Infectious Diseases Management in Asian Elephant : TB and EEHV-HD

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Infectious Diseases Management in Asian Elephants: TB and EEHV-HD

Infectieziekten Management in Aziatische Olifanten: TB en EEHV-HD (met een samenvatting in het Nederlands)

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To my mother and family

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General Introduction

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General Introduction

Conservation status of Asian elephants

The Asian elephant *(Elephas maximus)* is a mega-herbivore listed in the Appendix I of the Convention on International Trade in Endangered Species of Wild Fauna and Flora (CITES) and qualified as endangered species in appendix I. Eight Asian countries with wild elephants have ratified CITES (Bangladesh, China, India, Indonesia, Malaysia, Nepal, Sri Lanka and Thailand). Currently, around 40,000-50,000 Asian elephants live in 13 countries widely spread around 500,000 km^2 (Blake and Hedges, 2004; Choudhury, 2008). Another 13,000 elephants are maintained under human care (Asian Elephant Range States Meeting Report, 2017), where they are important culturally and economically.

Table 1. Estimated distribution of elephants in range countries (Asian Elephant Range State Meeting Report, 2017).

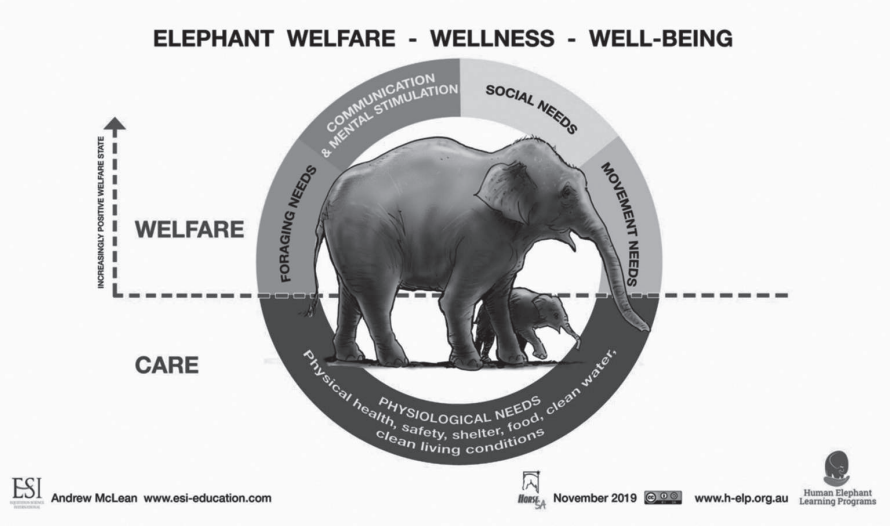
Country	Wild population	Captive population
Bangladesh	210-330	96
Bhutan	513	9
Cambodia	400-600	70
China	300	243
India	29,391-30,711	3,467-3,667
Indonesia	1,724	467
Lao PDR	600-800	454
Malaysia		
-! Peninsular	1,223-1,677	92
-! Sabah	2,040	23
Myanmar	2,000	4,382
Nepal*	70-170	171
Sri Lanka	5,879	230
Thailand	3,100-3,600	3,783
Vietnam	104-132	88
Total	47,554-50,580	13,575-13,775

*Nepal country report by Saragusty (2012)

At least since 4,000 years ago, wild elephants were caught and tamed, perhaps as many as 2-4 four million in that time span; about 100,000 have been captured in the last century alone (Sukumar, 1992). Asian elephants have been used for a variety of purposes, such as in the 5 timber and tourist industries (Santiapillai and Jackson, 1990; Lair, 1997). In addition, elephants have been kept in circuses, zoos and temples for religious ceremonies. One concern

today is that not all of these venues care for elephants in a way that meets their welfare needs or gives them a good quality of life (Bansiddhi et al., 2018). No matter where elephants live or what they are used for, management must meet physiological and psychological needs. Elephants need proper environments, safety, health care, mental stimulation, social interaction, and ability to express natural behavior. The needs of elephants are illustrated in Fig. 1.

Figure 1. Factors important to elephant welfare and well-being in captivity (McLean, 2019).



Currently, captive elephants in South East Asia (SEA) are managed under two systems: intensive and extensive; see Table 2. The physical requirements in an intensive management system were defined by J. Lehnhardt (in Fowler and Mikota, 2006), and concerns elephants kept in temples, tourist camps (mainly in cities) and villages, of which basic requirements, including food, water, and shelter are all provided by humans. The other system, extensive management, was described by Puangkham et al. (2005) and applies to elephants often found in logging camps, more natural eco-tourist camps, and patrol elephants in national parks or wildlife sanctuaries. In an extensive system, most food and water are available through freeroaming in natural habitats and are not generally provided by humans. Extensive elephant management systems have declined in recent years because there is not enough habitat for elephants, which is shared with livestock, wildlife, humans, and in some areas by wild elephants. Competition for shrinking habitats causes conflicts between humans and elephants, and is high throughout most elephant range countries. By contrast, elephants managed in intensive systems require less space as most of their provisions are provided from outside sources; they do not need to forage or be kept in large areas of land.

Almost all captive elephants in SEA are under a direct contact system of management (i.e., free contact), in which elephants and humans share the same space and are trained and managed by vocal commands and physical contact. Traditional methods of training young elephants typically involved the use of chains or ropes for restraint, food-water-sleep

deprivation, and beatings to get them to obey the commands of humans (Saragusty, 2012). These techniques are cruel and today are considered unethical and out of date. Rather, more elephant friendly training methods that are less fear-based are now being used to improve elephant welfare and life quality, and are safer to humans and elephants alike. For example, current taming methods utilize both negative and positive reinforcement to elicit proper behaviors, balancing elephant welfare and mahout training needs in free contact situations (McLean, 2019). However, mahouts (i.e., handlers) need to be highly skilled to maintain control in a more positive, less punitive way, to avoid increased risk of injuries or death (Saragusty, 2012). Therefore, modern western zoos are now managing elephants under a protected contact system to minimize negative interactions when humans and elephants share the same space (Maslow in Fowler and Mikota, 2006). Limiting contact between humans and elephants is intended to reduce the incidence of injuries to human caretakers. On the other hand, this system can make things more difficult because elephants can choose not to participate in activities, including those for medical procedures. Thus, training elephants with positive methods and establishing strong bonds, creating a relaxed training environment building good habits with repetition, is important for welfare (McLean, 2019). Captive elephants in range countries perform activities on command, often in association with work or other tasks. Encouraging elephants to display more natural behaviors is more acceptable today, and has been initiated in several places that allow elephants more free time, without chains, with social opportunities. However, it is important that elephants are trained for veterinary procedures, including drug administration, venipuncture, oral/trunk examination, foot care, vagina/rectal examination, urine collection and trunk wash sampling, regardless of whether elephants are managed under direct or protected contact. Recently, the use of protected or no contact systems are gaining popularity in Asia, particularly for bulls and un-tamed elephants, to avoid injury or even death of handlers.

An important consideration for elephant welfare is meeting social needs to avoid mental problems. In the past, a mahout and elephant might spend their whole lives together, with sons following in the footsteps of their fathers, and traditional knowledge passed on. Unfortunately, these kinds of relationships are not as common now, and mahouts change elephants often, especially those used for commercial purposes, like logging and tourism. Behavioral abnormalities, such as stereotypies, can develop as a result of poor social bonding (with humans or other elephants). Previously, under extensive systems with free contact, elephants were allowed to free-forage and interact with captive and/or wild elephants (Mar in Fowler and Mikota, 2006; Mumby et al., 2013), which is good for elephant mental health. Fewer examples of these extensive systems exist today, and elephants are mainly kept individually. The need for social and environmental enrichment for elephants to keep them mentally stimulated is now well recognized by elephant experts, but many handlers and elephant owners are not as concerned as they should (Bansiddhi et al., 2018). Elephants captured and trained by humans in countries like Myanmar, Laos and Thailand and may be considered semi-captive in that they have adapted to the captive environment in terms of maneuvering through the terrain, and knowledge of food and water resources, but many have poor social skills and problems with mothering or mating (Lair, 1997). Captive elephants often live longer and close bonds are critical for long-term relationships between humans and elephants. Yet elephants can suffer at the hands of humans, by overwork, malnutrition, and harsh training and control methods.

Because captive and wild elephants in extensive systems often live and share the same areas and resources, and can interact and breed, there is a legitimate concern that this co-mingling can result in shared diseases (Mumby et al., 2013), with potentially high risk to infection at the Human-Elephant-Livestock-Wildlife interphase.

Elephant management for veterinary care

Understanding the personality, habits and behavior of individual elephants is critical for working with them safely, especially under a direct contact regimen. For veterinary interventions, handlers must be able to command their elephants to allow vets to safely approach them, preferably in a quiet, secluded area. It is equally important to know whether elephants require chaining, roping or protection behind bars when working closely together with humans. In cases of poorly trained elephants or bulls in musth, chemical restraint using sedative drugs may be necessary. Types of α2- adrenoreceptor agonists (xylazine HCl, demetomidine, detomidine) with antidotes (α2- adrenorecept or antagonist; yohimbine HCl, tolazoline, atipamezole) are commonly used (Aik, 1992; Sarma et al., 2002), with the precaution that muscle relaxation and peripheral vasoconstriction and intestinal muscle dilatation may occur. Alternatively, azaperone and acepromazine HCl have been used without antidotes in cases of transportation, to decrease excitement, and calm calves in cases of maternal rejection (Puangkham, per comm.). For anesthesia, opioid derivatives, such as etorphine, carfentanil and thiofentanil have been used in Malaysia and Thailand (Edwin, 1997), but these are dangerous if used improperly, and are now illegal in many Asian countries, such as Laos. Long-acting tranquilizers also can have unpredictable results (Clausen and Angkawanish, pers comm, 2008). Sedatives and tranquilizer drugs are administered through an intramuscular route, and so long-handle syringes and dart guns are required. Both elephant and human safety are of concern, so handlers/vets must understand and be aware of the behavioral characteristics and physical state of each elephant, which would apply to management of wild elephants as well, whenever medical management is required.

Table 2. Description of terms applying to elephant management.

Resource system
• Intensive system: elephants are cared for more-or-less individually, fed prepared fodder and tethered or caged at night. • Extensive system: more traditional, elephants are hobbled or chained and released into a forest at night to forage and interact with tame and wild elephants.
Handling system
• Free contact management is contact without protection, and humans and elephants sharing the same space, • Protected contact management is where all interactions are behind barriers. • No contact is giving elephants maximum freedom from human contact, but requires sedation when humans need to work with them.

CHAPTER 1

Health management of captive Asian elephants

For the past five decades, Asian elephants in captivity have been classified as commercial animals without a rigid framework of health surveillance. Health care is one of the pillars for improving elephant well-being, and comprehensive regional disease surveillance is crucial. Improvements in preventive medicine, diagnosis and therapeutics are needed, and elephant conservation needs to take into account the ex situ / in situ interface

Preventive Medicine

In different regions, captive elephants are kept under varying conditions, but no matter the type of facility, quarantine protocols for incoming elephants are important and should be followed. The isolation area should be set up to house elephants for 4-8 weeks and suitable for handling and sanitization; in Asia, a natural area is recommended. For that period of time, fecal examination, vaccination, nutrition evaluation, and health screening should be performed. Elephant vaccination protocols vary in reliability depending on immune responses and the target disease, and so there is a need for further investigations. However, some diseases are routinely vaccinated for, such as hemorrhagic septicemia, tetanus, rabies, clostridium spp. (Miller et al., 2015), and anthrax in endemic areas. All deaths should be followed by routine necropsy for gross and histopathology findings, anatomy records, and sample collections. Handlers need to be well trained to prevent injuries by elephants during quarantine, and understand the individual behavior of elephants, checking for behavior changes, especially signs of stress or abnormal symptoms, and also monitoring feed intake and fecal characteristics as described by Phuangkum et. al. (2005).

General Health Problems

Asian elephants are the one most sensitive species suffering from habitat fragmentation and destruction (Olivier, 1978b), and likely will be severely impacted by climate change, temperature and rainfall, in various regions (Mumby et al., 2013). Diseases related to elephants have been studied (Angkawanish et al., 2009; Mar et al., 2012; Miller et al., 2015) and classified as: 1) non-infectious diseases: traumatic injuries comprised of abscesses, wounds, luxation, and generalized traumatic fractures; 2) gastrointestinal disorders comprised of obstruction of inlet of cardia, impaction/constipation of intestines, colic, as well as diarrhea; 3) malnutrition and weakness related with elephant activities and geriatric problems; 4) musculoskeletal problems including lameness, arthritis, metabolic bone and joint diseases; 5) ocular diseases, often reported as conjunctivitis and keratitis; 6) tusk problems with periodontitis, and susceptibility to tetanus infection; 7) obstetric problems, comprised of umbilical hernia (Pathak et al.,1990; Abou-Madi et al., 2004; Wiedner et al. 2008), dystocia (Thitaram et al., 2006), retained placenta (Hermes et al.,2008), uterine prolapse and cystocele (Angkawanish et al., 2016); 8) foot problems - nails crack, nail overgrowth and deformity,hyperkeratinization with predisposed lameness and pododermatitis; 9) parasitic diseases -flukes, gastric myiasis *(Cobboldia elephantis)* and filariasis; and (10) miscellaneous – poison, heat stroke etc. Serious infectious diseases for elephants include tuberculosis (TB), elephant endotheliotropic herpesvirus (EEHV), tetanus, and cow pox.

Wounds are common in elephants, especially in those that work, and most are treatable

and not a problem to manage. By contrast, high mortality disorders (both infectious and non-infectious) include acute infectious diseases like EEHV, generalized injuries, fractures, obstetrics, and gastrointestinal disorders. Several cases of dystocia and uterine prolapse are unusual obstetrical problems that have caused death in elephants (Angkawanish et al., 2016). Gastrointestinal problems are generally associated with inappropriate feeding practices, pesticides and fertilizer contamination. Elephants in poor condition often have problems with parasitic infestation, deep wounds, or infectious diseases (Angkawanish et al, 2009; Min Oo, 2012). Tusk and obstetric problems can become severe if elephants are not handled or treated properly (Thitaram et al., 2006; Hermes et al., 2008). Injuries, malnutrition and infectious diseases are major causes of death for elephants in Myanmar (Table 3). In Thailand, injuries and malnutrition are main causes of poor health (Angkawanish et al, 2009). Reported rates of infections in Thailand are only 4.3% of all health cases, but that is mostly due to lack of diagnostic tools. Gastrointestinal problems are another major health issue in Thailand, associated with poor husbandry and living conditions. Some of these problems can be reduced when more free foraging after work time is allowed, as has been demonstrated in timber camps (Mar et al., 2012).

Table 3. Health disorders and mortality in treated and untreated Asian elephants.

Health disorder and mortality in Asian elephants	Thailand[a]	Myanmar (calf)[b]	Myanmar (adult)[c]	Asia (without treatment)[d]	Asia (with treatment)[d]
Injuries (localized and generalized)	21.2%	46.8%	33.8%	0-70% (general) 20-100% (fracture)	0-10% (general) 10-85% (fracture)
Gastrointestinal disorders (noninfectious)	24%	N/A	18.3%	0-70% (general)	0-5% (general)
Malnutrition and weakness	14.1%	26.3%	N/A	0-20%	0%
Lameness and arthritis	13.8%	N/A	N/A	0-5%	0%
Ocular disorders	5.7%	N/A	N/A	0-10%	0-10%
Tusk problems	3.9%	N/A	N/A	N/A	N/A
Obstetric problems	5.7%	N/A	N/A	N/A	N/A
Foot problems	3.2%	N/A	N/A	0-50%	0-40%
Parasitic diseases	3.5%	N/A	N/A	0-50% (general)	0-10% (general)
Infectious diseases	4.3%	22.7%	23.8%		
- TB				0-60%	0-20%
- EEHV				0-80%	25-60%
- Tetanus				100%	70%
- Cow Pox				90%	10%
Perinatal	N/A	N/A	18.7%	N/A	N/A
Miscellaneous	N/A	4%	4.4%	N/A	N/A

a = health disorders (n=289), Thailand 2005-2008 (Angkawanish et al., 2009).
b = mortality in calves 0-5 years old (n=224), Myanmar 1960-1999 (Mar et al., 2012).
c = mortality (n=272), Myanmar 1965-2000 (Mumby et al., 2013).
d = mortality with/without treatment in Asia (health survey overview from 45 individuals in 8 Asian elephant range countries), (Miller et al., 2015).

Captive elephants living in high-density conditions, like tourist camps, may have high incidences of microbial exposure resulting in infection. Whether infected elephants show clinical signs depends on several factors: e.g., microbial loads, living condition, and general health status, which affects immune responses against infectious agents. Diseases in Asian elephants based on reports and personal experiences are summarized below and include parasitic diseases, fungal diseases, bacterial infection diseases, and viral infection diseases. Of concern is that 70% of emerging zoonotic diseases have wild animals as reservoir hosts (Kumar et al., 2013), and elephants are one of the potential reservoir hosts that live closely with livestock and as well as humans.

Parasitic diseases

Ectoparasite infestation

Asian range countries are in tropical zones, where external parasites thrive and can affect elephants. They present with severe pruritis and with solid swellings, and elephants often rub sub-abdominal regions on trees and other objects. The elephant louse, horse fly and screwworm fly are commonly found. Elephant lice, *Haematomyzus elephantis,* are blood sucking, usually found on skin around the eyes, behind the ears, along the tail, and ventral abdomen, and can be a cause of dermatitis, and sometimes secondary corneal infection when elephants scratch their itching eyelids. Tabanids or horse flies *(Tabanus spp., Haematopota spp.,* and *Chrysops spp.)* are related to diseases of blood parasite infections (Trypanosomiasis) in elephants, which are intermediate hosts for *Trypanosome evansi* (Desquesnes et al., 2013). Keeping these flies away from elephants is difficult; mahouts will make a smokey fire during the twilight period, which can be helpful because horse flies are diurnal species. Many infestations occur as a result of maggots that develop from the eggs laid by blowflies, or screw worms that are commonly present on chronic wounds. Daily wound cleaning and ivermectin injections can help eliminate these parasites.

Figure 2. *Haematopota spp.* potential vector of *Trypanosoma evansi,* collected from elephant in Thailand (Desquesnes et al., 2013).

Intestinal parasites

Gastrointestinal parasites have been found in elephants in several Asian countries (Agatsuma et al., 2004; Angkawanish et al., 2009; Oo et al., 2012, Abeysinghe et al., 2017 and Abeysekara et al., 2018). Nematodes – Strongyloides are commonly found in elephants with asymptomatic signs, ingestion of strongyloides larvae (L3) during foraging is the most common route. Flukes *(Fasciola jacksoni),* a type of trematode (amphistomes and paramphistomes) are often found in cases of emaciated and exhausted individuals, and in severe cases, anemia and ventral edema are sometimes found. Elephants may ingest immature flukes via water plants or water; the fluke then penetrates the intestinal wall, peritoneum, and liver. Seasonal fecal examinations to monitor internal parasites is recommended and in cases of fluke infection, oral administration of albendazole (7.5-10 mg/kg) for 3 days is recommended by Mar (Fowler and Mikota, 2006). Elephants with loose stools, anorexia, and weakness can present with gastric myiasis *(Cobboldia elephantis),* which is related to bot flies (Fowler and Mikota, 2006). Repellent powder and 1% ivermectin injections may be required for prevention and treatment. In Malaysia, ascariasis has been found in elephant carcasses, with whipworm infestations in Asian elephants reported as well (Fowler and Mikota, 2006).

Protozoa infection

In Thailand, co-infection of *Balantidium spp.* with *Giardia spp.* has been found in young elephants that show signs of abdominal pain, heavy bloody diarrhea, and die within a few days (Somgird, pers. comm., 2006). Evidence for *Toxoplasma gondii* infection has been found by serology surveys in captive elephants in Sri Lanka and Thailand without any clinical manifestations (Dangolla et al., 2006; Tuntasuvan et al., 2001). In a Spanish zoo, screening of 36 primate and 62 herbivore species found *Cryptosporodium spp.* oocytes in 14 primates, 18 ungulates and 1 elephant, although none showed clinical signs of parasitic infection (Gomez et al., 2000).

Blood parasites

Trypanosome evansi has the widest range of domestic and wild hosts, including cattle, buffaloes, sheep, goats, pigs, camels, horses, donkeys, mules and elephants, of which some are susceptible to developing disease (Desquesnes et al., 2013). This blood parasites has been found in elephants in range countries in Asia, including India, Myanmar and Thailand (Hin-On et al., 2004; Arjkampa et al., 2012, Oo et al., 2012; Desquesnes et al., 2013). Blood sucking insects with *T. evansi* can be vectors of infection. *Haematopota spp., Tabanus spp.* and *Stomoxys spp.* infections have been reported in elephants; transmission can be vertical, horizontal, iatrogenic, or per-oral (Desquesnes et al., 2013). Clinical signs include intermittent fever, edema around the face, neck, brisket and abdomen, anemia, loss of appetite and weight loss, cachexia, and death. To control the disease, elimination of both vectors and parasites is suggested, but may be difficult to achieve (Rathore et al., 2016). In Thailand, logging elephants were found to be infected with *Trypanosome evansi, Ancylostoma sp. Anoplocephala sp.* and *Strongyloides sp.,* and treated with diminazen aceturate (5 mg/kg), which reduced parasite numbers. However the disease re-occurred in 4 weeks and some of the elephants eventually died (Arjkampa et al., 2012). Hematocrits (HCT) and thin blood smears are useful to screen for the presence of

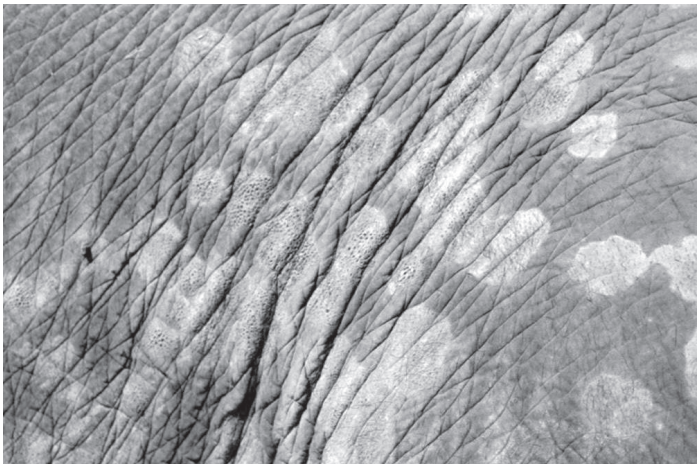
parasites, with confirmation by PCR or LAMP (loop mediated isothermal amplification). In general trypanosome was not infected to human except humans who lacking apoliprotein L-1 or with an abnormality of trypanolytic factor may get infection with trypanosomes (Joshi et al., 2005; Vanhollebeke et al., 2006).

Fungal infections

In mammals, more than half of fungal infections occurs on the skin, and are not uncommon in captive elephants. Because skin lesions often show co-infections with bacteria and fungi, it can be difficult to define the primary cause of infection and is sometimes misdiagnosed as scale dermatitis. *Aspergillus spp.* and *Trichophyton spp.* have been found in elephant skin lesions (Fowler and Mikota, 2006). *T. erinaceid* have been found in pruritic skin lesions of humans directly after contact with elephants in Thailand (Borges-Costa and Martins, 2014).

In Thailand, some captive elephants present skin lesions with a yeastlike odor, but the etiology remains unknown, although Qiao and others (2015) have reported that *Microsporum canis* was the pathogen causing skin disease in Asian elephants at the Chongqing zoo. Herbal medications, like leaf and cortex of neem *(Azadirachta indica)* boiled and applied as a paste have been shown to limit infection progression (BLES, pers comm, 2010). From a case study, a mixture of curcumin and red lime applied to fungal-like lesions decreased lesion size within 8 weeks (Boonprasert, pers. comm., 2005).

Figure 3. Fungal infection like lesions on elephant skin.



