.028	0.025				D		
Sep-14	29-48H	Mainos				0.056	0.035	0.021	0.628	0.028	0.023	0.046	0.02	0.033	0.034				D		
Sep-14	30A-24H	Dhetema	SUSP-ESAT24H	SUSP	SUSP	0.247	0.189	0.058	1.337	0.021	0.423	0.492	0.294	0.025	0.025	Neg	Susp	Neg	D	16	M
Sep-14	30A-24H	Dhetema				0.248	0.175	0.073	1.252	0.018	0.41	0.47	0.284	0.025	0.025				D		
Sep-14	30A-48H	Dhetema				0.273	0.348	-0.075	1.485	0.016	0.205	0.268	0.172	0.019	0.036				D		
Sep-14	31A-24H	Ladoma	POS	POS	POS	0.365	0.123	0.242	1.127	0.052	0.288	0.454	0.184	0.016	0.027	Neg	Neg	Neg	D	16	M
Sep-14	31A-24H	Ladoma				0.314	0.091	0.223	1.022	0.046	0.25	0.359	0.163	0.018	0.029				D		
Sep-14	31-48H	Ladoma				0.397	0.216	0.181	1.02	0.043	0.61	0.563	0.15	0.188	0.047				D		
Sep-14	31-48H	Ladoma				0.377	0.208	0.169	1.063	0.03	0.577	0.439	0.083	0.156	0.046				D		
Sep-14	32A-24H	Hwange	POS	POS	POS	0.576	0.162	0.414	1.389	0.114	1.785	1.64	1.608	0.279	0.05	Neg	Neg	susp	D	16	F
Sep-14	32A-24H	Hwange				0.503	0.152	0.351	1.05	0.11	1.754	1.214	1.491	0.216	0.065				D		
Sep-14	32-48H	Hwange				0.2	0.145	0.055	1.802	0.266	1.259	1.221	1.038	0.423	0.033				D		
Sep-14	32-48H	Hwange				0.17	0.147	0.023	1.764	0.262	1.198	1.203	1.033	0.42	0.034				D		
Sep-14	32-48H	Hwange				0.169	0.129	0.04	1.723	0.277	1.118	1.384	1.196	0.444	0.045				D		
Nov-15	32-24H	Hwange	SUSP ESAT 48 HRS	SUSP		1.764	2.156	-0.392	3.339	1.807	2.9115	1.4915				Neg	Neg		D		
Nov-15	32-48H	Hwange				0.3105	0.3755	-0.065	2.124	0.13	0.136	0.572							D		
Sep-14	33A-24H	Masuwe	NEG	NEG	NEG	0.203	0.18	0.023	1.393	0.081	0.083	0.106	0.08	0.051	0.386	Neg	Pos	Neg	D	15	F
Sep-14	33A-24H	Masuwe				0.197	0.169	0.028	1.347	0.075	0.082	0.101	0.075	0.045	0.4				D		
Sep-14	33A-48H	Masuwe				0.302	0.296	0.006	1.23	0.053	0.068	0.071	0.051	0.077	0.134				D		
Sep-14	34A-24H	Kariba	SUSP-ESAT24/48H	SUSP	SUSP	0.245	0.225	0.02	1.455	0.09	0.725	0.749	0.599	0.038	0.043	Neg	Pos	susp	D	14	F
Sep-14	34A-24H	Kariba				0.225	0.229	-0.004	1.415	0.09	0.713	0.786	0.64	0.044	0.042				D		
Sep-14	34A-48H	Kariba				0.294	0.336	-0.042	1.615	0.025	0.798	0.856	0.646	0.026	0.041				D		
Nov-15	34-24H	Kariba	SUSP-ESAT24/48H	SUSP		0.3695	0.749	-0.3795	2.3445	0.451	0.925	1.4065				Neg	Neg		D		
Nov-15	34-48H	Kariba				1.788	1.9105	-0.1225	2.1805	2.2295	2.4615	2.5625							D		

Sep-14	35A-24H	Deka	POS	POS	POS	0.288	0.139	0.149	1.334	0.019	0.915	1.411	0.22	0.139	0.065	Neg	Neg	susp	D	12	M
Sep-14	35A-24H	Deka				0.297	0.127	0.17	1.287	0.018	0.907	1.409	0.246	0.163	0.069				D		
Sep-14	35-48H	Deka				0.262	0.103	0.159	1.48	0.044	0.624	0.938	0.086	0.06	0.08				D		
Nov-15	35-24H	Deka	SUSP ESAT 24HRS	SUSP		2.4905	2.511	-0.0205	2.5195	0.0305	0.525	0.795				Neg	Neg		D		