Bacterial Infections

Tuberculosis (TB) in elephants

Tubercle bacilli were first described by Koch in 1882, and TB has since been recorded as a serious disease in domestic and free-living animals (Table 4). Mycobacteriosis is a *M. tuberculosis* associated-disease, which includes other members of the *M. tuberculosis* complex (MTBC) like *M. bovis, M. africanum, M. microti, M. canetti* (Brosch et al., 2002), *M. caprae* (Aranaz et al., 2003), and M. pinnipedii (Counsins et al., 2003). *M. tuberculosis* infection also occurs at a high rate in humans. Around one-third of the world population is infected, and 60 % of human TB cases live in Asia (WHO, 2019). Approximately 10 million

people are diagnosed with TB each year, and 1 Million die due to it. TB also occurring in other mammals, such as cattle *(M. bovis),* affects economy and has zoonotic potential. Prolonged and close contact with human TB may result in infection (Mikota et al., 2001; Michalak, 1998), especially in elephants that are in close contact with their keepers (mahouts) and family in countries such as Thailand, Myanmar, Sri Lanka, and India, where the health risk for humanelephant-human infection is high.

In zoos, TB has been reported in both African and Asian elephants, but with a higher incidence in Asian elephants. Most cases in elephants are caused by *M. tuberculosis* (Mikota et al., 2000), the first of which was in an African elephant in France (Urbain, 1938). The first Asian elephant with TB was reported in London by Garrod 1875 (Mikota et al., 2000). Between 1994-2013, *M. tuberculosis* was isolated from 57 captive Asian elephants in the US (Maslow and Mikota, 2015), with some being latent infections (Simpson et al., 2017). An outbreak of four different strains of *M. tuberculosis* in Asian elephants and other species (i.e. giraffes, rhinoceroses and buffalos) occurred at a Swedish zoo (Lewerin et al., 2005) and TB infection in a newborn calf, obtained from its Myanmar mother, was reported in an Australian zoo (Stephens et al., 2013; Vogelnest, 2015). In Asian range countries, free roaming elephants have been diagnosed with *M. tuberculosis* in India (Chandranaik et al., 2017; Zachariah et al., 2017) In Thailand, TB lesions were found in four elephants at necropsy. *M. tuberculosis* was isolated and confirmed by culture, PCR, and sequencing, and from another elephant by trunk washing (Angkawanish et al., 2010). Infection with *M. caprae* was found in a Borneo elephant in Japan (Yoshida et al., 2018). The disease transmission in elephants is long-term close contact with an infected elephant or humans may spread the disease via air droplets, mucous, and feces (Landolfi et al., 2015). Transmission between elephants and humans was first reported in the US when 13 employees tested positive from tuberculin skin tests after being exposed to TB-infected elephants in the quarantine barn at an elephant sanctuary in Tennessee (Murphree et al., 2011). Other examples of keeper staff exposure to elephants with TB have been reported at the Los Angeles Zoo, Oregon Zoo, Albuquerque Biopark and Taronga Zoo (Oh et al., 2002; Zlot et al., 2016; Miller et al., 2018; Simpson et al., 2017; Volgelnest et al., 2015).

Clinical signs of elephant TB range from no obvious signs to weight loss, inappetence, exercise intolerance, respiratory signs (characterized by harsh cough or labor breathing) and ventral edema (Mukundan et al., 2015). The best way to control the spread of TB in elephants is treatment with antibiotics, which unfortunately is expensive, debilitating and needs to be continued for at least 6 months. During treatment the elephant should be closely monitored, including regular blood sampling to measure serum drug concentration to adjust the dosage and monitor side effects (Lewerin et al., 2005). Treatment also does not guarantee that an elephant will become free of the disease (Mikota et al., 2001; Lewerin et al., 2005), and drug resistance can occur (Oregon Zoo, unpublished). Elephant TB is generally treated with first line antibiotics comprised of isoniazid (INH), rifampin (RIF), pyrazinamide (PZA), and ethambutol (ETH) (Isaza et al., 2005; Peloquin et al., 2006; Zhu et al., 2005). Fluoroquinolone and levofloxacin are recommended in advanced cases (Wiedner and Schmitt, 2007; Wiedner and Hunter, 2013; Yoshida et al., 2018). Treatment of elephants by these drugs in an Australian zoo were confirmed effective by serology tests (Dual Path Platform (DPP) VetTB assay)

(Vogelnest et al., 2015). By contrast, a case of MDR-TB resistance to isoniazid and rifampin to genitourinary and pulmonary TB was reported in the U.S. (Dumonceaux et al., 2011).

Table 4. Susceptibility and transmission of TB among several species (adapted from Une and Mori, 2007).

Species 1	No. of bacilli in lesions*	Species 2	Susceptibility to infection with human, bovine and avian TB**			Spread***
			Human	Bovine	Avian	
Primitive human	1		5	5	1	5
Monkey	2	Great apes,	3	2	3	5
		Asian	5	5	2	
		monkey African	4	4	2	
		monkey South	2	2	2	
Modern human	1	American monkeys	2	2	1	5
Elephant	3		3	3	1	?
Cattle	1		1	4	1	5
Chicken	4		1	1	3	4
Horse	3		1	2	1	1
Dog	2		2	2	0	0
Cat	3		1	4	2	1

* Bacilli number: 1= 1-9/100 fields, 2= 1-9/10 fields, 3= 1-9/field and 4= >9/field

** The degree of susceptibility infection by human, bovine and avian TB.

*** The degree to which cases of TB spreads naturally.
The rating scale is as follows: 0 = none, 1= not likely, 2= rare, 3= occasional, 4 = common, and 5= classic.

Mycoplasmosis

Mycoplasma spp. can be part of the normal flora of genital and urinary tract mucous membranes, and include *M. elephantis, M. orale, M. pneumoniae, M. proboscidea, M. salivarium, Acholeplasma laidlawii* and *Ureaplasma spp.* worldwide (Clark et al., 1978; Kirchhoff et al., 1996), but can also cause disease (Fowler and Mikota, 2006). Clinical signs include symptoms of arthritis, a delayed type of mycoplasma hypersensitivity; 60% of arthritis cases in elephants are associated with *mycoplasma spp.* (Clark et al., 1976; Sumithra et al., 2013), although a causative relationship between mycoplasma and arthritis in elephants has never been empirically established. Laboratory tests for mycoplasma can diagnose the disease, and treatment with antibiotics, such as tetracycline is effective.

Anthrax

Bacillus anthracis, which causes anthrax has been found in elephants (Evan, 1910), particularly under free-ranging conditions; no cases of anthrax in zoo elephants have been reported to date (Fowler and Mikota, 2006). Anthrax bacteria can spread between elephants, livestock, and humans (Walsh et al., 2019). The spores are quiescent and can remain in a vegetative state for years. The organisms then grow and multiply quickly in a suitable environment, i.e., slightly alkaline soils (Hugh-Jones and Blackburn, 2009). Elephants get infected when foraging in contaminated areas and exposure to organisms by ingestion, inhalation or wounds. When microbes enter the bloodstream they multiply quickly, and septicemia occurs. After death, the organisms can enter the environment and infect other animals. Thus, extreme precautions in fatal cases suggestive of anthrax are warranted. In one case, a human fatality by anthrax was associated with elephant ivory (Seideman and Wheeler, 1947). Clinical symptoms depend on the source and route of infection, including high fever with red mucous membranes, pupil dilation, ventral edema, anorexia, depression, ataxia, bloody diarrhea, dyspnea, paralysis, and death. Diagnosis includes observation of bloody fluids from orifices and a reddish color of the mucous in the carcass. In postmortem examination, haemorrhagic serous and mucous membranes around body organs and fluids in the cavity and spleen enlargement with dark color are present. Bacterial presence in blood and tissue samples is detectible through PCR. Treatment with high dosage of antibiotics when clinical signs are visible is generally unsuccessful, so destruction of the carcasses and surrounding vegetation is recommended (Fowler and Mikota, 2006).

Haemorrhagic septicemia (Pasteurellosis)

Haemorrhagic septicemia (HS) caused by *Pasteurella multocida* is an important disease of livestock in Asian countries. From 1960-2000 in India, HS caused 46-55% of bovine deaths with a high incidence in other countries in Southeast Asia (Benkirane and de Alwis, 2002). For example, elephant HS was reported in India and Sri Lanka (Harish et al., 2002; Yadav et al., 2012) and signs in elephants are acute and fatal, similar to anthrax and EEHV. Clinical symptoms are high fever, red mucous membranes such as in the eye lids, mouth, tongue, trunk, anorexia and death in young elephants. Disease is associated with contact with cattle or buffaloes, both direct and indirect. Currently, HS in elephants appears to be rare, and in the last decade, it has not been reported in Thailand (Tankaew et al., 2017). The diagnostic techniques available for HS include PCR, biochemical and serological tests (Fowler and Mikota, 2006; Tankaew et al., 2017). Treatment with high doses of broad spectrum antibiotics in the early stages can help, and vaccination in endemic areas is required.

Tetanus (Clostridium tetani)

Tetanus is a worldwide disease caused by *Clostridium tetani*, anaerobic bacteria. The causative agent is found in soil, and in human, horse and cattle feces. Contamination with the agent can cause disease, especially in deep wounds with debilitated tissues. Horses with tetanus present high mortality rates (between 59-80%), so preventive medication is suggested (Reichmann et al., 2008). Studies in elephants are ongoing, but it is clear that disease occurs in relation to foot and tusk infections in range countries where tetanus is common. In those situations, tetanus is a concern when there are deep wounds, usually in

feet punctured by hard objects such as nails, or tusk inflammations that create a lot of pain. Treatment of injured elephants commonly relies on spraying soil dust or mud into the area of pain, or packing the wounds or pulse cavity of tusks that are of high risk for infection. After infection, *Clostridium tetani* creates toxins, such as tetanospasmin, that cause clinical signs, including muscle spasms, rigid movement, snake-like head-tail elevation and erected ears, inability to ingest food and chewing, dyspnea, nictitating membrane prolapse, reluctance to lay down, and eventually death from respiratory paralysis. Chandrasekharan and others (1991) have tried treating tetanus with muscle relaxants and by eliminating the infection with antibiotics, neutralizing antibodies against tetanus neurotoxins, establishing hydro-electrolytic balance, and keeping the elephant in a quiet place for recovery. However, tetanus treatment in elephants is rarely successful; few cases have survived, with death normally occurring within 4-8 weeks. Currently, both prophylactic and post injury tetanus toxoid vaccination for elephants twice in 4 weeks is recommended (Lindsay et al., 2010; Natalia et al., 2011). Elephant foot problems are a concern in range countries, and there is always the potential for some of these to be associated with tetanus infection. Comparatively, tetanus associated with tusk infections is observed less frequently.

Clostridium spp. infection

Elephant gastrointestinal tract anatomy and physiology is similar to *Equine spp*., in that they are mono-gastric and hindgut fermenters. Thus, a normal constitution of microflora is needed for proper digestion. The majority of enteritis diseases in elephants are caused by the same agents as in horses: *Salmonella spp., Escherichia coli* and *Clostridium spp.* (Bojesen et al., 2006). *Clostridium spp.* infection can be harmful to the host when inflammation or damage of the intestinal mucosa is present, and production of exotoxin results in hemolysis and local tissue necrosis, whereas *C. botulinum* and *C. tetani* produce neurotoxins (Fowler and Mikota, 2006). *C. difficile* can cause fatal enterocolitis in Asian elephants while *C. perfringens* has been found in fatal ulcerative enteritis in African elephants (Bacciarini et al., 2001). *C. botulinum* also has been reported in elephants (Fowler and Mikota, 2006) and can be related to signs like flaccid muscle paralysis. *C. difficile* and *C. perfingens* are common diseases associated with enterotoxemia in elephants, the clinical signs of which include severe diarrhea, gas, colic, convulsion, paralysis of the posterior part, sudden death and pathological findings of hemorrhages of the mucosa of intestines. Diagnosis can be confirmed by PCR, but many cases of *C. botulinum* infection are not properly treated.

Salmonellosis

Salmonellosis is a zoonotic bacterial disease that affects the intestinal tract. As summarized by Windsor and Ashford (1972), infections have been observed in zoo and logging Asian elephants around the world. *Salmonella spp.* may be present as normal microflora in elephant intestines, but warm and cold blood animals act as reservoirs. The organism also can survive in the environment for months and cause disease in new hosts. The disease is often found in young elephants, usually when they are stressed from transportation, overcrowding, or during stressful training or post weaning periods. In Thailand, young orphaned elephants often suffer from diarrhea and *Salmonella spp.* are commonly found in fecal culture, potentially related to lack of transfer of maternal antibodies. *Salmonella typhimurium* can be fatal in

young elephants, presenting signs of ventral edema, weakness, recumbence, anorexia, and mild diarrhea (Fowler and Mikota, 2006). In general, salmonella organisms infect the terminal part of the small intestine and the proximal part of the large intestines, and are invasive to lymphoid cells in submucosa, resulting in diarrhea. Fecal culture in symptomatic animals, in addition to blood cultures, can diagnose the disease, especially in the early stages of septicemia. In addition, DNA probes and PCR techniques are also available. Symptomatic and supportive treatments are needed to control diarrhea, balance the electrolytes and prevent dehydration. Increasing the number of good flora in the intestines through use of appropriate probiotics and antibiotics may be helpful.

Colibacillosis

Escherichia coli is common in the normal flora of the large intestine of most vertebrate species, although it can become pathogenic. Enterotoxins, siderophores, shiga-like toxins, cytotoxic/necrotizing factors, and hemolysins are excreted by pathogenic strains, which can cause water and electrolyte imbalances, diarrhea, hypovolemia, metabolic acidosis, metalloprotein inhibition, protein synthesis inhibition, and cell membrane damage (Fowler and Mikota, 2006). Between hosts, *E.coli* is transmitted via direct contact or ingestion of fecal contaminated food and water. It can survive for several days to several years in a suitable environment, including in water, sediments and soil (Chiyo et al., 2014). Elephant *E.coli* infection has been reported as a secondary infection and coinfection with other enteric bacteria or hemolytic bacteria. Thus, clinical signs and pathology lesions might not be easily distinguished from other enteritis diseases, however several techniques for toxin measurements are available. Symptomatic and supportive treatments are required. *E.coli* is considered a zoonosis between elephants and humans, although direct transmission has not been confirmed.

Leptospirosis

Leptospirosis occurs worldwide, and it is endemic in mammal species in Thailand. The pathogen causing the disease is *Leptospira interrogans,* a zoonotic infection spread mainly via urine and water contamination and muddy soil, entering via skin wounds or mucous membranes. The clinical signs in humans are fever, anorexia, depression, muscle pain, renal or liver failure in acute cases. High susceptibility hosts to *Leptospira spp.* develop acute disease and transmission rarely occurs, whereas low susceptibility host species present with mild or subclinical signs and can maintain the disease as a chronic infection, associated with leptospirosis shedding and a seropositive state. A study in elephants in Thailand showed 58% seroprevalence of leptospirosis among asymptomatic elephants, suggesting that leptospirosis may be endemic in this species (Oni et al., 2007). However, further investigations, especially in elephants, are required.

Ulcerative pododermatitis

Elephant foot problems are a concern in range countries, and causes are related to harsh work activities, poor diets, excessive body weight, and keeping on poor substrates (Miller et al., 2015). Foot problems can cause foot rot and thus are susceptible to infections by bacterial agents such as *Streptococcus spp., Staphylococcus spp., Klebsiella spp., Proteus spp., Corynebacterium spp.* and *E.coli.,* which induce ulcerative pododermatitis as described

in Sri Lanka and Bangladesh (Fowler and Mikota, 2006). The disease can be painful and affect elephant well-being. Moreover, severe pododermatitis can be a cause of elephant recumbences and finally death when the elephant is not able to stand. In western zoos, foot and joint problems often are a cause for euthanasia (Fowler and Mikota, 2006). Foot care management is needed to prevent the disease and daily wound cleaning with mild corrosive solution can help.

Scale dermatitis

Over the past two decades, some elephants in Thailand have developed dermatitis lesions and fungal-like lesions. In Cambodia, *Dermatophilus congolensis* has been isolated from elephants with scale-like lesions (Martelli in: Fowler and Mikota, 2006), similar to those in horses and cattle. However, affected elephants present normal behavior and no other clinical signs, except for scales on the skin, and generally scale dermatitis is not transmissible, hence the cause of disease is unknown, and a therapy is not available.

Figure 4. Examples of elephant scale dermatitis.

Viral Infections

Elephant Endotheliotropic Herpesvirus (EEHV)

In 1988, a young Asian elephant in a circus died of an unknown cause in Switzerland. During the 1990s, 20 cases of elephants with similar symptoms were observed in Europe and North America (Long, et al., 2016). EEHV was eventually recognized as the cause of death in many young Asian elephants (generally <10 years of age) and the disease was named in 1999 (Richman et al., 1999). Since that time, 28 and 29 fatal cases of EEHV hemorrhagic diseases (HD) have been reported in Europe and North America, respectively. The first EEHV case in Asia was reported in Cambodia (Ried, 2006), with the next nine cases confirmed in south India (Zachariah. et al., 2013). Several other Asian countries have reported fatalities due to this disease (Bouchard et al., 2014). In Thailand, between 2006 and 2018, 58 EEHV cases were confirmed by molecular techniques (Boonprasert et al., 2019). The clinical signs and pathological findings included sudden-onset, lethargy, tongue, cyanosis, edema, head swollen, organ and tissue hemorrhage, with most affected elephants dying within 1-3 days due to hypovolemic shock and heart or organ failure. This virus infects and replicates in endothelial

cells of capillaries, and consequently causes vascular damage. The severity depends on the subtype of viral infection: EEHV1A, EEHV1B, EEHV4 cause fatality in Asian elephants, whereas EEHV5 infection is found in healthy adult Asian elephants. In contrast EEHV2, EEHV3, EEHV6 and EEHV7 are found in lung and skin nodules of African elephants (Long, et al., 2016). Routes of transmission of infection are still unknown. Diagnosis of the virus can be done by conventional and real time PCR from blood, tissues and secretions. In addition, an ELISA is available for serodiagnosis (van den Doel et al., 2015; Angkawanish et al., 2019).

Rabies

One of the oldest zoonotic diseases is still present worldwide - rabies, and all mammals are susceptible. In human, most rabies victims are children and more than 95% of human cases originate from dogs, the main source of the disease (Mindekem et al., 2013). The causative agent is classical rabies virus (RABV) phylogenic group I in the Lyssavirus genus. Rabies occurs in urban and wildlife species and is endemic in Africa and Asia. Rabies is transmitted by bites of rabid animals, reservoirs hosts with viruses present in saliva. Clinical signs are encephalomyelitis and neurological symptoms as a consequence (Lupulovic et al., 2015). In Asia, rabies in elephants has been reported in India, Sri Lanka and Thailand (Gopal and Rao, 1984; Wimalaratne and Kodkara, 1999; Chuaplaivech et al., 2005). Humoral immune responses have been detected in elephants after vaccination with rabies vaccine, but protection against the disease was not shown (Miller and Olea-Popelka, 2009). Clinical signs are variable depending on the stage of infection. Fowler and Mikota (2006) described that in the initial stages of infection elephants may change behavior, including anorexia and progression to an excitement stage (period of encephalitis symptoms) that presents as hyperesthesia to visual and auditory signals, increased aggression, incoordination, convulsion and autonomic nerve stimulation. The subsequent period of neurologic symptoms or the paralytic stage includes lethargy, paresis, tenesmus, flaccid, ataxia, swaying, paralysis and coma. Histopathological examination of the brain and confirmation by rabies virus specific immunofluorescent antibodies (IFA) is the standard method of diagnosis at necropsy. Definite diagnosis is based on viral isolation in tissue culture. Many diseases have clinical signs like rabies, such as encephalomyocarditis, tetanus, *Clostridium botulinum* infection, heavy metal poisoning, α-swine herpesvirus infection, and EEHV, so proper diagnosis is critical. Rabies is almost 100% fatal in mammals. Treatment in both Asian and African elephants has consisted of a series of two doses of monovalent inactivated rabies virus vaccine that are available to evoke humoral immunity for 24 months (Isaza et al. 2006; Miller et al. 2009). In endemic areas, annual vaccination against rabies in domestic dogs and elephants is required. Veterinarians and animal handlers must also be routinely vaccinated against rabies.

Elephant pox

Zoonotic pathogens in the genus Orthovirus include small pox, monkey pox (MPXV), vaccinia virus, buffalo pox (VACV), and cowpox (CPXV) viruses that are restricted to different hosts. Since 1960, several pox virus outbreaks in elephants have been reported (Dathe et al., 1966; Dym et al., 1973; Wisser, et al., 2001), suggestive of CPXV as causative agents to elephant pox incidents. CPXV infects a broad range of natural host species including humans, cats, cattle, elephants, and zoo animals, with rodents as reservoir hosts (Essbauer et al., 2010).

Transmission to elephants is by direct contact with an infected reservoir host or other infected elephants. Elephant pox should be considered an occupational zoonotic that can be transmitted to humans and vice versa (Fowler and Mikota, 2006; Kurth et al., 2008). Clinical signs vary depending on the strain, route, site of administration and dose of infection and range from conjunctivitis to systemic illness and death. Pox lesions are present on skins and mucous membranes (Kurth and Nitsche, 2012). In one case study, congenital CPXV infection was reported in a pregnant elephant vaccinated with modified vaccinia viruses (MVA) at days 293 and 322 of pregnancy; she had a stillborn calf that showed pox lesions (Wisser et al., 2001). Kurth and others (2008) have reported that circus elephants and rats are sources of infection, which can be transmitted to elephant keepers that develop into skin lesions. Diagnosis of elephant pox infection is based on clinical signs and lesions, detection of viral particles in histology, and on serology, PCR and viral isolation. However, in the early stages of infection, erythema lesions, edema, papules or nodules with blisters can be confused with other diseases. Elephant pox mainly occur in non-vaccinated elephants, so preventive programs, including vaccination with the MVA strain, boosted with vaccinia Lister (Elstree) strain (Fowler and Mikota, 2006), may be effective. Comprehensive pest control, particularly of rodents, is also important. Elephant handlers must be immunized and health checks conducted annually. A review of CPXV outbreaks over the years is presented in Table 5, while Table 6 summarizes the species from which CPXV have been isolated in and whether transmission to humans occurred.

Table 5. Incident of CPXV infection in elephants (modified from Wisser, et al.,2001).

Elephant Species	Year of outbreak	Reference
Elephas maximus	1971	Gehring and other(1972)
Elephas maximus	1980	Pilaski and other(1983)
Elephas maximus	1986	Pilaski and other(1988)
Loxodonta africana	1988	Pilaski and other(1992)
Elephas maximus	1994	Pilaski and other(1995)
Elephas maximus	1994	Pilaski and other(1996)
Elephas maximus	1998	Wisser and other(1998)

Table 6. Different species that CPXV has been demonstrated in by serological evidence and/or virus isolation and transmission to humans (modified from Essbauer, et al., 2010).

Species	Virus isolated	Proven transmission to humans
Cat (*Felis sylvestris, F.Catus*)	Yes	Yes
Dog (*Canis lupus familiaris*)	Yes	No
Fox (*Vulpes vulpes*)	No	No
Wild boar (*Sus scrofa*)	No	No
Cow (*Bos Taurus*)	Yes	Yes
Horse (*Equus caballus*)	Yes	No
Black rhinoceros (*Diceros bicornis*)	Yes	No
White rhinoceros(*Ceratotherium s. simum*)	Yes	No
Tapir (*Tapirus indicus*)	Yes	No
Asian elephant (*Elephas maximus*)	Yes	Yes
African elephant (*Loxodonta Africana*)	Yes	No
Wood mice (*Apodemus sylvaticus*)	No	No
House mice (*Mus musculus*)	No	No
Common rat (*Rattus norvegicus*)	Yes	Yes
Ground squirrel (*Citellus fuvus*)	No	No
Common shrew (*Sorex araneus*)	No	No

Foot and mouth disease (FMD)

FMD is a highly contagious disease of livestock (Pfeiffer, 1993) caused by a virus (FMDV). Seven serotypes of FMDV, identified by cross protection and serological tests, include type A, O, C, Asia 1, and South African Territories (SAT) 1, 2 and 3 (Murphy, 1999). Each serotype can be infective and cause clinical signs in animals, like high body temperature and blanched epithelium, followed by formation of vesicles and erosion after loss of the epithelium. Cloven hooved animals are susceptible to FMDV; thus, livestock animals such as pigs, cattle, buffalo, sheep and goats are the most likely to be affected. In wildlife, water buffalos, deer, elk, antelopes, camels, giraffes and elephants can also be infected with FMDV (Piragina, 1970; Chakraborty and Majumder, 1990; Murphy, 1999). FMDV has spread throughout many parts of the world, including Africa, Asia, Middle East and South America. In Asia, wildlife may be affected by FMDV from domestic animals and vice versa (Nyamsuren et al., 2006; Corn et al., 2009). FMD in Asian elephants was first reported in 1964 (Fowler and Mikota, 2006), and in 1988, the FMDV serotype Asia 1 was isolated from an Asian elephant in India, which was infected from cattle and buffaloes in the area (Rahman et al., 1988). In 1993, four elephants were infected by FMDV serotype O while living in the suburbs of Bangkok. Disease

investigations determined that the infection originated from a local swine farm (Sirivan and Pemayodhin, 1993). Elephants are occasional hosts for FMDV; it can be transmitted through direct contact with infected animals, vectors, or contamination of food, water and soil. In 2008, the northern region of Thailand had a FMD outbreak in dairy farms, and an elephant that was occupationally exposed to food contaminated with FMDV infected cattle secretions was diagnosed with the disease. Within a week, FMD like clinical signs were present, and by serology the FMDV serotype O was confirmed (Yano et al., 2018). Clinical signs included vesicular lesions extending into the trunk, on the lips, tongue, hard and soft palate, coronary band on the soft tissue of the feet and soft tissue around the nails, anorexia, lethargy and lameness at initial stages, thereafter salivations, and ulcerative wounds in oral, trunk and around the nails were present. Generally, animals recover without treatment, but an isolation program and supportive management is required.