Nov-15	35-48H	Deka				0.0315	0.027	0.0045	1.425	0.0135	0.022	0.035							D		
Sep-14	36A-24H	Bonface	SUSP-ESAT48H	SUSP	SUSP	0.139	0.091	0.048	1.528	0.016	0.163	0.143	0.062	0.024	0.023	Neg	Neg	Neg	E	29	M
Sep-14	36A-24H	Bonface				0.128	0.092	0.036	1.486	0.018	0.17	0.134	0.056	0.027	0.017				E		
Sep-14	36-48H	Bonface				0.271	0.172	0.099	2.282	0.051	0.202	0.531	0.088	0.089	0.118				E		
Sep-14	37A-24	Sondela	SUSP-ESAT24/48H	SUSP	SUSP	0.141	0.12	0.021	1.559	0.036	0.267	0.45	0.166	0.038	0.076	Neg	Neg	Neg	E	18	M
Sep-14	37A-24H	Sondela				0.14	0.119	0.021	1.515	0.031	0.267	0.149	0.164	0.033	0.078				E		
Sep-14	37-48H	Sondela				0.15	0.147	0.003	0.663	0.045	0.164	0.435	0.074	0.021	0.086				E		
Nov-15	37-24H	Sondela	NEG	NEG		0.5995	0.507	0.0925	1.4955	0.013	0.0155	0.03							E		
Nov-15	37-48H	Sondela				0.0515	0.092	-0.0405	1.5925	0.0155	0.0345	0.061							E		
Sep-14	38A-24H	Mary	POS	POS	POS	1.142	0.452	0.69	1.928	0.072	0.528	0.61	0.492	0.585	0.967	Neg	Susp	Neg	E	22	F
Sep-14	38A-24H	Mary				1.09	0.461	0.629	1.885	0.078	0.518	0.616	0.498	0.618	0.968				E		
Sep-14	38A-48H	Mary				1.282	0.74	0.542	1.954	0.03	0.528	0.643	0.174	0.383	1.111				E		
Nov-15	38-24H	Mary	SUSP PPD 48 HRS	SUSP		0.4105	0.393	0.0175	1.5795	0.2975	0.024	0.1715				Neg	Susp		E		
Nov-15	38-48H	Mary				1.4535	0.729	0.7245	1.8735	0.046	0.0535	0.063							E		
Sep-14	39A-24H	Themba	NEG	NEG	NEG	0.077	0.068	0.009	1.662	0.033	0.115	0.161	0.032	0.025	0.045	Neg	Neg	Neg	E	16	F
Sep-14	39A-24H	Themba				0.077	0.068	0.009	1.647	0.03	0.108	0.15	0.035	0.033	0.04				E		
Sep-14	39-48H	Themba	INVALID			0.079	0.067	0.012	1.924	0.816	0.047	0.028	0.11	0.023	0.063				E		
Nov-15	39-24H	Themba	NEG	NEG		0.043	0.0375	0.0055	0.864	0.027	0.0225	0.026							E		
Nov-15	39-48H	Themba				0.2465	0.117	-0.03	1.6305	0.1935	0.131	0.097							E		
Sep-14	40A-24H	Mouse	POS		POS	0.353	0.167	0.186	1.774	0.042	0.159	0.358	0.07	0.032	0.034	Pos	Neg	Neg	E	16	F
Sep-14	40A-24H	Mouse				0.354	0.186	0.168	1.678	0.047	0.147	0.314	0.067	0.036	0.042				E		
Sep-14	40A-48H	Mouse				0.223	0.584	-0.361	1.754	0.035	0.12	0.194	0.279	0.029	0.051				E		
Nov-15	40A-24H	Mouse 1 24h 2015				0.986	0.7785	0.2075	1.37	0.0195	0.052	0.153				Neg	Neg		E		
Nov-15	40A-48H	Mouse (1) 48h 2015				0.165	0.27	-0.105	2.0325	0.021	0.0655	0.0655							E		
Nov-15	40A-24H	Mouse 2 24h 2015				1.598	1.541	0.057	1.5185	0.014	0.8325	1.2605							E		
Nov-15	40A-48H	Mouse (2) 48h 2015				0.5625	0.16	0.4025	2.2935	0.0705	0.0745	0.128							E		
Sep-14	41-24H	Tata	NEG	NEG	NEG	0.067	0.087	-0.02	1.554	0.057	0.076	0.066	0.066	0.044	0.048	Neg	Pos	Neg	E	13	F
Sep-14	41-24H	Tata				0.069	0.084	-0.015	1.616	0.047	0.058	0.065	0.057	0.033	0.046				E		
Sep-14	41-48H	Tata				0.094	0.069	0.025	1.755	0.042	0.095	0.056	0.036	0.03	0.058				E		
Sep-14	42-24H	Bop	POS	POS	POS	0.36	0.255	0.105	1.834	0.047	0.547	0.761	0.314	0.043	0.036	Neg	Neg	Neg	F	60	M
Sep-14	42-24H	Bop				0.409	0.266	0.143	1.811	0.05	0.605	0.772	0.307	0.039	0.051				F		
Sep-14	42-48H	Bop				0.722	0.549	0.173	2.462	0.106	0.486	0.443	0.267	0.053	0.035				F		
Sep-14	43-24H	Danny	POS	POS	POS	2.195	0.432	1.763	1.952	0.77	2.171	2.083	1.759	0.98	0.617	Neg	Neg	Neg	F	55	M
Sep-14	43-24H	Danny				2.116	0.436	1.68	1.966	0.737	2.175	2.099	1.785	0.934	0.62				F		
Sep-14	43-48H	Danny				1.685	1.676	0.009	2.601	0.043	2.269	2.474	1.79	0.084	1.163				F		
Sep-14	44-24H	Marula	POS	POS	POS	0.39	0.2	0.19	2.083	0.038	0.675	0.658	0.339	0.027	0.027	Neg	Neg	Neg	F	35	M
Sep-14	44-24H	Marula				0.42	0.172	0.248	2.205	0.049	0.663	0.583	0.331	0.038	0.038				F		
Sep-14	44-48H	Marula				0.398	1.196	-0.798	2.591	0.02	0.801	0.701	0.269	0.188	0.028				F		
Sep-14	45-24H	Madinda	POS	POS	POS	0.653	0.294	0.359	1.627	0.076	0.506	0.759	0.405	0.052	0.056	Neg	Pos	Neg	F	35	M
Sep-14	45-24H	Madinda				0.667	0.283	0.384	1.64	0.062	0.505	0.716	0.359	0.043	0.051				F		
Sep-14	45-48H	Madinda				0.638	0.601	0.037	2.822	0.026	0.792	1.039	0.472	0.027	0.08				F		

Sep-14	46-24H	Mashumbi	POS	POS	POS	1.023	0.682	0.341	1.706	0.078	0.146	0.397	0.091	0.025	0.021	Neg	Neg	Neg	F	35	F
Sep-14	46-24H	Mashumbi				1.025	0.631	0.394	1.688	0.074	0.145	0.388	0.076	0.032	0.021				F	35	F
Sep-14	46-48H	Mashumbi				0.275	0.557	-0.282	2.613	0.066	0.082	0.189	0.038	0.018	0.02				F		
Sep-14	47-24H	Liwa	NEG	NEG	NEG	0.111	0.134	-0.023	1.653	0.018	0.122	0.141	0.046	0.017	0.034	Neg	Pos	Neg	F	10	F
Sep-14	47-24H	Liwa				0.111	0.134	-0.023	1.721	0.019	0.128	0.139	0.046	0.017	0.035				F	8	F
Sep-14	47-48H	Liwa				0.142	0.156	-0.014	2.609	0.053	0.1	0.087	0.092	0.047	0.138				F		
Sep-14	48-24H	Chavaruka	SUSP-ESAT48H	SUSP	SUSP	0.488	0.393	0.095	1.226	0.027	0.064	0.117	0.057	0.032	0.022	Neg	Susp	Neg	F	5	M
Sep-14	48-24H	Chavaruka				0.536	0.446	0.09	1.339	0.038	0.073	0.133	0.066	0.041	0.036				F		
Sep-14	48-48H	Chavaruka				0.792	1.918	-1.126	2.333	0.1	0.296	0.745	0.105	0.049	0.352				F		
Sep-14	49-24H	Nandi	POS	POS	POS	0.823	0.522	0.301	2.369	0.096	1.731	2.12	0.945	0.06	0.061	Neg	Pos	Neg	F		
Sep-14	49-48H	Nandi				1.236	1.384	-0.148	2.319	0.079	2.194	2.344	1.523	0.043	0.165				F		
Sep-14	50-24H	Sekuti	POS	POS	POS	0.369	0.228	0.141	1.789	0.077	2.044	2.295	0.742	0.095	0.073	Neg	Neg	Neg	F		
Sep-14	50-48H	Sekuti				0.457	0.288	0.169	1.789	0.139	1.853	2.071	1.183	0.156	0.202				F		