Outline of the Thesis

The overall purpose of the studies described in this thesis was to contribute to improved health and sustainable management of Asian elephants in captivity. Our studies focused on the two most important infectious disease affecting elephants: i) TB due to infection with *M. tuberculosis;* and ii) EEHV induced hemorrhagic disease (EEHV-HD). In the general introduction (Chapter 1) on Elephant Health and Management, amongst other relevant diseases, TB and EEHV-HD are briefly introduced. Subsequently a more elaborate description of pathogenesis, diagnosis and treatment and/or prevention of these two diseases is provided (Chapter 2). The TB situation in elephants, worldwide it is estimated that 10% of captive elephants may be infected with *M. tuberculosis* , has motivated us to study the incidence and pathology of TB infection in elephants in Thailand. This study encompasses confirmation of infection of Asian elephants by bacterial culture, and subsequent genotyping of the *M. tuberculosis* isolates. In addition, using a serological assay (TB Stat Pak) antibody titers were assessed in course of time, including retrospectively (Chapter 3). To enable early measurement of cell mediated immunity (CMI), like in humans for early diagnosis of *M. tuberculosis* infection, an elephant interferon gamma release assay (IGRA) was developed. The IFNγ responses in vitro were measured in samples of elephants of unknown status as well as contact/suspected individuals and in non-infected, and infected elephants as negative and positive controls respectively (Chapter 4). To complement assessment of our CMI studies, we also investigated the diagnostic potential of determination of MTBC specific antibodies, known to be prominent in later stages of infection, when immune responsiveness has switched from the protective CMI (Th_1-type) to humoral immune responsiveness (Th_2-type), potentially indicating progression of disease towards development of clinical signs. Latent Class Analysis assesses three antigen-specific ELISAs and a binary test (TB Stat Pak) to predict serological TB statuses (positive, inconclusive and negative) of the elephant population and prevalence of *M. tuberculosis* infection. In addition, association of demographic factors with predicted serological TB statuses of individual captive elephants in Thailand was investigated (Chapter 5). Seroprevalence of antibodies specific for EEHV was determined by ELISA. Based on the results of this assay elephants were categorized as positive, inconclusive and negative to investigate association of these categories with potential risk factors for EEHV infection among elephants in Thailand (Chapter 6). Finally, findings from the TB and EEHV studies,

were summarized and implications for health care and sustainable management discussed and suggestions offered for future studies to understand factors related to these two devastating diseases and to identify better diagnostic tools (Chapter 7).

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CHAPTER 2

Important Infectious diseases in Asian elephants

CHAPTER 2

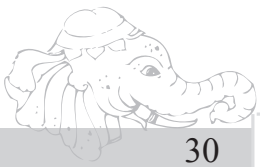
Important infectious diseases in Asian elephants

Among the many infectious diseases of Asian elephants (Chapter 1), tuberculosis (TB) and EEHV-HD are two of the most serious, not only because of the negative effects on animal health and population sustainability, but also the potential to severely affect the economy, particularly in relation to tourism. TB is a chronic and debilitating zoonotic disease transmitted upon close and prolonged contact between captive elephants and their handlers Thus, understanding the pathogenesis and diagnostic options of the disease as well as epidemiology and demographic factors related to TB infection in elephants is a necessity to control it. The other critical disease affecting elephants is endotheliotropic herpesvirus hemorrhagic disease (EEHV-HD), which is highly fatal to young Asian elephant calves worldwide. This viral disease was first identified 20 years ago, but there is still limited information related to its pathogenesis, epidemiology, diagnosis and treatment. Our studies were designed to gain a better understanding of TB and EEHV infection in elephants, information that is needed to create sustainable management plans for elephants, both captive and wild populations.

Tuberculosis

Mycobacterium tuberculosis and other members of the mycobacterium complex (MTBC) including *M. bovis, M. africanum, M. canetti* and *M. microti* cause TB and share a 99.9% similarity in DNA sequences (Garnier et al., 2003). Mycobacteria are aerobic, non-motile and acid-fast rods of 2-4 µm in length and 0.2-0.5 µm in width. Cell walls contain lipids, including mycolic acid, which help the bacilli, once phagocytosed, to survive destruction in macrophages, by generation of reactive oxygen (ROI) and nitrogen intermediates (RNI) and acid and alkaline compounds. The lipids also protect bacilli from lysis by complement in plasma, and play a role in resistance to antibiotics. Likewise, due to the cell wall characteristics, mycobacteria can survive in the environment for approximately 45 days. Hence M. tuberculosis infection is a difficult disease to eradicate and control, and today is considered a serious re-emerging zoonotic disease in elephants. Furthermore, it is a chronic, slowly progressing disease that often goes undiagnosed because many elephants are asymptomatic until death. So, it is easy for TB to be spread between elephants and also between humans and elephants.

M. tuberculosis is the main causative agent of the disease in Asian elephants (Paudel et al., 2014; Landolfi et al., 2014). The first elephant TB case was described in 1875 in a London zoo (Garrod, 1875), followed by numerous other cases in captive elephant facilities globally (Fowler and Mikota, 2006; Lewerin et al., 2005). In the U.S., 57 cases were confirmed between 1994-2013, while a serological survey of 446 elephants (1994-2011) found a prevalence of 12.4% (Maslow and Mikota, 2015). In Asia, serological testing and bacterial culture has confirmed *M. tuberculosis* in elephants in India, Nepal, Laos, Myanmar and Thailand (Maslow and Mikota, 2015; Paudel et al., 2018). In addition, *M. tuberculosis* infection has been reported in isolated Asian elephant populations, such in Australia and the Malaysian Peninsular (Vogelnest et al., 2015; Ong et al., 2013). In captivity, the infection in elephants is a public health concern for handlers, and when exhibited to the public, it poses a risk for transmission.

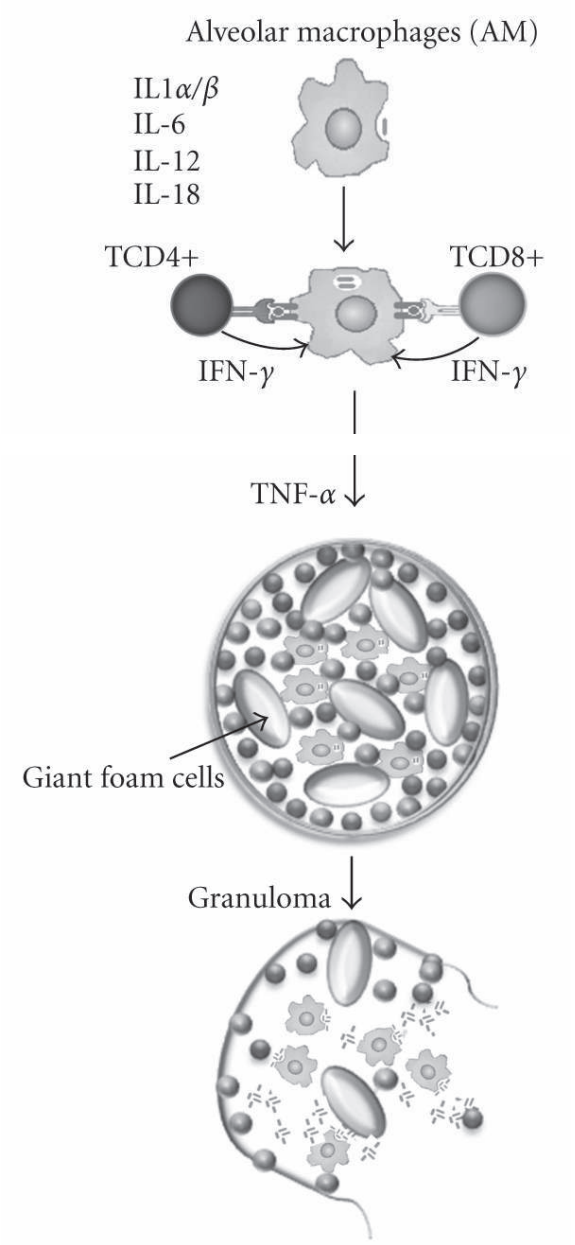


Immune responses to M. tuberculosis in human and non-human primate models

Limited studies on elephant TB have been conducted; hence, knowledge gained from human and non-human primate studies is used to describe and understand the phenomenon (Fig. 1). TB is an intracellular pathogen and stimulates both cellular (CMI) and humoral immune responses. Phagocytosed bacteria arrest phagosome maturation, while T-lymphocytes, together with macrophages, multinucleated giant cells, neutrophils, and dendritic cells become organized in granuloma's that control mycobacterial multiplication and spread, however the bacteria are not fully eradicated. Cytokines produced by CD4+ T-cells, like the proinflammatory TNF-α and IFN-γ, and anti-inflammatory IL-10 are important in this process (Miranda et al., 2012). In addition, infected macrophages present mycobacterial antigens to CD8+, γ/δ and CD1 restricted T cells that secrete lymphotoxins, that contribute to the equilibrium in the granulomas by killing infected macrophages (Kaufman, 2002; Zuniga et al., 2012; Miranda et al., 2012). These processes can result in a latent infection, with granulomas remaining in situ for decades (Zuniga et al., 2012). Gradually the lesions may become caseous and calcified, and bacilli may spread to form multiple additional granulomas. About 10% of latent infections can be reactivated by disturbance of the immunological balance (Fowler and Mikota, 2006). As a consequence mycobacteria may be shed into the environment (Kaufman, 2002).

To distinguish between latent and active TB, microanatomy and immunology analyses are required (Landolfi et al., 2014). Several studies suggest that high levels of IgG antibodies indicate high risk latent TB (Zuniga et al., 2012). For elephants, Landolfi and other (2014) described that, during active disease production of antibodies predominates, whereas CMI responsiveness with prominent production of TNF-α and IFN-γ wanes, and control of the infection is lost. This is in accordance with the shift in immune responsiveness from Th1- to Th2-type described in human TB (Miranda et al., 2012).

Figure 1. The immune response to *M. tuberculosis* (modified from Zuniga et al., 2012).



Inhalation of *M. tuberculosis*

- Infection with *M.tb*
- Bacilli elimination

Inflammatory cell recruitment

- IL-12 and IL-18 secreted by alveolar macrophages co-activate Th_1 cells
- IFN-γ activates macrophages CD_4, CD_8 and NK cell
- TNF-α control bacilli growth and granulomas formation

Granulomas formation (latent TB)

- Th_1 cell response
- Balanced activating and regulatory T cell responses (Th_1, Th_2, Th_{17} and T_{Reg})
- Stop *M.tb.* proliferation, chronic cytokine stimulation, granuloma formation

Progression to active TB

- Disturbance of immune balance; Th_2 cells, become activated and protective Th_1 activity wanes, *M.tb.*-specific antibodies produced
- Granuloma organization destroyed, disease is reactivated, and *M.tb* spreads

Transmission of M. tuberculosis and pathogenesis of TB in elephants

Transmission routes of *M. tuberculosis* in elephants are unclear, but most likely inhalation is the principle route, as it is in various host species (Une and Mori, 2007). The disease develops in lymph nodes of the upper and lower respiratory tract after animals have been exposed to bacilli containing aerosol or droplets from animals or humans with active TB. In addition, ingestion of bacilli via direct contact or contaminated food and water may occur as well also spread (Pollock and Neil, 2002). Transmission between elephants and other animals (Table1), from elephants to humans and vice versa, all have been reported (Michalak

et al., 1998; Lewerin et al., 2005; Murphree et al., 2011; Stephens et al., 2013; Yakubu et al., 2016; Zlot et al., 2016). Prolonged and close contact with infected animals or humans poses a threat for a high risk of infection. In elephants, the transmission by fomites has not been demonstrated, whereas vertical transmission was observed in a newborn calf in an Australian zoo (Vogelnest et al., 2015). In cattle infected with *M. bovis,* the progression of disease relies on the number of pathogenic bacilli and route of infection. In addition, the state of natural resistance of animals and waning of protective immunity, both age-dependent factors, must be considered in disease development (Orme, 1987a. cited by Pollock and Neil, 2002). In addition, increased stress (Brown et al., 1995) and/or nutrition deficiency (Griffin et al., 1993), as well as other immune compromising conditions can affect immune responses to bacilli (Rook and Hernandez-Pando, 1996 and Monies and Head, 1999) and may, once established, result in progression of disease or conversion from latent to active states.

Clinical symptoms and pathology of TB in elephants

Several cases have been documented that were confirmed positive based on antibody detection assays, but no clinical signs were apparent at the time of diagnosis (Stephens et al., 2013; Zlot et al., 2016). Clinical signs are not always present in infected elephants, but generally are observed in the advanced stages of disease, and include weight loss, exercise intolerance, occasional cough, serosanguinous or mucopurulent nasal discharge, and hypoxia (Angkawanish et al., 2010). At necropsy, elephants with advanced infections often present with significant granuloma formation and caseation lesions in the lung, liver, and kidneys, and mandibular, thoracic and abdominal lymph nodes (Angkawanish et al., 2010 and Mikota and Maslow, 2011

Table 1. *Mycobacterium tuberculosis* complex and hosts (modified from Lecu and Ball, 2011).

MTBC	Main Host	Wild and zoo hosts
M. tuberculosis	Human, non-human primates	Elephant, non-human primates, baiza oryx, addax, goats, birds, lowland tapir, giraffes, springboks, mongoose, rhinoceros, addra gazelle
M. bovis	Cattle	All ruminants, badgers, possums, meerkats, big cats, canids, rodents, non-human primates, wild boars, elephants, camelids, rhinoceros, onager, horse, birds
M. africanum	Human	Cattle, swine, non-human primates
M. microti	Vole, camelids	New world monkeys, big cats
M. pinnipedii	Pinnipeds	Camel, tapir, big cats
M. caprae	Goat, sheep, swine	Swine, cattle, wild boars, red deer, white tailed deer, camel, bison
M. canetti	Human	unknown
Dassie bacillus variant	Hyraxes	meerkats

Diagnosis

Globally 10 million people are diagnosed with TB annually, a number that appears to be consistent over recent years (WHO, 2019). Symptoms of human TB are not obvious during the latent stage of infection, but with early diagnosis and antibiotic treatment for 6 months, most newly diagnosed TB cases can be cured and further transmission stopped. Reduction of the number of new TB cases each maybe achieved by reducing health-related risk factors (e.g. smoking, diabetes, HIV infection), providing preventive treatment to people with latent TB, and taking action on broader elements related to TB infection and disease (e.g. poverty, inadequate housing and poor nutrition) (WHO, 2019).

In elephants, proper diagnosis and treatment of TB is still not well established. The gold standard method is direct detection of the mycobacterium organism by either Ziehl Neelsen staining and microscopy, or through identification by polymerase chain reaction (PCR) on bacteria harvested from culture of trunk wash fluid, or tissue homogenate at necropsy. In addition, by spoligotyping or using variable number of tandem repeats (VNTR) analyis, strains of mycobacteria can be identified when DNA is available from culture or PCR (Angkawanish et al, 2010). However, bacterial culture is not a sensitive test and high rates of false negative results are common. Thus, understanding immune responses to *M. tuberculosis* is critical for development of better, more sensitive diagnostic tools to assess and monitor *M. tuberculosis* infection. CMI responses constitute the early part of immune responsiveness in *M .tuberculosis* infection (Fig. 2), so CMI measurement is key during the post-exposure period. Intradermal tuberculin tests are not reliable in elephants; therefore, lymphocyte proliferation assays (LPA) have been development (Lecu and Ball, 2011). Assays to assess in vitro responses of type-1 T cells, based on analyzing concentrations of the cytokine IFN-γ, have been developed for early detection (Angkawanish et al, 2013; Paudel et al, 2016). In addition, assays to assess humoral immune responses during later stages of infection (de la Rua-Domenech et al., 2006) are now available as commercial kits, such as the DPP® TB Vet Assay for Elephants (Lyashchenko et al., 2006). Detection of antibodies using in-house ELISAs with a combination of pathogen specific antigens have also been conducted in elephants (Verma-Kumar et al., 2012). So far, none of these assays have been completely validated. Clinical signs are important for diagnosis, but they normally present only in advanced cases or active TB. So, proper diagnosis of latent or active TB is best done using a combination of several diagnostic tests interpreted in parallel, including the IFN-γ assay, serological assays and specific-mycobacterium organism detection available for elephants.

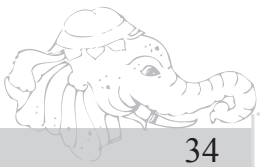
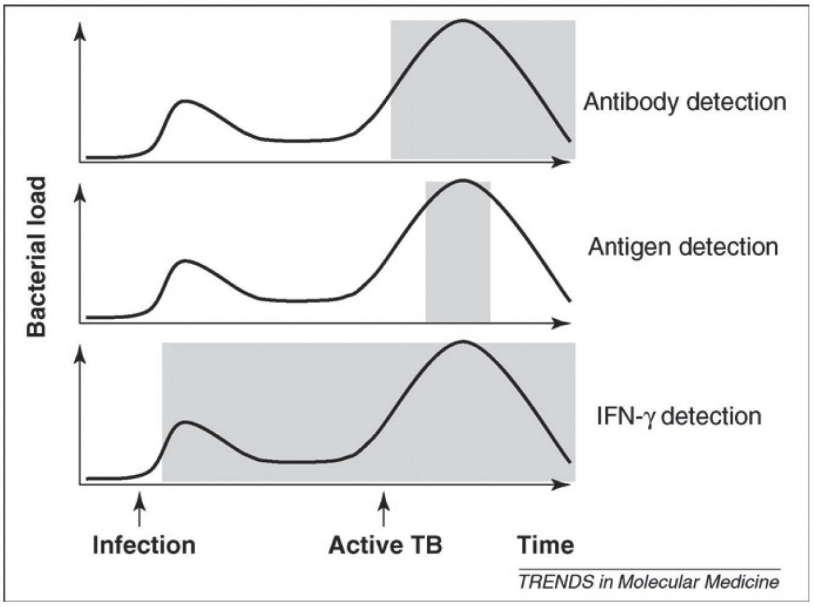


Figure 2 Immune response during the period of progression of *M. tuberculosis* infection (Andersen et al., 2007).



Treatment

The zoonotic aspect of TB, including concerns over shedding and public transmission, and the development of drug resistance, are important considerations for treatment of elephants. Treatment often ensues after a positive trunk wash culture. Initial treatment with the first line medicines consists of isoniazid (INH), rifampin (RIF), pyrazinamide (PZA) and ethambutol (ETH), and quinolones; 270 doses within 12 months are suggested by the guidelines for the elephant TB control (2012). *M. tuberculosis* infection has been successfully treated (Stephens et al., 2013; Vogelnest et al., 2015); however, the drugs have adverse side effects and drug resistance has been documented (Lewerin et al., 2005; Maslow et al., 2005). In an Australian zoo, *M. tuberculosis* infection of an elephant from Thailand was successfully treated with first line anti-TB drugs, with only mild side effects noted (Vogelnest et al., 2015). In the U.S., fluoroquinolone and enrofloxacin in combination with INH and PZA have been effective (Simpson et al., 2017). On the other hand, an infected elephant in Thailand experienced severe side effects after 14 days of treatment with isoniazid, including anorexia, ataxia and depression. The treatment was discontinued and instead, isolation with a monitoring program was chosen (Angkawanish and Sirimalaisuwan, pers comm 2011). Recently, an infected elephant was euthanized in the U.S. due to the development of TB drug resistance (Miller et al., 2018).

TB guidelines for elephants

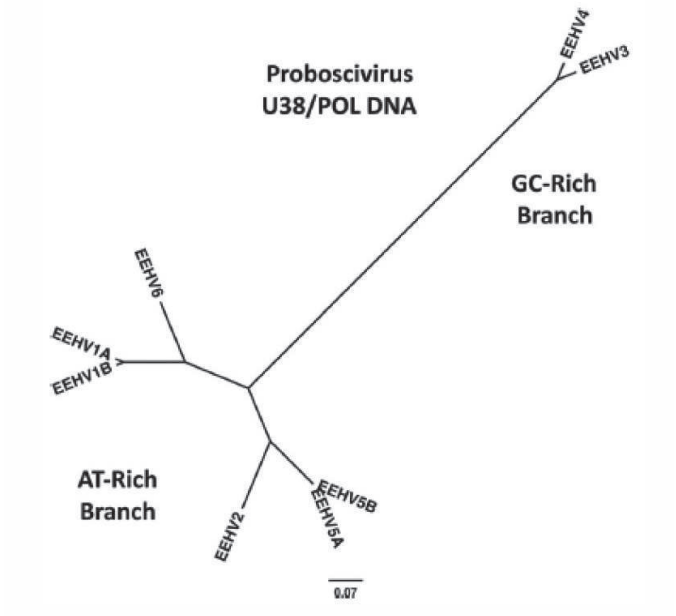
The guidelines for elephant TB control were developed in a collaboration among the United States Department of Agriculture (USDA), the American Association of Zoo Veterinary (AAZV), zoos, circuses and elephant experts. The recommendation for diagnosis and treatment was first published in 1997 and updated guidelines are available online at www.usaha.org (Mikota and Maslow, 2011). In Europe, regulations state that M. tuberculosis and M. bovis

are reportable diseases, and guidelines have been developed in compliance with those in the U.S. (Lecu and Ball, 2011). In Thailand, TB task forces have been created by experts from the Zoological Park Organization (ZPO), National Elephant Institute (NEI), Department of Livestock Development (DLD), universities, and elephant caretakers.

Elephant Endotheliotropic Herpesvirus - Hemorrhagic disease (EEHV-HD)

Currently hemorrhagic disease caused by EEHV infection, with a mortality rate of 85%, is one of the major causes of fatalities in Asian elephant calves aged 1-8 years. Since 1999, deaths of more than 100 young captive and wild Asian elephants has been attributed to EEHV based on diagnosis by molecular techniques (Richman et al., 1999; Latimer et al., 2011; Hayward, 2012). These herpesviruses deviated from all other mammalian herpesvirus families around 100 million years ago, including alpha (α), gamma (γ) and beta (β) herpesviruses. Only Probosciviruses in the β- herpesviruses subfamily are considered pathogenic. Instead, Zong et al., (2014) classified the EEHV members of the probosciviruses genus as a subfamily of delta (δ) herpesvirinae (Fig.3). Moreover, based on DNA sequence information, EEHVs differentiated into two branches, including an AT-rich branch and a GC-rich branch that diverged 35 million years ago, close to the time that elephantids diverged from mastodons (Fig. 4). Herpesviruses are comprised of seven species: EEHV-1A and -B, EEHV-2, EEHV-3, EEHV-4, EEHV-5, EEHV-6 and EEHV-7. EEHV-1A and -B, EEHV-4 and EEHV-5 have been found in Asian elephants while the others have shown to be present in the African species (Long et al., 2016)

Figure 3. Relationship between EEHV-1, EEHV-2, EEHV-3, EEHV-4, EEHV-5 and EEHV-6 considering the conserved U38(POL) DNA gene (Zong et al., 2014).



EEHV-1 may be subdivided into EEHV-1A and EEHV-1B strains that both cause hemorrhagic disease, however EEHV-1A infections predominate (Zong et al., 2014). EEHV-2, as well as EEHV-3, EEHV-4 and EEHV-6, may cause acute lethal hemorrhagic disease

(Garner et al., 2009; Sripiboon et al., 2013; Zong et al.,2014; Bronson et al., 2017). African elephants infected with EEHV-7 present with skin nodules (Atkins et al., 2013; Zong et al., 2014) (Table 2). Thus, whereas EEHV-2, EEHV-3, EEHV-6 and EEHV-7 are infections that result in relatively mild symptoms in African elephants naturally, EEHV-1A and EEHV-1B are deadly in Asian elephants, with milder or no symptoms also associated with EEHV-4 and EEHV-5 although found one Asian elephant fatality associated with EEHV-5 (Wilkie et al.,2014). It is now believed that EEHV is not an emerging disease, but has been present in elephant populations for tens of millions of years, usually remaining in an intangible latent state (Long et al., 2016.

Figure 4. Phylogeny-based classifications of EEHV species within AT-rich and GC-rich branches (modified from Long et al., 2016).

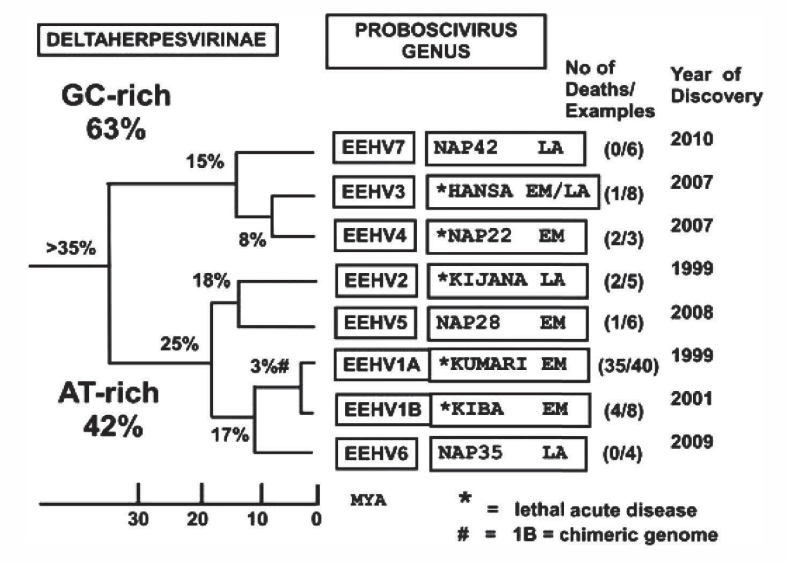


Table 2. Host species, disease characteristics and tissue sources of 11 (sub)types of EEHVs classified by PCR based DNA analysis (modified from Long et al., 2016).

strains	Host species	Lethal acute HD	Mild viremia	TW shed	Saliva	Lung nodules	Skin nodule
EEHV-1A	EM	+	+	+	+		
EEHV-1B	EM	+	+	+	+		
EEHV-2	LA	+			+		
EEHV-3B	LA		+	+	+		+
EEHV-4A	EM	+					+
EEHV-4B	EM		+	+			
EEHV-5A	EM	+	+	+			
EEHV-5B	EM		+	+			
EEHV-6	LA		+		+	+	
EEHV-7A	LA					+	+
EEHV-7B	LA						+

EM are *Elephas maximus;* LA are *Loxodonta Africana;* HD is hemorrhagic disease; TW is trunk wash.

Clinical symptoms and pathology

In the latent phase of infection, herpesviruses are commonly found in neurons, monocytes, lymphocytes and secretory glands of a host, as well as in pulmonary lymph nodes of African elephants (e.g., EEHV-2 and EEHV-3) (Zong et al., 2014). In the active phase, after reactivation, herpesviruses are shed via virus particles containing fluids. However, EEHV-1 has been found in cutaneous papilloma, oral and vestibular lesions of Asian elephants without any other clinical illnesses (Schaftenaar et al., 2010; Hardman et al., 2012). It appears that around 20% of Asian elephant calves are susceptible to the disease (Long et al., 2016). The pathogenesis of EEHV is unclear, although, the clinical term "acute hemorrhagic disease" implies per acute onset (1-7 days), pyrexia, anorexia, dyspepsia and dullness. Moreover, the degree of severity of cyanosis of the tongue and facial edema (Zachariah et al., 2013) depends on the strain of EEHV and the host's health and immune status. EEHV-1A is the main cause of severe hemorrhagic disease (>90%) in Asian elephant calves between 1-8 years of age, predominantly in calves aged 1-4 years (Richman and Hayward, 2012). Four EEHV strains were causes of death in Asian elephants, while strain of EEHV2 and EEHV3 caused death in African elephants (Long et al., 2016; Bronson et al., 2017) (Table 2). Necropsies reveal extensive haemorrhages of pericardium and endocardium, pericardial effusion, petechial haemorrhage of peritoneum, hepatomegaly, intestinal haemorrhage and ulceration, and specific necropsy finding of haemorrhages in gastrointestinal, respiratory and cardiovascular organs are potentially caused by EEHV-4 (Sripiboon et al., 2013).

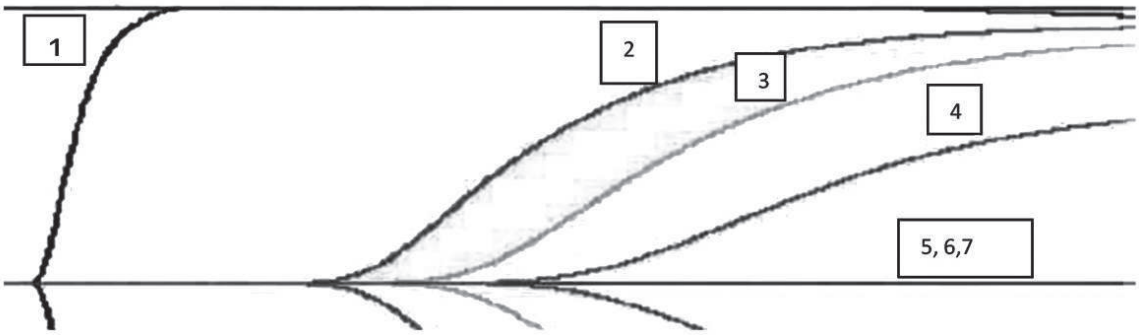
Diagnosis and monitoring

Because of the acute mortality associated with EEHV, early detection is key to monitoring disease development. Measuring viral loads is the most effective way of early diagnosis and monitoring efficacy of treatments. Weekly real time quantitative PCR (qPCR) on DNA isolated from blood is recommended, with viral DNA loads higher than 5,000 viral genome copies per milliliter suggesting the need for treatment (Ling, 2015). Viral loads in healthy elephants have been documented e.g. Stanton and others (2010) detected EEHV-1A DNA at level of 1,200 copies/ml from trunk wash samples in Asian elephants with no symptoms or health consequences. Based on DNA and IFN-γ analyses EEHV has been shown unclear related during pregnancy (Bennett et al., 2015); however, shedding and transmission between a cow and calf have never been reported (Ackermann et al., 2017). Currently, European and U.S. elephant facilities are performing high level monitoring of EEHV through behavioral observations and qPCR. The disease is a particular threat to elephant populations in Asia, and the use of serology and qPCR assays has shown promising in identifying EEHV cases. However, diagnosis is more difficult in range countries due to lack of laboratory capacity, ability to sample young calves, and timeliness of sample analyses. Seen the fact that EEHV cannot be grown in vitro yet diagnostic procedures and even vaccine development has to rely on recombinant antigens. The whole genome of EEHV-1 has been sequenced and core genes were shown to be present in all herpesviruses (Ehlers et al., 2006). A herpesvirus envelop protein, glycoprotein B (gB), has recently been a target for disease detection, with recombinant gB produced for detection of antibodies to EEHV in an ELISA (van den Doel et al., 2015). Loop-mediated Isothermal Amplification (LAMP), a new technique to amplify DNA, also shows promise in diagnosis of the disease (Takehana et al., 2019).

Treatments

Recently, monitoring viral loads has shown promise in identifying elephants at risk for hemorrhagic disease, in addition to monitoring clinical signs (mild to severe degree symptoms). Stanton and others (2012) successfully treated three elephants that showed subclinical and clinical signs of EEHV infection; qPCR measured viral DNA at levels of 10,000-1,000,000 copies/ml in blood. On the other hand lethal cases occurred with high EEHV viral DNA loads in blood between 10,000,000-75,000,000 copies/ml and severe clinical signs (Long et al., 2016). High viral loads may be too much to cure (Long et al., 2016), so early detection is key. The treatment of suspected cases includes anti-viral drug developed for humans, such as acyclovir (ACV) and famciclovir (FCV). Both drugs target the viral thymidine kinase (TK) enzyme, with ganciclovir (GCV) also targeting protein kinase (CPK), although efficacy of these therapeutics is still unclear (Ehlers et al., 2006). EEHV infection inducing dissemination of intravascular coagulation (DIC) in internal organs may cause elephant deaths due to vascular shock. Theoretically, preventing DIC can be helpful, although it was only conducted once in an elephant as plasma transfusion (Bansiddhi et al., 2015) (Fig. 5). For human hemorrhagic diseases such as Ebola and dengue fever, glucocorticoid steroid administration appears to be beneficial, particularly among patients with severe disease (Dokuzoguz, 2013). A 6-month-old calf survived EEHV-HD after an aggressive treatment with acyclovir and one shot of corticosteroid (Sripiboon et al., 2017). In the advanced stages of EEHV-HD, a broad range of antibiotics can be used in young calves to prevent septicemia (Sripiboon et al., 2017).

Figure 5. Comparison of blood agglutination curves in an EEHV-1A infected young calf with multiple blood transfusions from the mother and Eptacog- α (Novo-7, Novo Nordisk A/S) administration, usually used for treatment and prevention of bleeding (modified from Schaftenaar, pers comm.2015).



1=mother line, 2=calf with mother's blood transfusion at 100ul/ml, 3=calf with mother's blood transfusion at 50ul/ml, 4= calf with Eptacog-α (Novo-7) administration, and 5, 6,7=calf without treatment.

EEHV working group

More recently, the EEHV Advisory Group and the Association of Zoos and Aquariums Taxon Advisory Group (AZA-TAG) have been monitoring and providing advice about EEHV sampling, diagnosis, treatment and research, available online at www.eehvinfo.org. In Europe, a European EEHV Research group was established to work closely with stakeholders and to keep connections and exchange of knowledge with other parts of the world. In Asia, the EEHV Working Group was founded in 2014 and guidelines for elephant care takers and owners have been published. In Thailand, an EEHV task force has been conducting research and work with the globalization network of EEHV experts. These EEHV task forces are a promising response to a deadly disease that threatens the sustainability of Asian elephant populations around the globe. It will only be through a coordinated effort among experts regionally and also internationally that advances can be made in developing new tools for understanding the epidemiology of disease and developing effective treatments.

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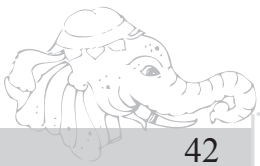

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CHAPTER 3

Mycobacterium tuberculosis infectious of domesticated Asian elephants, Thailand.

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Abstract

Four Asian elephants *(Elephas maximus)* were admitted to the National Elephant Institute, Thailand, with clinical signs of tuberculosis. Animals 1, 2, and 4 showed positive results in a serological test for tuberculosis. Trunk wash culture, the golden standard of diagnosis, and further analysis revealed that animal 2 was infected with *Mycobacterium tuberculosis.* Elephants 1, 3, and 4 died with clinical signs of apnea, collapse and heart failure. After necropsy pulmonary tuberculosis was confirmed by histopathology and culture of lesional tissues. Cultured mycobacteria were further classified as *M. tuberculosis* by identification of the 16S rRNA gene, the 16S-to-23S rDNA internal transcribed spacer (ITS), and by sequencing of the *gyrB* gene. In addition, the MIRU-VNTR in ETR-A genotyping and definition of single nucleotide polymorphism in the *gyrB* gene confirmed that the infection in these elephants stems from different sources in Thailand. The screening of large numbers of elephants for tuberculosis is recommended.

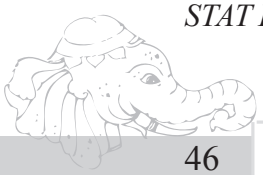
Key words: Asian elephant, Thailand, Tuberculosis.

Introduction

Tuberculosis (TB) is a major infectious disease in humans, but also affects the health of domestic and free ranging animals, and as a consequence e.g. livestock production and wildlife conservation programs (Thoen et al.,2009). During the last two decades infections of captive African and Asian elephants with *M. bovis* as well as *M. tuberculosis* were diagnosed in several countries worldwide (Mikota et al.,2001, Payeu et al., 2002, Michalak et al.,1998 and Lewerin et al.,2005). In conjunction transmission of infection to mammals and veterinary personnel was shown, this indicates that *M. tuberculosis* may be spread amongst species living closely together (Une Y. and Mori T.,2007). In 2005, two Asian elephants in Thailand showed comparable clinical signs of TB and lesions. Although tuberculous granulomas from lung and acid fast bacilli were identified in both elephants, *M. bovis* was isolated from only one of them (Mahasawangkul et al.,2005). Seen the continuous rise in numbers of Asian elephants reported infected with *M. tuberculosis* complex worldwide occurrence of this infection in Thailand is deemed inevitable. In the present paper we report results of various diagnostic approaches and describe pathological findings of animals with clinical signs likely to be attributed to *M. tuberculosis* complex infection. The different diagnostic approaches applied in parallel, encompassed the serologic test Chembio® *TB STAT Pak,* bacterial culture from trunk wash samples and internal lesional tissue after post mortem combined with staining of acid fast bacilli and PCR. DNA sequencing to identify *M. tuberculosis* (complex) species using clusterW multiple alignment was performed for all elephants in this report.

Materials and Methods

Four elephants were referred to the elephant hospital at the National Elephant Institute (NEI), Lampang, Thailand for clinical signs usually associated with Tuberculosis, in the period 2005-2008. Physical examination of elephants 1, 3, and 4 showed signs of weakness and chronic weight loss; elephant 2 had serous nasal discharge. To confirm tuberculosis the following diagnostic assays were used in course of time after admission. The Chembio® *TB STAT Pak* test to detect antibodies against specific *M. tuberculosis* antigens was performed at



time points and intervals indicated in results according to manufacturer's instructions. Bacterial cultures from trunk wash samples of three elephants (1, 2, and 4) were done according to the Guidelines for the Control of Tuberculosis in Elephants, *2003* (www.aphis.usda). Animal 3 that died within a week after admission was too weak for this procedure. Briefly, triplicate trunk wash samples taken prior to daily water intake in a week's period, were packaged in cooler boxes and send to the lab within 6 hours. Samples for bacterial culture, including trunk wash and lesional tissue collected at post-mortem (PM) were kept in -20°C for a week. PM examinations were done on animals 1, 3, and 4 at 21 months, 7 days and 33 months after admission respectively and lesional tissues were collected for bacterial culture, acid fast bacilli staining (Ziehl Neelsen) and histopathology. Bacteria cultured from trunk wash and tissue samples were taken from culture plates and further identified by PCR reactions to determine the 16S rRNA gene (1200 bp) and the 16S-to-23S rDNA internal transcribed spacer (ITS; 420 bp) as characteristics for *M. tuberculosis* complex members. Primer sets forward 5'-AgA gTT TgA TCC Tgg CTC Ag-3' and reverse 5'-ACg gCT ACC TTg TTA CgA CTT-3',forward 5'-TTg TAC ACA CCg CCg gTC a -3' and reverse TCT CgA TgC CAA ggC ATC CAC C-3' were used, respectively (Weisburg et al.,1991 and Jones et al.,2007). Starting with 2 ul of the extracted DNA preparation, thermal cycling began with pre-denaturation at 95°C for 3 minutes, and was followed by 40 cycles of 94°C for 30 seconds, 53°C for 30 seconds, and 72°C for 30 seconds. This was followed by a period of 5 minutes at 72°C for product extension. The *gyrB* genes consisting of 1,020 bp were amplified and sequenced using previously described methods (Kasai et al.,2000). The mycobacterial interspersed repetitive unit variable number of tandem repeats (MIRU-VNTR) typing of the ETR-A locus was performed according to protocols of Fleche et al (2002). The PCR products were analysed by 1% agarose gel electrophoresis followed by ethidium bromide staining and subsequent sequencing using ABI 3070. Unambiguous sequences were compared with data available in GenBank by using NCBI BLAST (www.ncbi.nih.gov/BLAST) and then analyzed by ClustalW version 1.4.

Table 1. Primers used to identify bacteria cultured from trunk wash and tissue samples from domesticated Asian elephants, Thailand, 2003–2008*.

Primer	Forward	Reverse
16s rRNA	5′-AgA gTT TgA TCC Tgg CTC Ag-3′	5′-ACg gCT ACC TTg TTA CgA CTT-3′
ITS	5′-TTg TAC ACA CCg CCg gTC a-3′	5′-TCT CgA TgC CAA ggC ATC CAC C-3′
gyrB	5′-TCG GAC GCG TAT GCG ATA TC-3′	5′-ACA TAC AGT TCG GAC TTG CG-3′

*ITS modified from (*7*,*8*); *gyrB* modified from (*9*). ITS, internal transcribed spacer; *gyrB*, gyrase B.

Results

Serology

Animals were tested using the *TB STAT Pak* test in course of time after their admission to the NEI (table 1). Serum of Elephant 1 was found to be negative in the test after admission while a positive result was obtained 10 months later. Serum of elephant 2 showed a negative result after admission and a positive result 23 months later. Serum of Elephant 3 was found negative 1 and 7 days after admission, the elephant died few hours after the second test. Retrospectively using a stored serum sample taken 4 months prior to admission of the animal a negative result was also found in the test. Serum of elephant 4 showed a positive result at admission.

Bacterial cultures and microscopic observations

Elephant 1. Bacteria could not be grown from trunk wash samples, but at necropsy elephant 1 revealed tuberculous lesions, purulent exudates and multifocal calcified nodules in the respiratory tract and mediastinal lymph nodes, as well as in liver, kidney and spleen. The histopathological examination showed caseous necrosis, infiltration of lymphocytes, accumulation of macrophages and giant cell in the lung tissue, lymph nodes and liver, and the Ziehl Neelsen stain identified acid fast bacilli in lung tissue and lymph nodes. Mycobacteria were cultured from lesional tissue and subjected to further analysis as described below.

Elephant 2. Bacteria were cultured from a trunk wash and the colonies, positive for acid fast bacilli staining (Ziehl Neelsen), were subjected to further analysis as described below.

Elephant 3. The animal was severely ill and since it was in lateral recumbency from the first day of admission, it could not be subjected to trunk washing. The animal died 7 days later (table 1). Postmortem examination showed tuberculous lesion in respiratory organs including lung, upper trachea and mediastinal lymphnodes (table 2). Histopathological examination showed caseous necrosis and accumulation of macrophages and giant cells but only in the lung and lymph nodes, the Ziehl Neelsen stain was performed and revealed acid fast bacilli. Mycobacteria were cultured from lesional tissue and subjected to further analysis as described below.

Elephant 4. Non-tuberculous mycobacteria *(M. avium)* were identified in trunk wash cultures initially. At postmortem examination tuberculous lesions were found in respiratory organs and mediastinal lymph nodes. Histopathological analysis showed macrophage accumulation and edema in the lung tissue (table 2). The Ziehl Neelsen staining did not show acid fast bacilli (0/7). Mycobacteria were cultured from lesional tissue and subjected to further analysis as described below.

Molecular confirmation

The 16S rDNA and ITS sequencing confirmed that bacteria cultured from lesional tissue of elephants 1, 3 and 4 as well as a trunk wash culture of elephant 2 are members of the *M. tuberculosis* complex (data not shown). The first trunk wash derived bacteria from elephant 4 were confirmed to be *M. avium* complex (table 1). The *gyrB* sequencing showed that isolates from elephants 2, 3, and 4 (917 bp) were identical to *M. tuberculosis* strain ATCC 27294 (table3), in addition identical to strains KZN1435, F11, H37a, H37v, and CDC 1551 (data not show). The *gyrB* sequence of elephant 1 differed at position 482 (C replaced G) which is identical to *M. tuberculosis* strain KPM KY679, that belongs to the ancient TbD_1 positive strain (Kasai et al.,2000, Gutierrez et al.,2005 and Niemann et al.,2000). The sequences of the ETR-A locus clearly showed that different types of *M. tuberculosis* were present in elephants 2, 3 and 4, having three, two and four repeats of the typical 75bp sequence, respectively (data not show).

Table 2. *gyrB* gene sequence comparisons of 4 *Mycobacterium tuberculosis* isolates from domesticated Asian elephants, Thailand, 2003–2008*.

	Gene position						
Organism	41	122	482	677	776	816	824
M. tuberculosis ATCC	C	G	G	T	C	G	C
M. tuberculosis TbD1	C	G	C	T	C	G	C
M. africanum	C	G	G	T	C	T	C
M. canetti	C	G	G	T	C	T	T
M. microti	T	G	G	T	C	T	T
M. bovis	C	A	G	T	T	T	T
M. caprae	C	A	G	G	T	T	T
Elephant 1 isolate	C	G	C	T	C	G	C
Elephant 2 isolate	C	G	G	T	C	G	C
Elephant 3 isolate	C	G	G	T	C	G	C
Elephant 4 isolate	C	G	G	T	C	G	C

*Nucleotide variability at relevant positions of the *gyr B* gene in the genome of mycobacteria isolated from the 4 infected elephants as compared with those in established *M. tuberculosis* ATCC 27294 (GenBank accession no. GQ247736.1), *M. tuberculosis* KPM KY679 (accession no. AB014215.1), *M. africanum* (accession no. AB014192.1), *M. canetti* (accession no. AJ749915.1), *M. microti* (accession no. AB014205.1), *M. bovis* (accession no. AB018554.1), and *M. caprae* (accession no. AJ276122.1). Modified from Gutierrez et al. (*11*). Shading corresponds to sequence stretch in the strains that are identical to the sequences in *M. tuberculosis*: yellow, performed ATCC strain; blue, position 482 performed TbD1 strain; tan, performed other *M. tuberculosis* complex strain. *gyrB*, gyrase B; ATCC, American Type Culture Collection.

Discussion

This is the first report to present *M. tuberculosis* in Thai Elephants (n=4) as definitely confirmed, by bacterial culture from lesional tissue after necropsy (animals 1, 3 and 4) or from trunk wash samples (animal 2, still alive). The clinical signs shown by the 4 animals varied considerably. The two extremes being nasal discharge only in animal 2 that is still alive and on the other hand severe clinical signs and lateral recumbency in animal 3 that died soon after admission to the hospital. In the latter animal antibodies could not be shown, which may indicate an anergic status of the mycobacteria specific immune response (Van Rhijn et al.,2008). Histopathology showed that this animal was severely affected by the infection. Elephant 2 is still alive and trunk wash samples were shown to contain mycobacteria by culture. In addition serum samples of the animal showed repeatedly positive results in the *StatPak* test. The other two elephants (1 and 4), showing anorexia and (thus) chronic weight loss as well as comparable lesions upon necropsy, showed variable results in diagnostic assays. Trunk wash bacterial culture that is considered as the gold standard for confirmation of *M. tuberculosis* complex infections in elephants, has its limitations as described elsewhere (Lyashchenko et al.,2006). Here it was shown that from only 2 out of 60 trunk wash samples mycobacteria could be grown from 4 animals that were in the end all shown to be infected with *M. tuberculosis., M. tuberculosis* complex specific immune responses may differ in species and at least will differ in course of time after infection (Van Rhijn et al.,2008). The present studies are the first to confirm *M. tuberculosis* infection in elephants in Thailand, and indicate that serological tests nor individual other observations could unequivocally identify infected animals. Rather, only the combination of the different diagnostic observations in course of time after infection holds promises. Sequence analysis of 16S and ITS used to differentiate *M. tuberculosis* complex and non-tuberculoid mycobacteria (Gutierrez et al.,2005), clearly indicated the presence of *M. tuberculosis* complex bacteria in each of the elephants. The nucleotide sequence polymorphism in the *gyrB* gene of mycobacteria (Kasai et al.,2000, Gutierrez et al.,2005 and Niemann et al.,2000) confirmed the identity of M. tuberculosis for all four elephants. Since the sequences of *M. tuberculosis* and *M. africanum* subtype II are identical based on *gyrB* gene (Niemann et al.,2000) only phenotyping based on growth characteristics on bromcresol

purple medium, could confirm *M. tuberculosis* as the causative agent of tuberculosis in our four elephants. *M. tuberculosis* may be classified into ancestral and modern strains based on the presence or absence of a *M. tuberculosis* specific deletion (TbD1) (Broch et al.,2002). *M.tuberculosis* isolated from the elephant 1 had a *gyrB* gene sequence identical (100%) to *M. tuberculosis* strain KPM KY679 which was identified as the ancient TbD1 positive strain (Table 3). The other three elephants (2, 3 and 4) were infected with strains, according to the *gyrB* gene sequencing, identical (100%) to *M. tuberculosis* strain ATCC 27294 which is a modern type potentially related to major epidemics like the Beijing, Haarlem, and African *M. tuberculosis* clusters (Broch et al.,2002), from the sequence results, we might assume that *M. tuberculosis* in elephants of this report were transmitted from human, because the *gyrB* gene nucleotide sequences of isolates from the infected elephants were identical to those of human *M. tuberculosis* strains. Still it could be shown that the source of infection of these elephants is from different origins according to MIRU-VNTR typing of the ETR-A gene *M. tuberculosis* strains in elephant 2, 3 and 4 differ number of 75bp, respectively.Annual health checks of mahouts and veterinarians in contact with the infected animals for more than 4 years at the elephant hospital, NEI did not identify individuals positive by chest x-ray in the context of the tuberculosis control program in Thailand. This may imply low zoonotic potential of tuberculosis from elephants to humans exposed to the infected animals for longer than 4 years. To control the possibility of *M. tuberculosis* complex transmission from humans and other species to wild animals including elephant or vice versa assays that enable early diagnosis of infection is necessary. Since no single assay unequivocally defines the infectious status, combination of diagnostic approaches is essential. Further investigation of the tuberculosis transmission from other species to elephant or vice versa is necessary in order to control this disease in this Proboscis species. Since *M. tuberculosis* infection was confirmed in four Asian elephants, the surveillance and monitoring of this disease in Thailand would help to understand its epidemiology, essential for control and prevention of tuberculosis in Asian elephants.

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Supplemental materials

Supplement Table 1. Comparison of results of bacterial culture from trunk washes at various times during hospitalization and from tissue samples obtained at necropsy as well as of serology (TB *Stat Pak* test) in course of time (indicated in months: m).

Elephant ID	Serology results (sampling times)		Bacteriological result (sampling times)	
	Negative	Positive	Negative (trunk washes)	Positive
1	m 1	m 11 m 13 m 20 m21	m12 m 13 m 14 m 20 m 21	Necropsy at m 21:Lesional tissues (lung and mediastinal lymph nodes)
2	m 1	m 23 m 33 m 35 m 43 m 45 m 49	m34 m 35 m 36 m 43 m 46 m 47 m 48	Trunk wash m42
3	Before 4 m m 1	-	Trunk wash was not done	Necropsy at m 1:Lesional tissues (lung)
4	-	m 1 m 11 m 18 m 24 m 31 m 33	m 12 m 13 m 16 m 18 m 25 m 31 m 32	Trunk wash m 24 (*M. avium*)Necropsy at m 33: Lesional tissues (trachea and mediastinal lymph nodes)

Supplement Table 2. Clinical signs at and during hospitalization and gross and microscopic lesions as well as ZN positive bacilli observed at and after necropsy.

Elephant ID	Clinical signs	Gross lesions	Microscopic lesions	ZN* stain
1	Chronic weight loss, weakness, anorexia, dyspnea	Generalized lymphadenopathy, Purulent exudates and multifocal calcified pulmonary nodules. Hepatic congestion, together with renal multifocal micro abscesses.	Lung: severe chronic diffuse caseous necrosis, fibroplasias, infiltration of lymphocytes and macrophages. Lymph nodes:Chronic diffuse caseous necrosis lymphadenitis. Liver: severe centrilobular heamorrhage and necrosis multifocal caseous necrosis, diffuse lymphocyte and macrophage accumulation around blood vessels	Positive (100/HPF**)
2 (still alive)	Good condition, serous nasal discharge	NA	NA	Positive (50/HPF**)
3	Chronic weight loss, weakness, anorexia, depression	Multifocal calcified pulmonary nodules. Frothy, purulent exudates and ulcer in the upper trachea.	Lung: Chronic diffuse caseous necrosis, fibroplasias, Infiltration of lymphocytes and macrophages. Lymph nodes: mild degree of lymphocytic depletion.	Positive (10/HPF**)
4	Chronic weight loss, weakness, anorexia.	Pulmonary heamorrhage and edema, mediastinal lymph nodes enlargement, splenomegaly with multifocal micro abscesses.	Lung: pulmonary edema, infiltration of neutrophils.	Negative

* Ziehl Neelsen staining for acid fast bacilli in either of the samples of lung and mediastinal lymph nodes.
** HPF: High Power Field.

CHAPTER 4

The elephants interferon gamma assay : A contribution to diagnosis of Tuberculosis in elephants.

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Summary

Mycobacterium tuberculosis (M. tb) shown to be the main causative agent of tuberculosis in elephants worldwide. *M. tb* may be transmitted from infected humans to other species including elephants and vice versa, in case of prolonged intensive contact. An accurate diagnostic approach covering all phases of the infection in elephants is required. Since *M. tb* is intracellular pathogen and cell mediated immune (CMI) responses are elicited early after infection, the skin test is the CMI assay of choice in humans and cattle. However, this test is not applicable in elephants. The interferon gamma (IFN-γ) assay is considered a good alternative for the skin test in general, validated for use in cattle and humans.

The present study aimed at development of IFN-γ assay applicable for diagnosis of tuberculosis in elephants. Recombinant Elephant IFN-γ (rEpIFN-γ) produced in eukaryotic cells was used to immunize mice and generate the monoclonal antibodies. Hybridoma's were screened for IFN-γ specific monoclonal antibody production, subcloned, and antibodies were isotyped and affinity purified. Specificity of the antibodies was confirmed by Western blot. The optimal combination of capture and detection antibodies selected was able to detect rEpIFN-γ in concentrations as low as 1 pg/ml. The assay showed to be able to detect the native elephant IFN-γ, elicited in positive control cultures (PWM, PMA/I) of both Asian and African elephant whole blood cultures (WBC). Preliminary data were generated using WBC from non-infected elephants, M. tb infection suspected elephant, and culture confirmed *M. tb* infected elephant. The latter showed measurable production of IFN-γ after stimulation with ESAT6/CFP10 PPDB and PPDA in concentration ranges as elicited in WBC by MTBC specific antigens in other species.

Hence the IFN-γ assay presented potential as diagnostic tool for the detection of elephant tuberculosis. To validate the assay will require its application in large populations of non infected and infected elephants.

Key words: elephant, tuberculosis, IFN-γ

Introduction

For thousands of years elephants have been trained for the purpose of human use. As a result, especially captive Asian elephants *(Elephas maximus)* are often in prolonged contact with humans and are prone to infection with Mycobacterium tuberculosis *(M. tb)*, as first reported in 1875 (Michalak et al., 1998). *M. tb* may be transmitted from infected humans to other species including elephants and vice versa, in case of prolonged intensive contact (Une and Mori, 2007, Angkawanish et al., 2010 and Murphee et al., 2011). Infection has an impact on animal welfare and constitutes a risk for humans, hence for the tourist industry and may have economical consequences. In the last two decades elephant tuberculosis has been reported from all around the world (Michalak et al., 1998, Mikota et al., 2001, Payer et al., 2002, Oh et al., 2002, Lewerin et al., 2005, Une and Mori, 2007, Angkawanish et al., 2010 and Murphee et al., 2011), hence an accurate diagnostic approach covering all phases of the infection is required. The gold standard being bacterial culture from trunk washes and affected tissues at necropsy is insensitive (Mikota et al., 2001 and Lyaschenko et al., 2006). Diagnostic assays detecting antibodies specific for *M. tuberculosis* Complex (MTBC) antigens

in serum have their limitations, especially due to cross-reactivity caused by non-pathogenic mycobacteria (Greenwald et al., 2009). Moreover, they do not form part of the initial immune responsiveness. Since *M. tb* is an intracellular pathogen, cell mediated immune (CMI) responses shown to be elicited early after infection are considered to contribute to protection. The skin test, the CMI assay of choice for diagnosis of tuberculosis in humans and cattle, is not applicable in pachyderm species. However, the interferon gamma (IFN-γ) assay is currently in the process of replacing the Mantoux test in humans (Schiller et al., 2010) and is considered a good alternative for the skin test. Briefly, in humans the typical cytokine profile indicates that at initial stages of infection *M. tb* specific immunity is predominantly Th_1 mediated. Tumor necrosis factor (TNF-α) and IFN-γ are produced and activate macrophages to kill engulfed mycobacteria. In addition, killing of macrophages infected by mycobacteria, may be mediated by CD_4 cells, producing lymphotoxin and by perforin and granulysin produced by CD_8, γδ T-cell and CD_1 restricted T-cells (Kaufman, 2002). Activity of these cells is facilitated by IFN-γ and TNF-α. Currently, IFN-γ assays have been developed for humans (Taggart et al., 2004), cattle (Vordermeier et al., 2001 and Vordermeier et al., 2006), domestic cats (Rhodes et al., 2008), lions (Maas et al., 2012) and rhinoceros (Morar et al., 2007).

The present study aimed to develop a capture ELISA for the quantification of IFN-γ in Asian as wells as African *(Loxodonta africana)* elephants after stimulation with crude protein extracts of mycobacteria (PPD-B, PPD-A) and MTBC specific antigens (ESAT-6, CFP-10). Initial results confirmed detection of IFN-γ in concentration ranges comparable to those elicited by MTBC specific antigens in whole blood cultures of other species (Andersen et al., 2007, Morar et al., 2007 and Maas et al., 2012). Thus the IFN- γ assay an important tool for early detection of MTBC infection in many species, may be added to the diagnostic potential for tuberculosis in elephants.

Materials and Methods

1. Expression of eukaryotic recombinant elephant IFN-γ (rEpIFN-γ)

Eukaryotic recombinant elephant IFN- γ was produced by U-Protein Express BV, Utrecht, The Netherlands. The gene encoding rEpIFN-γ (Morar D, PhD Thesis University of Pretoria, 2009; Figure 1) was synthesized by GeneArt and cloned into a pUPE expression plasmid, that was transiently transfected into HEK293 EBNA cells as described by Durocher et al., 2002. Expression products were harvested from culture supernatants six days post-transfection.

Fig. 1. Nucleotide and translated amino acid sequence of the synthetic gene for recombinant elephant IFN-γ (rEpIFN-γ) used in this study. (a) The real nucleotide sequence of the EpIFN-γ (Jasmin) gene. Selected primers used for the cloning are underlined (Morar, 2009). (b) The predicted protein sequence resulting from (a). (c) The synthetic nucleotide sequence that was ligated into pUPE expression vectors. (d) The predicted protein sequence resulting from (c).

(a)

GGCCTAACTCTCTCTGAAACAATGAATTTTACAAGTTATATCTTAGCTTTTCAGCTTTGC
ATCATTTTGGGTTCTTCTAGCTGCTACTGCCAGGCTACTTTTTTGAAAGAGATACAGAAC
CTAAAGGAATATCTTAATGCAACTGATTCAGATGTAGCGGATGGTGGGCCTCTTTTCATA
GATATTTTGAAGAACTGGAAAGAGGAGAGTGACAAAAAAATAATTCAGAGCCAGATCGTT
TCCTTTTACCTCAAAATCTTTGACAACTTGAAAGACAACCAGGTCATCCAAGAGAGCGTG
AAGACCCTTGAGGAAGACCTCTTTGTTAAGTTCTTCAATAGCAGCTCCAGCAAACGGGAT
GACTTCCTAAAAGTGATGCAAACTCCGGTAAATGACCGGAACGTCCAGCGCAAAGCCATA
AGTGAGCTCTCCAAGGTGATGAATGACCTGTCACACAGATCTAACGGGGCAAACGAAAAA
GGAGACAGTATTCGTTTCGAGGCCGGAGAGCATCAGAATAATGG

(b)

MNFTSYILAFQLCIILGSSSCYCQATFLKEIQNLKEYLNATDSDVADGGPLFIDILKNWK
EDSDKKIIQSQIVSFYLKIFDNLKDNQVIQESVKTLEEDLFVKFFNSSSSKRDDFLKVMQ
TPVNDRNVQRKAISELSKVMNDLSHRSNGAKRKRRQYSFRGRRASE

(c)

CAGGCTACTTTTTTGAAAGAGATACAGAAC
CTAAAGGAATATCTTAATGCAACTGATTCAGATGTAGCGGATGGTGGGCCTCTTTTCATA
GATATTTTGAAGAACTGGAAAGAGGAGAGTGACAAAAAAATAATTCAGAGCCAGATCGTT
TCCTTTTACCTCAAAATCTTTGACAACTTGAAAGACAACCAGGTCATCCAAGAGAGCGTG
AAGACCCTTGAGGAAGACCTCTTTGTTAAGTTCTTCAATAGCAGCTCCAGCAAACGGGAT
GACTTCCTAAAAGTGATGCAAACTCCGGTAAATGACCGGAACGTCCAGCGCAAAGCCATA
AGTGAGCTCTCCAAGGTGATGAATGACCTGTCACACAGATCTAACGGGGCAAACGAAAAA
GGAGACAGTATTCGTTTCGAGGCCGGAGAGCATCAGAATAA

(d)

GSQATFLKEIQNLKEYLNATDSDVADGGPLFIDILKNWK
EDSDKKIIQSQIVSFYLKIFDNLKDNQVIQESVKTLEEDLFVKFFNSSSSKRDDFLKVMQ
TPVNDRNVQRKAISELSKVMNDLSHRSSNGAKRKRRQYSFRGRRASE **AA**

2. Production of monoclonal antibodies

Immunization Two mice (BALB/c females; Charles River, Someren, Netherlands) were immunized intraperitoneally with 50 µg rEpIFN-γ in 50µl PBS mixed with 50µl Specol (Stimune® Prionics®, the Netherlands) on days 0, 21 and 42. On days 63 and 64 the immune response of the mice was boosted intravenously with 50 µg rEpIFN-γ in PBS.

Cell fusion and cloning. On day 67 spleen cells of the immunized mice were fused with mouse myeloma cells according to routine procedures. Sp2/0 using 40% polyethylene glycol 4000 (Art.9727 – Merck®). For two weeks hybridoma's were cultured in OptiMEM (Invitrogen®) medium supplemented with 10% Fetal Clone I (Hyclone®, USA), hypoxanthine, aminopterin, thymidine and supernatants were screened by indirect ELISA (see below). Finally, selected hybridoma cultures were subcloned by a BD Influx Cell Sorter (BD Biosciences®, USA).

Isotyping and affinity purification. Monoclonal antibodies (MoAbs) were harvested and isotyped using the Beadlyte Mouse Immunoglobulin Isotyping Kit (Millipore®, The Netherlands) and the Luminex 100TM System according to the manufacturer's instructions.

MoAbs were affinity-purified, using GammaBindPlus Sepharose according to the manufacturer's instructions (GE Healthcare Europe GmbH). Subsequently, 1 mg of each MoAb was biotinylated with Biotin-ϵ-aminocaproic acid N-hydroxysuccinimide ester (biotin-X-NHS) according to the manufacturer's instructions (Roche® Diagnostics, The Netherlands).

Indirect ELISA. Hybridoma supernatants were screened for rEpIFN-γ specific antibodies by indirect ELISA. All incubations were done on a microtiterplate-shaker at 300 rpm at room temperature (RT). Ninety-six well ELISA plates (Costar®, Corning Incorporated,

USA) were coated with rEpIFN-γ (5µg/ml, 50 µl/well) in phosphate buffered saline (PBS) for 1 hour, Antigen was discarded and the plates were blocked with 200 µl/well of block buffer (Roche® Diagnostics, The Netherlands) for 30 minutes. Block buffer was discarded and 50µl of hybridoma supernatants were added to the wells and incubated for 1 hour. Subsequently plates were washed 3 times with phosphate buffered saline containing 0.1% Tween 20 (PBST) and, polyclonal rabbit anti-mouse IgG-HRP 1:6000 (Southern Biotech® Alabama, USA), was added (50µl/well). After 1 hour incubation, plates were washed 4 times with PBST and once with tap water. Finally, 100µl/ well substrate 2,2'-azino-di (3-ethylbenzthiazoline-6-sulfonate) in a glycine/citric acid buffer (ABTS) (Roche®Diagnostics, The Netherlands) was added. After 30 minutes of incubation, colour development was measured at 405 nm (Biorad microplate reader 550®, USA).

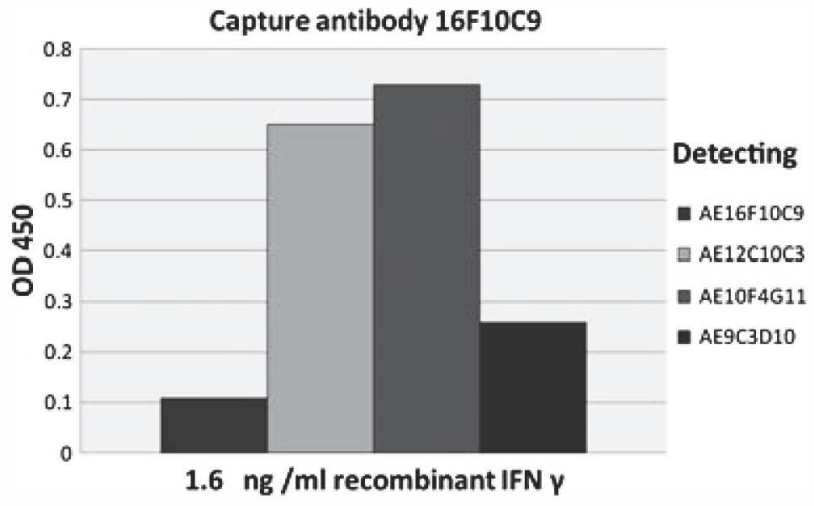
In addition, after purification and biotinylation of monoclonal antibodies, indirect ELISAs as described above were performed to select the MoAbs (biotinylated) with the highest affinity to rEpIFN-γ. For this, rEpIFN-γ was coated at 2 µg/ml (50 µl) and (biotinylated) MoAbs were added in 4-fold dilutions from 10 to 0.0024 µg/ml. Selection of monoclonal antibodies for the IFN-γ capture ELISA. To select the optimal antibody tandem for the IFN-γ capture ELISA plates were coated with 50 µl (5 µg/ml) of capture monoclonal antibodies (non-biotinylated) per well. After incubation for 30 minutes and washing, rEpIFN-γ was added in 50 µl (1 µg/ml) per well, after subsequent incubation for 30 minutes and washing, biotinylated detection monoclonal antibodies were added in 50 µl (1 µg/ml) per well. After incubation for 2 hours and washing, the conjugate streptavidin-peroxidase (Biosource®) 1:2,000 was added. In subsequent assays to fine tune selection of antibody combinations rEpIFN-γ dilution series of 200-0.2 ng/ml were used in the same ELISA set up. The most sensitive combination was selected and used in the capture ELISA.

Table 1. Monoclonal antibodies specific for elephant IFN-γ and their reactivity in indirect ELISA. Monoclonal antibodies (n = 11) specific for recombinant elephant IFN-γ and their relative affinities.

MoAb	Reactivity
AE16D7	+
AE11A7 [a]	+
AE10F4	++
AE12C10	++
AE15A4	++
AE11D3	++
AE13B4	+
AE11G9 [a]	++
AE16F11	++
AE9C3	++
AE16F10	+
Strong reactivity	++
Moderate reactivity	+

MoAb, monoclonal antibody; IFN-γ, interferon gamma. [a]Interaction with HIS-tag.

Fig. 2. Combinations of capture and detection antibodies in the capture ELISA. Performance of capture antibody AE16F10C9 with four different detection antibodies is shown, at a concentration of 1.6 ng/ml recombinant IFN-γ. Capture antibody AE16F10C9 and detection antibody AE10F4G11 was the combination of choice in the optimized capture ELISA protocol.



Western blot assay. The rEpIFN-γ was loaded onto precast Criterion gels (Biorad®) at concentrations of 11.66 µg/ml and 21µg/ml with a marker (precision plus protein dual colour, Biorad®) in one of the lanes. To determine which antibodies were binding linear epitopes, the recombinant IFN-γ was heated to 100℃ for 10 minutes in laemmli buffer. The samples were run at 100V for 1 hour and separated proteins were transferred to nitrocellulose at 0.8 mA/cm_2 for 45 minutes. After transfer, the blot was placed in a roller bottle in 10 ml 0.05% Tween20/PBS and incubated for 1 hour on a bottle roller at RT. After discarding 0.05% Tween20/PBS, the blots were cut into strips and incubated in 10 ml (0.1 µg/ml) of the purified monoclonal antibodies: 16F10C9 and 10F4G11 in PBS and incubated for 10 minutes, washed 3 times with distilled water and incubated with goat anti mouse alkaline-phosphatase (Southern biotech®) 1:1,000 for 10 minutes. The incubation was followed by three wash steps, using water and the detection was performed by adding nitro blue tetrazolium chloride and 5-bromo-4-chloro-3-indolyl-phosphate, toluidine-salt in 67% (DMSO) (v/v) (NBT/BCIP substrate) (Roche®) at RT for 60 minutes. To stop the reaction, the nitrocellulose was washed with running water and the membrane was dried.

3. Whole blood stimulation

First, to optimize the assay two elephants were selected; an Asian elephant from Artis Zoo, Amsterdam and one African elephant from Beekse Bergen Safari Park, both in The Netherlands, that tested negative both in bacterial culture as well as the TB STAT PAK® assay. Second, based on a variety of results of mycobacterial culture and serology (TB STAT PAK, Chembio®) four Asian elephants were selected from the population of the National

Elephant Institute, Thailand for preliminary assessment of IFN-γ responses to tuberculosis specific antigens like ESAT6 and CFP10 to assess their potential for differentiation between infected and non infected elephants. Two of these elephants had never had contact with TB infected individuals and were found negative in the TB STAT PAK® assay for 2 years (non infected). One elephant, was suspected of TB because of a positive result in the TB STAT PAK® assay during more than 2 years, but its trunk wash culture was never found positive. The fourth elephant was shown to be infected with *M. tb* strain ATCC 27294 (modern type strain), in trunk wash culture (Angkawanish et al., 2012). Both Asian and African elephant blood samples were collected in heparinized tubes (vaccutainer®) 20 ml, kept at 4℃ and the samples were processed within 6 hours. Whole blood was diluted 1:1 with complete medium (RPMI1640 + Glutamax containing 5%FCS, 50U/ml penicillin, 50U/ml streptomycin, 5,000 U heparin, 5x10-5 M 2-mercaptoethanol and L-glutamine (Gibco®) and incubated in 24-well tissue culture plates at 37℃, 5%CO_2 for 48 hours with mitogens used as positive control stimulants including pokeweed (PWM-lectin, Sigma®) 5 µg/ml and phorbol myristate acetate plus ionomycin (PMA/I, Sigma®, 100 ng/ml and 2 ug/ml) and the antigens avian and bovine tuberculin (PPDA and PPDB, synbiotics®) 25 U/ml, the hybrid of ESAT6 and CFP10 protein (E6/CFP10, Staten Serum Institute, DK) 1 µg/ml and ESAT6 (Staten Serum Institute, DK) 1 ug/ml. Culture medium was used as the negative control. After incubation, blood samples were centrifuged at 3,200 RPM for 10 minutes and the supernatants were harvested and stored at -80℃ until tested in the optimized IFN- γ ELISA, described below.

4. Elephant IFN-γ capture ELISA, optimized protocol

ELISA plates (Greiner® Microlon, extra high binding 655061) were coated with 50 µl of 2 µg/ml capture antibody (MoAb 16F10C9) in PBS for 1 hour at RT. Plates were blocked with 1.3% casein in PBS (universal casein diluents, SDT®) for 1 hour at RT, emptied and washed 4 times with PBS/0.05% Tween-20. The supernatants of the whole blood cultures were diluted 1:1 with 1.3% casein buffer and added in triplicate. To produce a rEpIFN-γ standard curve a two-fold dilution series (500pg/ml – 0.5pg/ml in 1.3% casein buffer), was included in the assay. After 2 hours incubation at RT, plates were washed and 50µl/well biotinylated detection antibodies (MoAb 10F4G11) 1: 20,000 (0.025 µg/ml) diluted in 0.65% casein buffer, were added for 1 hour at RT. Plates were washed and streptavidin-peroxidase (SA-HRP80) diluted 1: 20,000 in 50 µl/well 0.5% casein buffer was added for 30 minutes at RT. Plates were washed, substrate 3,3',5,5'-Tetramethylbenzidine (TMB reagent) (SDT®, extra sensitive) was added and the color reaction was stopped after 10 minutes using 1% HCl (1M; 50ul/well) and optical density (OD) was determined at 450 nm.

5. Data analysis

Responses measured by the IFN- γ ELISA were considered positive when higher than twice the average OD value of the negative (medium) control responses.

Results

The IFN-γ assay

The rEpIFN-γ produced in the eukaryotic cell line was used to immunize mice for production of MoAbs needed for the development of the capture ELISA. In addition it was used as the positive control in that assay. Hybridoma's identified as producers of IFN-γ specific antibodies in the indirect ELISA (n=11) were subcloned (Table 1). These antibodies, all identified as IgG1, kappa isotype, were subsequently purified. Finally 7 MoAbs were selected based on specific rEpIFN- γ binding and good growth capabilities of the corresponding hybridoma's to find the optimal combination for the capture ELISA. The combination of MoAb AE16F10C9 as capture antibody and MoAb AE10F4G11 as detection antibody showed the highest sensitivity (Figure 2). The Western blot assay confirmed the binding of rEpIFN-γ by those two antibodies (Figure 3). The typical rEpIFN-γ titration curve capture MoAb (AE16F10C9) and the detection MoAb (AE10F4G11) in the capture ELISA using optimal conditions as described above is shown in Figure 4. Detection levels ranged between 1 and 10,000 pg/ml of rEpIFN-γ.

Fig. 3. Monoclonal antibodies (MoAbs) detect recombinant elephant IFN-γ in Western blot. Capture (left) and detecting (right) MoAbs of choice recognize eukaryotic recombinant elephant IFN-γ (MW 25 kD) in Western blot.

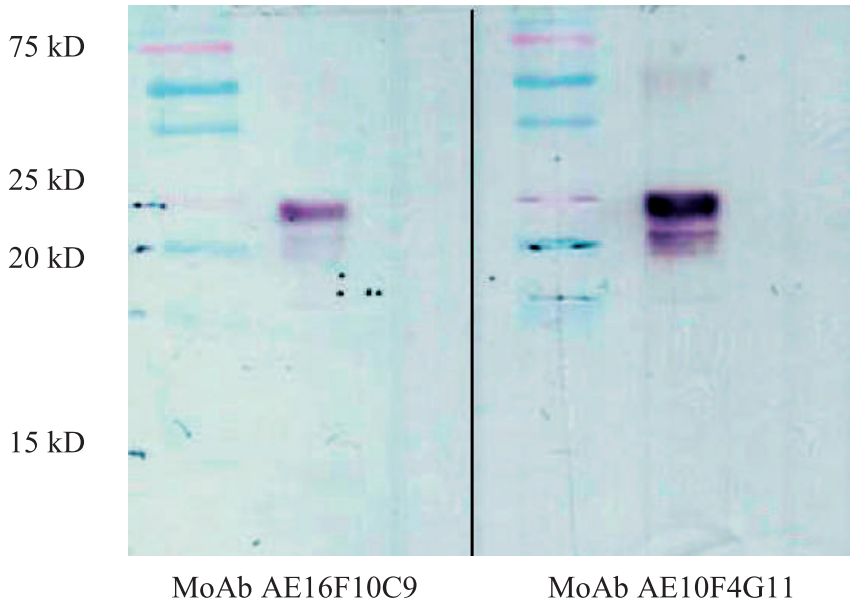
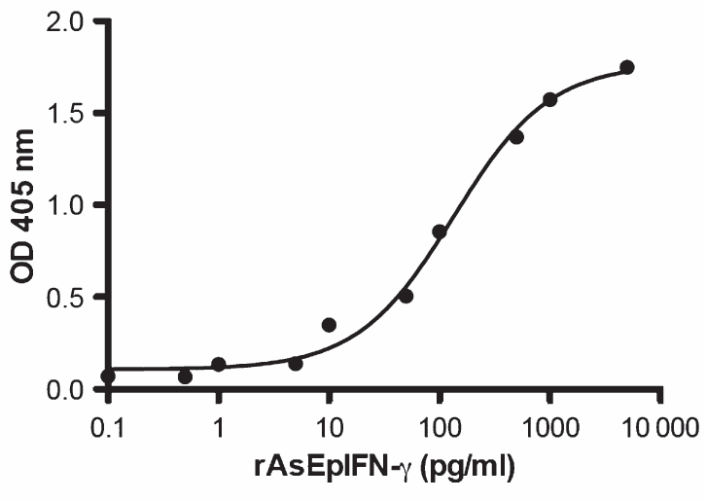


Fig. 4. Titration curve of recombinant elephant IFN-γ using the optimized capture ELISA. Eukaryotic recombinant elephant IFN-γ was titrated in the optimized capture ELISA with capture MoAb (AE16F10C9) and detection MoAb (AE 10F4G11). The lower detection limit was defined as 1 pg/ml.



Detection of native elephant IFN- γ

Assays using supernatants of whole blood cultures of the African and Asian elephants from MTBC free areas (NL) stimulated with PWM showed high OD with means of 1.00 and 2.36 respectively (Figure 5) as compared with the medium (negative control) stimulated cultures that showed mean OD's of 0.12 and 0.26. The assay showed to be applicable for both Asian and African elephants.

Fig. 5. Recognition of native African and Asian IFN-γ in the capture ELISA Whole-blood cultures (duplicates) of an Asian and African elephant were stimulated with mitogen in duplicate.

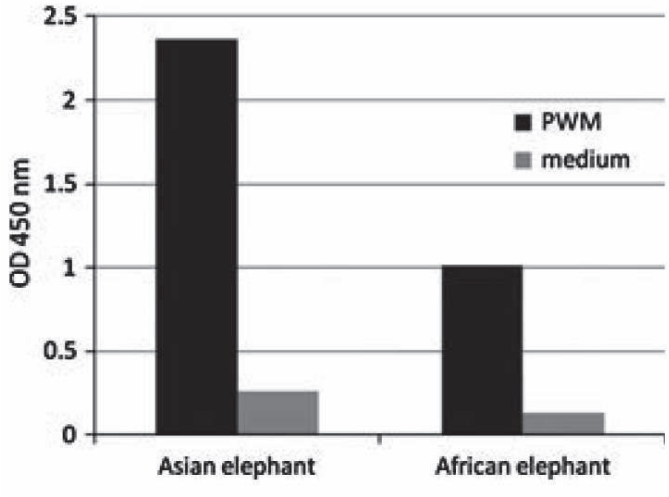


Table 2. Detection, of IFN-γ among four Asian elephants of different TB status. IFN-γ ELISA results (OD) in two non-infected, one TB-suspected and one *M. tb*-infected elephants after 24 h whole-blood stimulation (duplicates) with PWM, PMA/I as positive controls, and the MTBC-specific antigens ESAT6/CFP10 (EC) and PPDB. PPDA and medium were (negative) control stimulants.

Stimulant	Non-infected elephant 1	Non-infected elephant 2	TB-suspected elephant	M. tb-infected elephant
PWM	1.577 ± 0.24	1.058 ± 0.19	1.867 ± 0.19	2.569 ± 0.43
PMA/I	2.021 ± 0.02	1.976 ± 0.05	2.563 ± 0.12	2.572 ± 0.55
EC	0.103 ± 0.00	0.196 ± 0.03	0.129 ± 0.00	1.208 ± 0.02
PPDB	0.127 ± 0.03	0.164 ± 0.04	0.104 ± 0.03	0.530 ± 0.02
PPDA	0.132 ± 0.00	0.206 ± 0.01	0.123 ± 0.00	1.141 ± 0.10
Medium	0.080 ± 0.00	0.151 ± 0.03	0.062 ± 0.00	0.119 ± 0.00

IFN-γ, interferon gamma; *M. tb*, *Mycobacterium tuberculosis;* MTBC, *Mycobacterium tuberculosis* complex; PPD-B PPD-A, protein extracts of mycobacteria.

In Table 2 results of IFN- γ assay are shown that confirm its ability to detect native elephant IFN-γ to a relevant level. The positive controls PWM and PMA/I reveal high OD values in all 4 Thai Asian elephant samples. Samples from non infected elephants were found negative upon stimulation with ESAT6/CFP10 PPDB and PPDA. The IFN-γ ELISA of supernatants of whole blood culture of the elephant suspected of TB is negative (ESAT6/CFP10 borderline positive), whereas OD values after stimulation with ESAT6/CFP10 fusion protein, PPDB and PPDA are lower than the OD values of the non infected elephants. Finally, test results of the infected TB elephant were positive for all antigens ESAT6/CFP10 fusion protein, PPDB and PPDA with high OD450 values (1.208, 0.53 and 1.141, respectively).

Discussion

Availability of MoAbs specific for rEpIFN- γ and native elephant IFN- γ is an important pre-requisite for the development of an IFN- γ release assay for the diagnosis of tuberculosis in elephants. Antibodies produced showed cross-reactivity to both Asian and African elephant IFN- γ. The lower detection limit of the assay, 1 pg/ml rEpIFN- γ, seems adequate to assess responses elicited by MTBC specific antigens. In humans in highly endemic areas such as Ethiopia, ESAT-6 responses showed IFN-γ levels in the range 0-100 pg/ml in the lowest 30% of responders (Andersen et al., 2007). Similar levels were also observed in other species like cats (Rhodes et al., 2008).

In preliminary attempts to assess the assay in a TB setting whole blood samples of two non infected and one TB suspected elephant did not show responsiveness to ESAT6/CFP10, PPDB and PPDA, while those of the confirmed infected elephant showed IFN-γ production in response to these MTBC specific antigens as well as PPDA, the latter most likely due to cross-reactivity with environmental mycobacteria such as *M. avium* (Rhodes et al., 2008). The assay needs further optimization using pathogen specific antigens in addition to CFP10, ESAT6 and may have potential for differentiating between non infected and infected elephants (Vordermeier et al., 2001 and Andersen et al., 2007). In recent years, the IFN-γ assay has been complementing the tuberculin skin test in humans and cattle, facilitating early detection and follow up of disease progression. The IFN-γ assay is now established for both Asian and African

elephants, but needs further validation for its use in diagnosis which relies on its application in large populations of non infected, suspected and infected elephants.

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CHAPTER 5

Prevalence of *Mycobacterium tuberculosis* infections in captive Asian elephants *(Elephas maximus)* and association of infection with demographic risk factors.

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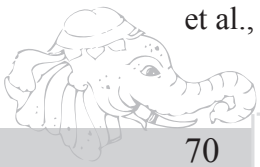
Summary

To assess elephant's serological TB statuses and to identify and quantify potential demographic risk factors for TB, three ELISAs specific for different mycobacterial antigens (ESAT6, CFP10, MPB83) and the TB Stat-Pak assay were used as surrogate serological markers for TB infection in elephants. In view of the low number of animals of which the infected status could be confirmed (4 out of 708) Latent Class Analyses was used to predict the serological TB status of each of 708 elephants as positive (17.3%), inconclusive (48.7%), or negative (34%) when assessed on a population basis. Correlation between test performance of the individual assays was high between the ELISAs, but low with that of the TB Stat-Pak assay. Risk factors, assessed based on cut off values determined by ROC analysis, included sex, BCS, age, working time, feed type, management system, camp size and region. Old age elephants were more likely to be of the positive serological TB status, than younger ones. Elephants working 7 hours per day and the ones in good condition BCS (7-11) were less likely to be of the positive serological TB status. In addition, fewer animals in the large camp size (31-50 elephants) were found to be positive in ELISA tests, compared to elephants in the other camp sizes. In this study, the North region had the lowest percentages of elephants predicted to be of positive serological TB status. The West region and to a lesser extend the other regions showed clearly higher percentages. Even though assays used in the present study have not been validated yet, results obtained showed promise as diagnostic tests, especially when used in combination, in identification of *M. tuberculosis* infected and non-infected elephants as well as assessment of demographic factors as potential risk factors.

Introduction

Tuberculosis (TB) is a re-emerging infectious disease that can be transmitted within and between humans and animal species by transfer of mycobacteria of the *Mycobacterium tuberculosis* complex (MTBC). Its main representatives are *M. tuberculosis* (Une and Mori, 2007), particularly infecting humans, and *M. bovis* hosted by a broad range of domestic and wild animals, as well as humans (Katale et al., 2012 ; Luciano and Roess, 2020. Infection of captive and wild Asian elephants *(Elephas maximus)* with *M. tuberculosis* has been shown worldwide (Angkawanish 2010, Mikota et al., 2015, Zachariah et al., 2017 and Chandranaik et al., 2017)) Transmission of MTBC from humans to elephants as well as other species and vice versa, is likely to happen in situations of close and prolonged contact and may occur via air, droplets, mucus and feces (Michalaak et al., 1998, Mikota, et al., 2001, Lewerin et al., 2005, Une and Mori, 2007, Angkawanish et al., 2010, Murphree et al., 2011, Stephens et al., 2013 and Zachariah et al., 2017). Transmission between domesticated and wild elephants may furthermore occur in direct contact at their interface (Muslow and Mikota, 2015, Yakubu et al., 2016, Zachariah et al., 2017 and Paudel et al., 2018) and, because mycobacteria can survive for more than 45 days in the environment, shared facilities and resources may be major risk factors for infection with *M. tuberculosis.* Especially in countries in South East Asia, like Thailand where TB prevalence in humans is very high (Oh et al., 2002), zoo employees, including ground keepers and custodians show a high incidence of interferon gamma release assay or tuberculin skin test positivity, indicating (latent) infection, related to their socio-economic background, thus identifying them as potential risk factors for the animals in their care (Yakubu et al., 2015).



MTBC infection in elephants is a chronic disease, and difficult to diagnose (Lyaschenko et al., 2012; Steinmetz and Rutten, 2016). The gold standard for TB diagnosis, definitely confirming infection, is bacterial culture. However, since this assay has a very low sensitivity (i.e., a high false negative rate) (Lyaschenko et al., 2006; Greenwald et al., 2009; Angkawanish, 2010) and can only be conducted in a very limited number of elephants, alternative diagnostic assay have to be considered. An Interferon Gamma Release assay (IGRA), the diagnostic method of choice in humans (Taggart et al., 2004 and Whitworth et al., 2013), is available for Elephants (Angkawanish et al.,2013 and Paudel et al., 2016) , but not yet validated, besides for large surveys it is cumbersome to use due to strict demands for sampling and limited testing facilities. In the present study, as an alternative, serological assays were assessed for discrimination between infected and non-infected animals (Verma-Kumar et al., 2012), The ultimate goal of the current study was to contribute to the design of control and prevention strategies for M. tuberculosis infection and its transfer to and within Asian elephants.

Like for humans, stressful conditions such as poor sanitation, starvation, and high workloads are considered to be risk factors for tuberculosis in elephants (Betts, 2002 and Sood et al., 2016). Furthermore other stressors like transportation, separation or isolation, and training could activate latent tuberculosis in elephants, aggravate active disease, and induce respiratory shedding of MTBC into the environment (Lewerin et al., 2005; Angkawanish et al., 2010; Stephens et al., 2013). Thus, potential risk factors considered to be associated with *M. tuberculosis* infection (Payeur B. et al., 2002; Yakubu et al., 2015; Miller et al., 2019) include the infection status of humans in close contact with elephants, and a number of intrinsic and extrinsic factors like elephant age, body condition score (BCS), exposure to infected animals or shared enclosures, water/food resources, working activities and time, camp size, and feeding management.

The aim of the present study using ESAT-6, CFP-10, and MPB83, as putative correlates of infection, in enzyme-linked immunosorbent assays (ELISAs) and the Stat-Pak assay, was to: 1) estimate the prevalence of *M. tuberculosis* infection in the captive elephant population in Thailand; and 2) assess associations of the TB status, predicted by latent class analysis (LCA, Kumar et al., 2012), with demographic characteristics; 3) assess cutoff values, sensitivity and specificity of elephant humoral immune responses to specific MTB complex antigens.

Animals, Materials and Methods

Elephants

The present study was conducted on captive Asian elephants *(Elephas maximus)* in Thailand that were visited by veterinarians for registration and health care purposes as part of a mobile elephant clinic project (MEC) between 2004 – 2009. Of 1,500 serum samples collected in this cross sectional survey, a randomly selected subset of 708 samples (47%) from individual elephants at 124 tourist camps in six regions of the country (13 provinces) were used for serological diagnosis. This sample set represented approximately 18.7 % of the captive elephant population in Thailand.

Elephant information provided by keepers and owners included: owner name/address; elephant name, age and sex; elephant ID; physical examination data; a description of the

elephants habits/behavior; elephant current and past work places and daily routines; camp sizes; and feeding types and general management.

Data collection

Blood was collected from an ear vein into 10-ml vacutainer™ tubes without EDTA, kept at 4°C and centrifuged within 12 hrs. Serum was harvested and stored frozen (-20°C) until further analysis using the Stat-Pak assay or three different ELISAs at the National Elephant Institute (NEI), Lampang, Thailand. Elephants were categorized into five age groups: young (less than 3 years), sub adult (4 – 10 years), adult (11-50 years), middle age (51-60 years) and retired (over 60 years) as described by Corvanich (1981). Working times were divided into four categories: no work (0 hour), limited work (2 hours/day), intermediate work (3 hours/day) and full day work (7 hours/day). Three feeding types were distinguished: natural feeding, provided (by man) feeding, and a combination of provided and natural feeding. Elephant management consisted of either intensive or extensive categories (Fowler and Mikota, 2006). The intensive management system is characterized by provided feeding, and prolonged times in proximity of and under the care of humans. With an extensive management system, elephants were more independent, and allowed to forage in the forest at night; hence, natural feeding is only supplemented by man. Elephant camp sizes were divided into four categories: small camps (1-9 elephants), medium camps (10-30 elephants), large camps (31-50 elephants) and very large camps (over 51 elephants). Body condition scores (BCS) based on visual observation of several body regions were categorized as poor (scores 1-2), thin (scores 3-4), optimal (scores 5-8) and obese (scores 8-11) (Wemmer et al., 2006).

Serological assays

The Elephant TB Stat-Pak, a lateral-flow test (Chembio Diagnostic Systems, Inc., Meldford, NY) employing specific antigens common to *M. bovis* and *M. tuberculosis* for the detection of antibodies was performed according to manufacturer's instructions (Lyaschenko et al., 2006; Landolfi et al., 2010). For quantitative assessment of antibodies specific for individual MTBC related recombinant antigens, indirect ELISAs were conducted as follows. First wells of 96-well Microwell™ maxisorb ELISA plates (Nunc, C96 446140) were coated overnight at 4°C with 50 µl of a 1 µg/ml solution (in PBS, pH 7.4) of one of three recombinant antigens: early secreted antigen target 6 (ESAT-6); culture filtrate protein 10 (CFP-10); and cell surface lipoprotein MPB83. Subsequently, plates were washed once with 0.1% Tween20 in PBS (pH 7.4). Finally, 150 µl of a 0.1% blocking buffer solution (Cat. No. 11 112 589 001, Roche™) in sterile water was added per well for 30 minutes at room temperature. After this blocking step, wells were washed once more and elephant sera added at a dilution of 1:800 in blocking buffer (50 µl/well). This dilution was shown to provide an optimal range in optical density (OD) signal between positive and negative control sera. Two control serum samples were analyzed in each plate: one from an elephant confirmed to be infected (positive control), and one from a healthy elephant in the Netherlands without a history of tuberculosis or contact with infected animals (negative control). After 60 minutes of incubation at room temperature, plates were washed three times with 0.1% Tween20 in PBS (pH 7.4) after which 50 µl/well of rabbit anti-elephant IgG, diluted 1:8,000 in blocking buffer was added for 30 minutes at room temperature. Plates were washed three times again, followed by addition of 50 µl/well

goat anti-rabbit IgG H+L (HRP) conjugate diluted 1:2,000 in 0.1% blocking buffer (KPL's HRP stabilizer cat. No. 54-15-01) and incubation for 30 minutes at room temperature, after which plates were washed five times again. Finally 50 µl/well of substrate [2,2'-azinobis 3- ethylbenzothiazoline-6-sulfonic acid (ABTS) tablets, Cat. No. 11112 422 001, dissolved in buffer (Cat. No. 11 112 597 001, Roche®] prepared according to manufacturer's instructions was added and the OD (405nm) read after 15 minutes.

ELISA values were expressed as sample to positive ratio [S/P ratio = (OD of sample – OD of negative control) / (OD of positive control – OD of negative control)] × 100.

Statistical analyses

The S/P ratios obtained in the ESAT6, CFP10, and MPB83 ELISAs as well as the positive/negative scores of the TB Stat-Pak assays were subjected to pairwise comparisons. Latent Class Analysis (LCA; Visser and Speekenbrink, 2010) was applied using ESAT6, CFP10, MPB83 and TB Stat Pak assays to predict the three serological TB statuses (positive, inconclusive, negative). The predicted statuses were cross tabulated with demographic factors and univariable logistic regression was applied to assess the strength of association between predicted *positive* versus *not positive* (inconclusive and negative combined) statuses and the respective demographic factors. Multivariable logistic regression with all demographic factors with backward elimination based on Akaike's Information Criterion (AIC) was applied to assess adjusted odds ratios. The analyses were done using the statistical program R (R Core Team, 2019) version 3.6.0. Determination of cut-off values, sensitivity and specificity for the three ELISAs were achieved in two steps: 1) an LCA model using 3 out of 4 tests, excluding the specific test itself, was used to predict the three serological statuses; and 2) Receiver Operation Curve analysis (Sing et al., 2005) was applied for each of the tests with the predicted *positive* versus *not-positive* TB statuses as the true status. Subsequently, the cut-off values defined, were used to dichotomize the S/P ratios of the tests and results were cross tabulated (Table 7) with the three predicted serologic TB statuses. The numbers of positive tests for each serum sample were also cross tabulated (Table 8) with the predicted serological TB status. The same procedure was applied to assess cut-off values for *negative* versus *not-negative* TB status.

Results

Test results of CFP10 and MPB83 ELISAs of 708 elephant samples in the cross-sectional study showed high correlation (>0.6). In contrast pairwise correlations of results of these ELISAs with those of the ESAT-6 ELISA were moderate, whereas correlation of results of each of the three ELISAs with those of the commercial TB Stat-Pak assay were very low (Table 1). Based on LCA, the proportions of the population of elephants in the predicted serological TB positive, inconclusive, and negative status groups were 17.3%, 48.7% and 34%, respectively (Tables 2 and 3). In the predicted positive group, only 18.8% of the animals was TB Stat-Pak positive. The mean and standard deviation for S/P ratios resulting from the ESAT6, CFP10 and MPB83 ELISAs decreased from predicted positive to predicted negative status (Table 3).

Table 1. Pairwise correlations of test results (Stat-Pak assay (pos/neg); ELISAs (S/P ratios) between the four serological tests.

Test	ESAT6	CFP10	MPB83
TB Stat-Pak	0.112	0.248	0.197
ESAT6		0.417	0.377
CFP10			0.642

Table 2. Percentages of elephants (n=708) per LCA predicted serological TB status and concordance with the TB Stat-Pak assay outcomes for each of the TB statuses.

Predicted serological TB status						
	Positive (17.3%)*		Inconclusive (48.7%)		Negative (34%)	
TB Stat-Pak	pos.	neg.	pos.	neg.	pos.	neg.
Proportion	18.8 %	81.2 %	3.9%	96.1%	1.4%	98.6%

*Estimated population prevalence of TB statuses by applied LCA model.

Table 3. Percentages of elephants (n=708) per LCA predicted serological TB status and concordance with the TB Stat-Pak assay outcomes for each of the TB statuses.

Predicted serological TB status						
Positive (17.3%)*			Inconclusive (48.7%)		Negative (34%)	
S/P ratio	Mean	SD	Mean	SD	Mean	SD
ESAT6	31.92	23.04	19.37	14.27	6.98	6.54
CFP10	41.63	17.77	20.04	8.29	7.50	5.41
MPB83	50.41	21.54	26.30	12.03	9.94	7.99

*Estimated population prevalence of TB statuses by applied LCA model.

The distributions of the three predicted serological TB statuses of 708 individual elephants across the categories of each demographic variable is presented in Table 4. Though actual numbers are low elephants with a high BCS (7-8 and 9-11) were more often serologically negative than elephants with lower BCSs. The proportion of elephants predicted to be serological TB positive tended to increase with age. The proportion of elephants with negative predicted serological status was highest in the North region. For the other demographic variables representation of the three precited TB status did not differ between categories.

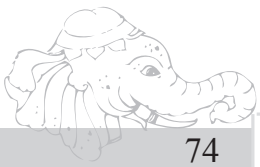


Table 4. Percentages of elephants (n=708) per LCA predicted serological TB status and concordance with the TB Stat-Pak assay outcomes for each of the TB statuses.

		Predicted serological TB status		
	Category	Positive n (%)	Inconclusive n (%)	Negative n (%)
Variable	Total	106 (15)*	357 (50)*	245 (35)*
Sex	Female	79 (15)	275 (51)	181 (34)
	Male	27 (16)	82 (47)	64 (37)
BCS	1-2	3 (27)	7 (64)	1 (9)
	3-4	3 (12)	12 (48)	10 (40)
	5-6	99 (15)	325 (51)	219 (34)
	7-8	0 (0)	12 (44)	15 (56)
	9-11	1 (50)	1 (50)	0 (0)
Age (y)	1-3	3 (13)	7 (30)	13 (57)
	4-10	7 (12)	31 (53)	21 (36)
	11-50	85 (14)	305 (52)	197 (34)
	51-60	8 (28)	11 (38)	10 (34)
	> 61	3 (30)	3 (30)	4 (40)
Working time (hr/d)	0	2 (20)	4 (40)	4 (40)
	2	0 (0)	0 (0)	1 (100)
	3	14 (30)	21 (45)	12 (26)
	7	90 (14)	332 (51)	228 (35)
Feed type	Natural-human	57 (14)	226 (54)	139 (33)
	Natural	17 (17)	44 (43)	41 (40)
	Human	32 (17)	87 (47)	65 (35)
Management system	Extensive	29 (13)	100 (47)	86 (40)
	Intensive	77 (16)	257 (52)	159 (32)
Camp (n) size	1-9	29 (18)	75 (47)	57 (35)
	10-30	45 (18)	129 (53)	70 (29)
	31-50	7 (6)	72 (60)	41 (34)
	> 51	25 (14)	81 (40)	77 (42)
Region	Central	4 (22)	12 (67)	2 (11)
	East	11 (9)	81 (63)	37 (29)
	North	16 (6)	115 (43)	138 (51)
	Northeast	5 (50)	3 30)	2 (20)
	South	31 (19)	83 (52)	46 (29)
	West	39 (32)	63 (52)	20 (16)

n = number; *Estimated individual percentages of TB statuses by applied LCA model.

Table 5 shows the results of the logistic regression modelling for each of the 8 demographic variables. Odds of elephants of both sexes having positive predicted serological TB status were similar. Individuals with a BCS higher than 6 were significantly less likely to have a positive predicted TB status compared to the 5-6 group. Older elephants (>50 years) were significantly more likely to have a positive predicted TB status than 10-50 years old elephants. Animals with a workload of 7 hours were significantly less likely to have a positive predicted TB status compared to those with a workload of 0-3 hours per day. Elephants in the North region were significantly less likely to have a positive predicted TB status than in the other regions (West, South and Central, East, and Northeast, respective). Elephants from large camps (31-50) were significantly less likely to have the positive predicted TB status compared to small camps (1-9). Differences in sex, feed type and management system did not show association with predicted serological TB status.

After reduction of the multivariable logistic regression model with all variables, only "region" remained in the final model, indicating confounding between "region" and other variables.

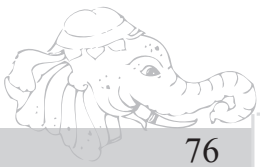


Table 5. Estimates of the strength of association (odds ratio and 95% confidence interval) for positive TB serological status with demographic factors using a univariable logistic regression model.

			Confidence Interval	
Variable	Category	OR	2.5%	97.5%
Sex	Female (Ref)	1		
	Male	1.1	0.7	1.7
BCS	1-2, 3-4	1.1	0.4	2.5
	5-6 (Ref)	1		
	7-8, 9-11*	0.2	0.01	0.9
Age category (y)	1-9	0.8	0.4	1.6
	10-50 (Ref)	1		
	51-100*	2.3	1.1	4.7
Working time (hours/day)	0-3 (Ref)	1		
	7*	0.4	0.2	0.8
Feed type	Natural-Provided (Ref)	1		
	Natural	1.3	0.7	2.3
	Provided	1.3	0.8	2.2
Management system	Extensive	1		
	Intensive	1.2	0.8	1.9
Camp size (n)	1-9 (Ref)	1		
	10-30	1.0	0.6	1.7
	31-50*	0.3	0.1	0.6
	> 51	0.7	0.4	1.3
Region	North (Ref)	1		
	Central, East, Northeast*	2.3	1.2	4.7
	South*	3.8	2.0	7.4
	West*	7.4	4.0	14.3

OR = odd ratio, * = significant

Estimated cut-off values between *positive* and *not-positive* (negative and inconclusive combined) of each of the serological tests, determined using LCA, and corresponding sensitivity and specificity based on Receiver Operation Curve (ROC) analyses (see supplemental Figure 1) are presented in Table 6. The sensitivity (Se) and specificity (Sp) measures were lowest for the ESAT6 ELISA, whereas Se was highest for the MPB83 ELISA and Sp was highest for the CFP10 ELISA. In addition, a second cut-off value was calculated to discriminate between *negative* and *not-negative* (positive and inconclusive combined) predicted TB statuses. For CFP10 particularly, both cut-off values were relatively similar, the histogram of CFP10 shows a very narrow peak hence a little shift to the right has a huge effect (supplemental Figure 2). For the other assay the cut-off values were lower for the second approach. Comparing the *negative* versus *not-negative* (inconclusive and positive) serological TB statuses similar values for sensitivity and specificity are observed except a much lower Se for CFP10.

Table 6. Receiver Operation Curve analysis: Estimated cut-off value and consequent sensitivity and specificity for each ELISA test based on the LCA predicted serological TB statuses.

ELISA Test	Cut-off 1*	Sensitivity1 (Se1)	Specificity 1 (Sp1)	Cut-off 2**	Sensitivity 2 (Se2)	Specificity 2 (Sp2)
ESAT6	18.83	61.5%	68.2%	11.21	63.4%	62.8%
CFP10	21.15	81.4%	75.7%	18.87	55.4%	77.7%
MPB83	28.39	88.0%	71.3%	21.22	83.4%	75.3%

* Cut-off value (S/P ratio) for predicted *positive* versus *not-positive* serological TB statuses at maximum sum of sensitivity and specificity, based on Latent Class Analysis with three tests.

** Cut-off value (S/P ratio) for predicted *negative* versus *not-negative* serological TB statuses at maximum sum of sensitivity and specificity, based on Latent Class Analysis with three tests.

Test results were dichotomized based on the comparison *positive* versus *not-positive* status in the ROC analysis (positive: S/P ratio > cut-off). In the elephants of the predicted positive serological TB status group, the percentage of positive ELISAs was higher than 71%, whereas the TB Stat-Pak was positive for 23% of the animals in this group (Table 7). The percentages of positive ELISAs in the group of elephants with inconclusive serological TB statuses were between 13.5% (CFP10) and 48.3% (ESAT6), whereas percentages were close to zero in the group with negative serological TB statuses, except for the ESAT6 ELISA which scored 21% positive. The TB Stat-Pak assay was positive in 4% and 2% of predicted inconclusive and negative samples, respectively. The proportion of negative ELISAs in elephants with predicted positive serological TB statuses was between 13 and 28%, in case of inconclusive statuses it was higher than the 52%, and higher than 79% in case of negative serological TB statuses. The TB Stat-Pak assay scored negative in more than 77%, 96% and 98% in predicted positive, inconclusive and negative serological TB statuses, respectively.

Table 7. Distribution (frequency and percentages) of dichotomized S/P ratios for each ELISA test per predicted serological TB status using the estimated cut-off value for a positive test for sera of 708 elephants.

		Predicted serological TB status*		
		Positive	Inconclusive	Negative
		92	288	328
Serological test	Single test status	n (%)	n (%)	n (%)
ESAT6	Negative	26 (28.3)	149 (51.7)	259 (79.0)
	Positive	66 (71.7)	139 (48.3)	69 (21.0)
CFP10	Negative	14 (15.2)	249 (86.5)	328 (100)
	Positive	78 (84.8)	39 (13.5)	0 (0)
MPB83	Negative	12 (13.0)	181 (62.8)	327 (99.7)
	Positive	80 (87.0)	107 (37.2)	1 (0.3)
TB Stat-Pak	Negative	71 (77.2)	276 (95.8)	321 (97.9)
	Positive	21 (22.8)	12 (4.2)	7 (2.1)

* Latent Class Analysis was used to predict the serological TB status per individual using four serological tests (ESAT6, CFP10, MPB83 and TB Stat-Pak).

n = number, % = percentage within predicted serological TB status per test

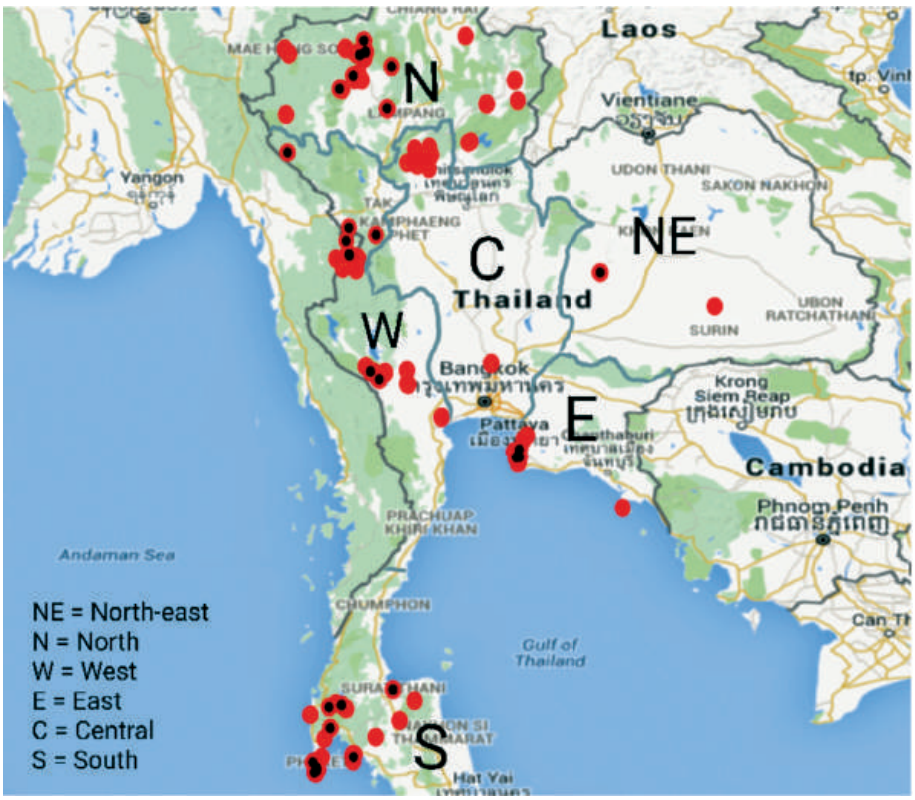
Most samples (93.5%) with a negative predicted TB status were negative in all four single serological tests (Table 8). In the group with inconclusive predicted TB status, about 75% of the samples had none or only one single test positive. In contrast, in the group of elephants with a positive predicted TB status, 95% of the samples were positive in two to four serological tests.

Table 8. Number of positive serological tests (out of 4) done (ESAT6, CFP10, MPB83 and TB Stat-Pak) of elephant samples versus the predicted TB serological status based on Latent Class Analysis.

	Predicted serological TB status		
	Positive	Inconclusive	Negative
Number of positive tests	n (%)	n (%)	n (%)
0	0 (0)	94 (26.3)	229 (93.5)
1	6 (5.7)	172 (48.2)	16 (6.5)
2	38 (35.8)	71 (19.9)	0 (0)
3	46 (43.4)	20 (5.6)	0 (0)
4	16 (15.1)	0 (0)	0 (0)

n= number of animals, % = percentage within predicted serological TB status

Figure 1. Spatial distribution of elephants in the study across Thailand. Red dots marked with a black spot indicate presence of at least one elephant to be of the predicted positive serological TB status.



Discussion

In the present study four different serological assays were assessed for their capability to discriminate between putative *M. tuberculosis* infected, seropositive for MTBC antigens, and non-infected (seronegative) animals (Verma-Kumar et al., 2012). By Latent Class Analysis (LCA), their ability to predict the serological TB status of each elephant was assessed, which resulted in groups of elephants qualified as predicted positive, inconclusive and negative, respectively. Correlations between test performance of the individual assays was modest to high between the three ELISAs, the latter especially between those employing MPB83 and CFP10 as antigens. It was low between the TB Stat-Pak assay and the ELISAs. Samples of animals predicted to be of positive serological TB status (17.3 % of the total population tested) showed highest S/P ratios in the MPB83 and CFP10, and less so in the ESAT6 ELISAs. In the predicted inconclusive group (49%), respectively the predicted negative group (34%) S/P ratios clearly tended to decrease. Hence, to predict negative TB statuses, CFP10 and MPB83 ELISAs are useful, but to predict a positive TB status most accurately parallel interpretation of the results ELISAs detecting MPB83, ESAT6, and CFP10 specific antibodies (Greenwald et al., 2009; Duncan et al., 2009) is required. By contrast, the TB Stat-Pak test scored positive for only 23% of animals of the predicted positive serological TB status. Although the decreasing trend (positive versus inconclusive versus negative) was similar to that observed in the ELISAs, the TB Stat-Pak assay seemed more discriminatory between positive and the other predicted TB statuses (Greenwald et al., 2009). Taken together, results from the three ELISAs and the TB Stat-Pak assay suggest that to identify or screen for non-TB infected elephants, a single test may suffice. However, to confirm a potential TB infected animal unequivocally, using all four tests in parallel with at least 2 positive results might be better as also clearly shown by the data represented in table 8.

The prevalence of *M. tuberculosis* infected elephants based on the percentages classified as "predicted positive serological TB status" is at least 15% and including animals classified as inconclusive up to 65% of elephants may be infected.

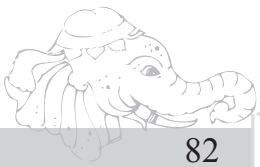
In addition to assessment of the potential of the four serological assays for diagnosis of MTBC infection of elephants, classification of elephants in either of the three predicted serological statuses was related with each of eight potential risk factors for M. tuberculosis infection. Single demographic variables BCS, age, working time, camp size and region seem to be associated to predicted serological TB statuses but in a multivariable analysis only region remained associated. The demographic variables are also related to each other which is explained at the end of paragraph below.

In addition, the strength of these associations were determined (Tables 4 and 5). Elephants with a high BCS (7-11) appear to be at low risk for TB, as compared to low BCS elephants. Low BCS may be associated with poor management, which can in turn predispose elephants to sensitivity to *M. tuberculosis* infection (Bett et al., 2002; Risco et al., 2016). Likewise old aged elephants were more likely to be positive in serological tests, potentially due to a long lifespan hence prolonged contact with infected elephants (Vynnycky and Fine, 1997), and/or caretakers potentially infected with *M.tuberculosis.* (Une and Mori, 2007; Murphree et al., 2011; Lassausaie et al., 2015; Yakubu et al., 2016; Paudel and Tsubota, 2016; Mikota

et al., 2016; Burke et al., 2017). The finding that the western region of Thailand had a high percentage of elephants positive in serological tests, coincides with the highest incidence of human TB in Thailand (Tschirhart et al., 2016; WHO, 2019). Finally, immune responses to control infections like TB can diminish with age (Wang et al., 2012), and may be one reason why older elephants were more likely to be seropositive for TB.

Elephants working a full 7 hours each day were at lower risk for TB as compared to elephants that partook in no or limited work activities. Exercise is important for mental and physical health (Boomershine and Zwilling, 2000; Sood et al., 2016), where elephants participating in work activities showed better body condition and metabolic health than those that did not work (Norkaew et al., 2018). Elephants in large camps (over 30 elephants) were more likely to be negative in serology. In general, larger camps have been in operation longer and are more likely to have standardized management protocols, including sufficient land, nutritious food, clean water, appropriate works loads, good sanitation, and often an on-site veterinarian (Norkaew et al., 2018; Bansiddhi et al., 2019) in comparison to camps of smaller sizes, mostly also due to better economic support. Assessment of other factors in the study, including sex, feed type, and management system did not reveal any differences in TB risk. Some factors like feed type and management, categories human feed and intensive care as well as natural feed and extensive care are closely related, resulting in uncertainty in interpretation. Risk factor results can be generalized to the population of elephants in tourist industry of which the elephants tested were representatives.

In conclusion. The seroprevalence of elephant in Thailand is at least 15%, possibly up to 50% if inconclusive results are included. Unfortunately, no manageable risk factors could be identified in the current study, as a seropositive TB status was related to region and age. Even though assays used in the present study have not been validated yet, results obtained showed promise for their use as diagnostic tests. Especially when used in parallel interpretation, identification of *M. tuberculosis* infected elephants seems valid, while assessment of demographic factors as potential risk factors can be based on LCA approach of multiple unvalidated tests.



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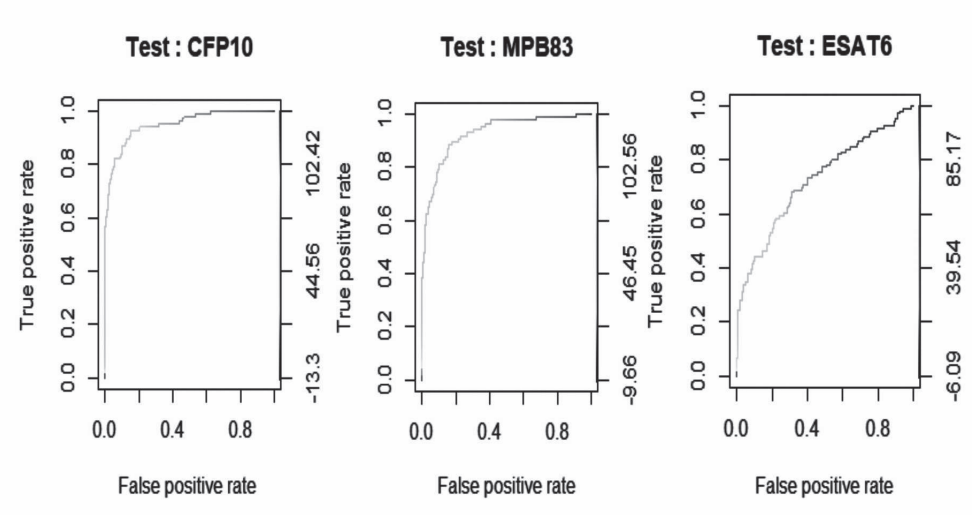
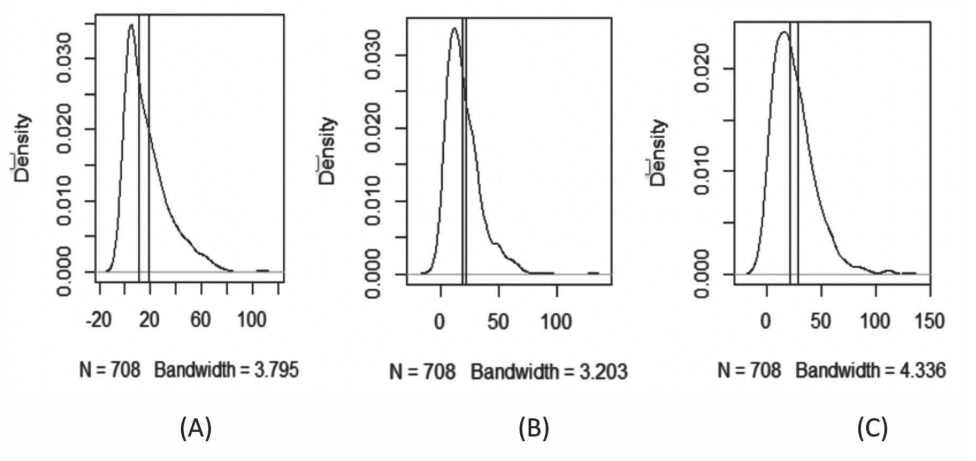
Supplemental Material

Receiver Operation curves (ROC) to estimate the cut-off value, sensitivity and specificity for each test based on the predicted TB state ("TRUTH") using the LCA-method (4 tests).

Supplemental Figure 1. Receiver Operation Curves (ROC) for four serological assays (ESAT6, CFP10 and MPB83 ELISAs and TB Stat-Pak) based on a Latent Class Analysis to predict three serological TB states. Predicted positive status was used as the true TB status for the ROC analysis.



Supplemental Figure 2. Density plots of S/P ratios of ELISA tests: (A)ESAT6, (B)CFP10 and (C) MPB83 respectively. Two vertical lines indicate estimated cut-off values for separating negative and inconclusive samples (left)and between inconclusive and positive.



CHAPTER 6

Evidence of high EEHV antibody seroprevalence and spatial variation among captive Asian elephants *(Elephas maximus)* in Thailand.

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Virology Journal. 2019,16:33,1-9.

Abstract

Background: Elephant endotheliotropic herpesviruses (EEHV) can cause an acute highly fatal hemorrhagic disease in young Asian elephants *(Elephas maximus),* both *ex situ* and in *situ.* Amongst eight EEHV types described so far, type 1 (subtype 1A and 1B) is the predominant disease-associated type. Little is known about routes of infection and pathogenesis of EEHV, and knowledge of disease prevalence, especially in range countries, is limited.

Methods: A large cross-sectional serological survey was conducted in captive elephants (n= 994) throughout Thailand using an EEHV-1A glycoprotein B protein antigen specific antibody ELISA.

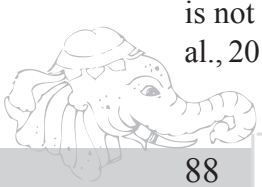
Results: Antibody seroprevalence was 42.3%, with 420 of 994 elephants testing positive. Associations between seropositivity and potential risk factors for EEHV infection were assessed and included: elephant age, sex, camp cluster size, management type (extensive versus intensive), sampling period (wet vs. dry season) and location of camp (region). Univariable regression analysis identified management system and region as risk factors for the presence of EEHV antibodies in elephants, with region being significant in the final multivariable regression model. Prevalence was highest in the North region of the country (49.4%).

Conclusions: This study produced baseline serological data for captive elephants throughout Thailand, and showed a significant EEHV burden likely to be maintained in the captive population.

Keywords: Elephant endotheliotropic herpesvirus, EEHV, Asian elephant, Glycoprotein B ELISA, Seroprevalence, Risk factor

Background

A number of infectious diseases significantly impact elephant population sustainability, particularly in captivity (Schmitt et al., 2003). of great concern over the past two decades is infection with the elephant endotheliotropic herpesvirus (EEHV), which can cause hemorrhagic disease (HD). First recognized in 1999 (Richman et al., 1999), eight genetically-distinct subtypes have been identified, at least six of which are associated with high mortality (Long et al., 2015). In Asian elephants, EEHV-1 (subtypes 1A and 1B) is the most common and virulent, while EEHV-3, -4 and -5 are infectious, but rarely fatal (Garner et al., 2009; Denk et al., 2012; Sripiboon et al.,2013 and Seilern-Moy et al., 2015). Hemorrhagic disease primarily affects Asian elephants under 10 years of age, particularly those between 1 and 4 years, as well as African elephants (Richman et al., 2007). Onset of EEHV-HD is rapid, often with few early clinical signs, resulting in death within a few hours to days after presentation of the first clinical signs in ~80% of cases that present with the disease (Hayward, 2012). Clinical signs are initially nonspecific, but can include lethargy, lameness and colic, later progressing to include swelling of the head and thoracic limbs, oral ulceration, and cyanosis of the tongue as widespread endothelial cell necrosis occurs (Stanton et al.,2013). Since its discovery, EEHV has been the cause of 60% of deaths of young captive-born Asian elephants in western zoos, affecting almost one in four Asian elephant calves born in zoos globally (Hayward, 2012). EEHV is not only present in *ex situ* collections, but has been observed *in situ* in India (Zachariah et al., 2013), Thailand (Sripiboon et al.,2013), Laos (Bouchard et al.,2014), Cambodia, Myanmar,



Nepal and Sumatra (Long et al., 2015). Overall, more than 100 deaths from EEHV have been confirmed globally (Long et al., 2015 and Richman et al., 2014), with many more cases likely going undiagnosed.

In Thailand, an earlier study sampled pharyngeal lymph nodes and found no evidence of EEHV in 31 Asian elephants based on PCR (Hildebrandt et al., 2005). More recently, EEHV infection was confirmed in 15 cases using a semi-nested PCR technique, of which 72% was EEHV-1A (Sripiboon et al., 2016). Today, EEHV-1A is considered the major threat among young Thai elephants (Lertwatcharasarakul et al.,2015), even though not all elephants infected with EEHV develop symptoms. For example, in Thailand, EEHV-1A was detected in 29 healthy Asian elephants from trunk swab samples (Sariya et al., 2012). Likewise, positive EEHV results have also been reported for healthy elephants in western zoos (Hardman et al., 2012 and Stanton et al., 2010). Therefore, it is now recommended that all elephant calves between 1 and 8 years be screened weekly using real time quantitative PCR (qPCR) to monitor viral loads and numbers of monocytes and platelets (Hardman et al., 2012; Stanton et al., 2010; Richman et al., 2000; Stanton et al.,2012 and Howard et al., 2018). Elephants with a high viral load and low monocyte and/or platelet counts should be treated immediately with antiviral and antibacterial drugs, as well as with supportive agents to maintain circulatory homeostasis, prevent inflammation which can lead to vascular shock (fluid therapy, plasma transfusion, glucocorticosteroids), and manage pain (NSAID's) (Howard et al., 2018). Unfortunately the efficacy of medications is not only inconsistent, but has yet to be clearly documented. A vaccine against EEHV is not available yet. Although routine testing using qPCR is generally done in a handful of western zoos, testing can be problematic in range countries because of high costs, and limited equipment and expertise. Despite progress made in viral screening by PCR, especially for symptomatic animals, little is known about the pathogenesis and transmission of this disease or about numbers of elephants exposed or infected. Thus, the current lack of sero-epidemiological data presents a significant gap in knowledge of the EEHV disease burden and susceptibility in a species of commercial and cultural importance to Thailand.

Determination of EEHV antibody titers to establish seroprevalence rates among various elephant populations is based on detection of antibodies against an EEHV-1A viral envelope protein, glycoprotein B (gB). Screening of Asian elephants in U.S. and European zoos showed that nearly 80% of PCR positive animals were seropositive for this protein (van den Doel et al., 2015). The aim of the present study was to assess EEHV seroprevalence in a large cross-sectional survey of elephants in Thailand using the EEHV-1A gB protein antigen ELISA (van den Doel et al., 2015), and to obtain preliminary data on factors potentially associated with infection throughout the captive population in Thailand.

Materials and Methods

Animals

The serological survey was conducted retrospectively on serum samples collected between January 2010 - February 2015, and comprised elephants (n = 994) in private, tourist, and logging camps that were included in a health screening program under the Mobile Elephant Clinic Project in Thailand, led by trained veterinarians and staff of the National Elephant Institute (NEI), Lampang, Thailand. Animals tested represented ~25% of the total

population of captive elephants in the country. Only elephants deemed healthy (asymptomatic) by veterinarians during the routine examinations were included in this study.

Data and sample collection

Elephant information recorded consisted of owner name, elephant name, sex, age, microchip number, copy of official registration identity card, present address and health information. Study variables are described in Table 1. Male and female elephants were categorized into three age groups: <11 years; 11-50 years; and >50 years. Camps were divided into two types of management systems: intensive and extensive, as defined by Mar (Fowler and Mikota, 2006). With intensive systems, elephants are managed individually or in small groups, are fed entirely by humans through prepared fodder, and are tethered at night. They may participate in work activities, such as trekking, but often in a more urban setting. Extensive management involves more traditional activities, like logging, forest trekking, and bathing, and releasing elephants into the forest by long chains or hobbles at night to forage and potentially interact with other captive conspecifics. Camps were grouped into six geographical regions, and whether they were close to the border with neighbor countries. Camps, within a radius of 2 km, that shared resources like a river, road, land area, or working area during the day were clustered and categorized based on the number of elephants: small (<10 elephants/cluster), medium (10-50 elephants) and large (>50 elephants). Over the 5-year survey, elephants were sampled throughout the year, with data grouped according to wet (April-October) and dry (November-March) seasons in Thailand.

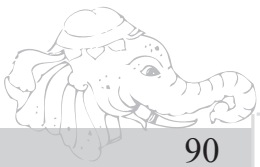


Table 1. Study population demographics, and potential risk factors in association with EEHV antibody seroprevalence of elephants in Thailand (n = 994).

Risk factors	**Category**	**Number** (proportion male/female)
Sex	Female	678 (0.68)
	Male	316 (0.32)
Age category	<11 years	73 (0.45/0.55)
	11-50 years	797 (0.30/0.70)
	>50 years	124 (0.29/0.71)
Management type (province [1])	Extensive: (CM, LP, CR, SKT, Tak, CP)	505 (0.34/0.66) (n=286, 125, 8, 16, 68 and 2 respectively)
	Intensive: (AY, NKPT, RBR, PJ, CBR, Trat, BRR, NKSM, SR, SRTN, PNG, Smui, SKL, CPo)	489 (0.29/0.71) (n=76, 27, 36, 1, 166, 41, 1, 3, 56, 15, 63, 2, 1 and 1 respectively)
Region	Central	76 (0.25/0.75)
	East	207 (0.16/0.84)
	North	435 (0.36/0.64)
	Northeast	62 (0.50/0.50)
	South	82 (0.46/0.54)
	West	132 (0.25/0.75)
Camp cluster [2]	Small cluster (<10 elephants)	19 (0.20/0.80)
	Medium cluster (10-50 elephants)	372 (0.30/0.70)
	Large cluster (>50 elephants)	603 (0.32/0.68)
Border contact with Myanmar	Yes	77 (0.19/0.81)
	No	917 (0.32/0.68)
Sampling period (months)	April-October	824 (0.30/0.70)
	November-March	170 (0.38/0.62)

[1] *CM*=Chiang Mai; *LP*=Lampang; *CR*=Chiangrai; *SKT*=Sukhothai; *Tak*=Tak, *CP*=Chaiyapum; *AY*=Ayuttaya; *NKPT*=Nakhonpathom; *RBR*=Ratchburi; *PJ*=Prajuobkirikhan; *CBR*=Chonburi; *Trat*=Trat; *BRR*=Burirum; *NKSM*=Nakhoratchsima; *SR*=Surinth; *SRTN*=Suratthani; *PNG*=Phang-nga; *Smui*=Smui; *SKL*=Songkla and *CPo*=Chumporn

[2] Defined as number of camps (i.e., those within a radius of 2 km) that shared resources like a river, road or land area, or working area during the day.

A 5- to 7-ml blood sample was collected from an auricular vein. Samples were transferred to blood collection tubes and kept at room temperature for 1-2 hours before centrifugation to harvest serum. Serum samples were stored frozen (-20°C) at the laboratory research unit of the NEI until analysis. One blood sample per elephant was used for this cross-sectional study, and randomly selected when multiple samples were available. Serum samples were

thawed, diluted 1:100 and 1:200 in phosphate-buffered saline, and assessed for the presence of antibodies using the EEHV gB specific capture ELISA described by (van den Doel et al., 2015). Results were expressed as OD ratios (OD sample/OD background) for both serum dilutions (1:100 and 1:200).

Statistical analysis

A single serum dilution (1:100 and 1:200) was considered positive when the OD ratio was >3, undetectable at <2, and inconclusive between 2 and 3 (van den Doel et al., 2015). An animal was deemed seropositive when one or both of the serum dilutions were scored as positive. Results of univariable logistic regression analyses of the potential risk factors [elephant age, sex, camp cluster size, management type (extensive versus intensive), sampling period (wet vs. dry season) and location of camp (region) for the likelihood of an EEHV positive sample were expressed as the OR, CI and P-value. All potential risk factors were included in multivariable logistic regression analysis to create the model, with the exception of management type due to a high correlation with region. Region was chosen to be included in the full model because the model fit was better than the full model with management type. The AIC was used in a backward procedure to select the best model (smaller AIC is better). All analyses were performed using R version 3.3.0; 2016-5-3 (R Core Team, 2014).

Results

Descriptive analysis

Animals were housed at 96 camps in 20 provinces throughout Thailand (Fig. 1); however, it was not possible to sample all elephants at every camp because elephants often were working or otherwise not available when visited by the veterinarian. We sampled one to 57 elephants per camp: at 48 camps, five or more elephants were sampled; at 25 camps, two to four elephants were sampled, and at 23 camps, only a single elephant was sampled. The interval between the first and last sampling date within each camp varied from 0 to >500 days, although for 60 of the 96 camps (including 23 single sample locations), sampling was done within an interval of 30 days. Due to these limitations we cannot qualify any camp as (fully) EEHV-negative or EEHV-positive. Supplemental figure1 established from the present test results, using OD ratio >3 respectively OD ratio > 4 as cut offs! gives an impression of the proportions of positive and negative animals)

Fig. 1 Locations of camps or clusters of camps with captive elephants (N = 994) enrolled in the present study are indicated with red dots. Red dots marked with a black dot indicate sites that had at least one EEHV antibody seropositive elephant.

Study population demographics are presented in Table 1. Amongst the 994 elephants sampled, two-thirds were female. The average age of the total population was 33.1 years (median=34.0), with females averaging 33.8 (sd=14.3; range, 2–76) years, and males averaging 28.9 (sd=14.8; range, 3–66) years. The majority (80.2%) of elephants were within the adult age category (11–50 years), with 7.3% in the <11 years and 12.5% in the >50 years age categories. The average number of elephants sampled per camp was 10.2 (sd=13.3; range, 1-57), and per province was 49.7 (sd=71.6; range, 1 - 286). Elephants were fairly equally distributed between the two types of management systems (Table 1), with most extensive-managed camps located in the North region (86.1%). By contrast, intensive management systems were found in all regions except the North (Table 2). Most elephants resided in large, clustered areas (60.7%), as compared to small (1.9%) and medium (37.4%) clusters (Table 2). Less than 10% of the study subjects lived along the border with Myanmar in the West and North regions (Table 1). The majority of samples were collected during the wet season (April-October, 82.9%) (Table 1).

Table 2. Numbers of elephants sampled in intensive and extensive management systems within six geographical regions in Thailand.

Region	Management System	
	Extensive [a]	Intensive [b]
Central	0	76
East	0	207
North	435	0
Northeast	2	60
South	0	82
West	68	64

[a] Elephants are managed using more traditional methods, including daily species-specific activities, and releasing elephants into the forest (by long chains or hobbles) at night to forage and interact with tame and/or wild conspecifics (U Mar, 2006).

[b] Elephants are managed individually or in small groups, are fed entirely by humans through prepared fodder, and are tethered at night (U Mar, 2006).

Prevalence

Serology results for both serum dilutions are shown in Supplement Table 1. A summary of serology results for separate analyses of 1:100 and 1:200 dilutions is shown in Table 3. of the samples designated as positive using a 1:100 dilution, ~88% were also positive at 1:200. Inconclusive results were similar between the 1:100 and 1:200 dilutions (287 versus 281), respectively, whereas for the undetectable group, the 1:100 dilution identified 407 samples, of which only 285 remained undetectable at 1:200. Eighteen undetectable samples at 1:100 became positive at 1:200, as did 102 inconclusive samples. Based on the criteria that a sample was considered positive if at least one serum dilution was positive, 42.3% were seropositive (420 of 994), 57.7% were undetectable (574 of 994) and none were inconclusive (Table 3).

Table 3. Comparison of antibody seroprevalence based on an EEHV1A glycoprotein B protein antigen specific ELISA of elephants sampled throughout Thailand between 2010-2015 (n=994) using serum dilutions of 1:100 and 1:200.

Dilution 1:100	Dilution 1:200			
	Positive[1]	Inconclusive[2]	Undetectable[3]	Totals (1:100)
Positive	263 (*) (87.7%)	37 (*) (12.3%)	0 (0%)	300
Inconclusive	102 (*) (35.5%)	140 (48.8%)	45 (15.7%)	287
Undetectable	18 (*) (4.4%)	104 (25.6%)	285 (70%)	407
Totals (1:200)	383	281	330	994

[a] Optical density (OD) ratio (OD sample/OD background) $\geq$3

[b] OD ratio between 2 and 3

[c] OD ratio <2

* At least one sample dilution was positive

Risk factor analysis

Descriptive serology test results related to potential risk factors (Table 4) were used in the univariable logistic regression analysis (Table 5). Variables used for the modeling were elephant age, sex, camp cluster size, management type (extensive versus intensive), sampling period (wet vs. dry season) and location of camp (region). Border contact samples were unevenly distributed and applied to only two regions (North and West), so those data were not included

in the univariable model. Female elephants trended towards being less often seropositive than males (OR=1.29; p=0.06), but there were no differences between the age groups. Elephants from camps utilizing extensive management systems had higher seroprevalence than those managed more intensively, with some regional differences. Compared to the North, elephants in the Central, Northeast and East regions had a lower odds for a positive sample. No association was found for the EEHV status of the sample with camp cluster size and sampling period. In the multivariable logistic regression analysis, only region remained in the model after backward elimination of the variables and therefore the results are the same as from the univariable model with region (Table 5).

Table 4. Antibody seroprevalence of elephants throughout Thailand (n=994) between 2010-2015 based on an EEHV1A glycoprotein B protein antigen specific ELISA, and the proportion of samples testing positive or negative relative to potential EEHV risk factors.

Potential risk factors	Positive[a]	Negative
• Sex		
Female (*n*=678)	273 (40.2%)	405 (59.7%)
Male (*n*=316)	147 (46.5%)	169 (53.4%)
• Age category		
<11 years (*n*=73)	32 (43.8%)	41 (56.2%)
11-50 years (*n*=797)	331 (41.5%)	466 (58.5%)
>50 years (*n*=124)	57 (45.9%)	67 (54.0%)
• Management type		
Extensive (*n*=505)	238 (47.1%)	267 (52.9%)
Intensive (*n*=489)	182 (37.2%)	307 (62.8%)
• Region		
Central (*n*=76)	17 (22.4%)	59 (77.6%)
East (*n*=207)	78 (37.7%)	129 (62.3%)
North (*n*=435)	215 (49.4%)	220 (50.6%)
Northeast (*n*=62)	21 (33.9%)	41 (66.1%)
South (*n*=82)	36 (43.9%)	46 (56.1%)
West (*n*=132)	53 (40.2%)	79 (59.8%)
• Camp cluster[b]		
<10 (*n*=19)	11 (57.9%)	8 (42.1%)
10-50 (*n*=372)	164 (44%)	208 (55.9%)
>50 (*n*=603)	245 (40.6%)	358 (59.4%)
• .Border contact		
Yes (*n*=77)	25 (32.5%)	52 (67.5%)
No (*n*=917)	395 (43.0%)	522 (57.0%)
• Evaluation period		
Apr-Oct (*n*=824)	337 (40.9%)	487 (59%)
Nov-Mar (*n*=179)	83 (48.8%)	87 (51.2%)

[a] Samples were considered positive if at least one dilution (1:100, 1:200) was positive (OD ratio ≥3) . All other combinations were defined as negative.

[b] Defined as number of camps (i.e., those within a radius of 2 km) that shared resources like a river, road or land area, or working area during the day.

Table 5. Univariable regression analysis of potential risk factors for the presence of EEHV antibodies in elephants sampled throughout Thailand between 2010-2015 (*n*=994) based on an EEHV1A glycoprotein B protein antigen specific ELISA.

Potential risk factors	Prevalence (%)	*p*-Value	OR	95% CI
Sex				
Female (*n*=678)	40.2	Ref	1	NA
Male (*n*=316)	46.5	0.06	1.29	0.98-1.68
Age category (year)				
<10 (*n*=73)	43.8	Ref	1	NA
10-50 (*n*=797)	41.5	0.70	0.91	0.56-1.48
>50 (*n*=124)	45.9	0.77	1.09	0.61-1.95
Management type				
Extensive (*n* 505)	47.1	Ref	1	NA
Intensive (*n* 489)	37.2	0.00	0.66	0.51-0.85
Region				
North (*n*=435)	49.4	Ref	1	NA
Central (*n*=76)	22.4	<0.00	0.29	0.16-0.51
East (*n*=207)	37.7	0.00	0.61	0.44-0.86
Northeast (*n*=62)	33.9	0.02	0.52	0.29-0.90
South (*n*=82)	43.9	0.36	0.80	0.49-1.28
West (*n*=132)	40.2	0.06	0.68	0.46-1.01
Camp cluster[a]				
<10 (*n*=19)	57.9	Ref	1	NA
10-50 (*n*=372)	44.0	0.24	0.57	0.21-1.44
>50 (*n*=603)	40.6	0.13	0.49	0.19-1.24
Evaluation period				
Apr-Oct (*n*=824)	40.9	Ref	1	NA
Nov-Mar (*n*=170)	48.8	0.37	1.24	0.76-2.01

Ref: reference category, *NA:* not applicable, *OR:* odds ratio, *CI:* confidence interval
[a]Defined as number of camps (i.e., those within a radius of 2 km) that shared resources like a river, road or land area, or working area during the day.

Discussion

The present study was the first to conduct a large cross-sectional survey of EEHV seroprevalence among captive elephants in Thailand. Using an EEHV-1A gB protein antigen ELISA (van den Doel et al., 2015), over 40% of elephants tested were found to be seropositive. Although animals were healthy at the time of blood collection, a significant number appeared to have been exposed to EEHV based on antibody seroprevelance, most likely maintaining

this virus within the population. Because it was not possible to sample every elephant at each camp, we could not determine if there were any 100% seropositive or seronegative camps in Thailand. However, the vast majority of seronegative elephants resided at camps with seropositive ones and so could be susceptible to infection in the future. In the study of van den Doel (2015), some elephants maintained significant titers for prolonged periods, while others were intermittently seropositive. One seropositive elephant was categorized as healthy at the time of blood collection, but had presented with EEHV-like symptoms a few weeks before. This finding may indicate a prior EEHV infection, but that could not be confirmed. Results suggest that routine serological surveys may help identify prior viral exposure, which would otherwise go undetected as many exposed elephants are asymptomatic.

One of the characteristics of herpes viruses is their ability to go into latency. By certain unknown stimuli these latent viruses may be reactivated (Bissinger et al., 2002). If reactivation does not occur over a long period of time, antibodies may drop to levels near to or below the detection limit of the ELISA. This makes it difficult to conclude that inconclusive or seronegative elephants are actually free of EEHV. The elephants in this study were all over 1 year of age, so maternal antibodies were not likely present to influence the outcome of the ELISA. As there is no vaccine against EEHV available, all antibody titers that were detected are assumed to be the result of previous exposure to EEHV. Each elephant with antibodies against EEHV should be considered as latently infected and a potential periodical shedder (Bissinger et al., 2002 and Bennett et al., 2015). Camps that consist of only seronegative animals are at risk of infection if a seropositive elephant is added to that camp; however, a false seronegative status may be the result of the absence of virus reactivation over a prolonged period, or insensitivity of the ELISA to detect a significant titer. As a consequence animals newly introduced into a camp are at risk of infection depending on the presence of even only one animal classified as EEHV infected. Model building initiated by submitting sex, age, regions, camp cluster size and sampling period (without management type) to multivariable analyses gave rise to the final multiple logistic regression model that identified “regions" as the most potent risk factor to EEHV in Thailand. More specifically, our study revealed that the Central, Northeast, East, West and South regions were lower in prevalence compared to the North. This result confirmed a higher incidence of EEHV in northern regions (Boonprasert et al., 2017) based on sample tissue submissions and reported elephant deaths. Specifically, between 2006-2017, 32 clinical cases of EEHV-HD in Thailand were confirmed by PCR techniques, and of those, a third (n=11) were found in the North. Overall, two thirds of EEHV antibody seropositive elephants were found in North, South and West regions of Thailand. By contrast, only two cases (2/32) occurred in the Central region, an area with only a few facilities close together, with limited exchange of animals from the outside. Understanding spatial differences in seroprevalence is complicated, however, by uncontrolled/unregistered elephant movements and transfers, particularly among facilities within those regions, and so needs further study.

The type of elephant management system was a significant risk factor to positive EEHV antibody seroprevalence in the univariable model, with 47% antibody seroprevalence in extensive systems compared to 37% in more intensive systems. The North and West regions include areas along the border with Myanmar, and contain more than half of the captive elephant population in Thailand. Although there is clinical evidence of EEHV-HD in captive

elephants in Myanmar, there has been no molecular confirmation to date (Charernpan P., personal communication, National Elephant Health Service, DLD Thailand, 2017). However, given the close contact and/or transport of captive elephants between the Thai-Myanmar borders, transmission of the viral disease to elephants in the North and West of Thailand from Myanmar is possible, similar to what has been documented for foot and mouth disease viral transmission across these regions (Gleeson et al., 2002 and Nardo et al., 2012). Captive elephants in Myanmar are maintained in more natural habitats (extensive care system), particularly at night. Most are allowed to forage in nearby forests while on long chains, so there is potential for more interaction between wild and captive elephants in that country, whereas in Thailand, captive and wild elephants are found to cohabitate mainly in western regions.

Captive elephants in the South also had a relatively high antibody seroprevalence. In general, these were working elephants from the North and West that are taken to rest at their owner's home in the South during the low tourist season. Conversely, elephants in the Central, East and Northeast regions live in more urban areas, closer to humans, where land and especially forest, is limited, and generally they are not exchanged between camps. The camps in intensive management systems also are less likely to transport elephants or recruit elephants from outside those regions than those in extensive systems. That may limit the degree of exposure to the virus, and agrees with our finding that most elephants living in isolated areas were seronegative. It is likely the seropositive elephants that experienced recent infection or reactivation might be related to camps with frequent or with rare viral reactivations.

Elephants sampled in this study were involved in tourism or logging, which requires tame elephants; hence, the higher numbers of adults than other age categories, and females being more prevalent than males. Trending towards significance (OR=1.29) was a sex effect, with more males being seropositive, although the relevance of this is unknown. By contrast, elephant age, camp cluster size, and sample collection period were not significant risk factors for EEHV antibody seroprevalence. Our finding that 42.3% of captive elephants in Thailand were seropositive for EEHV antibodies suggest a high rate of viral exposure in this population. Extrapolating to the total captive population in the country (n=4,016 elephants), upwards of 1,600 may have been exposed to the virus. The EEHV antibody seroprevalence survey showed that “region” was a significant risk factor associated with the disease incidence, particularly in the North, which is likely to be related to management or perhaps genetic relationships. Since the first diagnosed case of EEHV HD in 1999, this disease has resulted in elephant deaths, particularly calves, around the world, although it is more sporadic than epidemic in captive populations. Long et al. (2015) suggested that disease severity is related to primary infection, and that around 20% of young elephants are susceptible.

Finally, a potential limitation of the EEHV-1A gB protein antigen ELISA may be that it has insufficient sensitivity to detect low antibody titers, which could lead to an underrepresentation of seropositive animals. Van den Doel et al. (2015) suggested that one or both OD ratios should be >3 to indicate true seropositivity, while a cut off OD ratio >4 for both would be stricter. Hence, in addition to the analysis presented in table 4, we examined the distributions of positive and negative test results in camps using cut off OD ratios of >3 respectively >4 for both dilutions (Supplement Figure 1) and found they were similar with a peak around OD ratio 2. Both distributions showed a small elevation around OD ratio 5

(dilution 1:100) or OD ratio 6 (dilution 1:200), which might indicate distribution in a population with recent reactivation of virus or recent infection, whereas the elevation around OD ratio 2 might be the mode of an uninfected population. Obviously a more strict definition of positive animals e.g. both OD ratios >4 could lead to classifications of animals with low antibody titers as EEHV-negative. Comparison of the risk factor analysis for individual elephant data, criterion for qualification as a positive animal "one or both OD ratios >3" (Table 5) with risk factor analysis using the more stringent qualification criterion OD ratio >4 showed the same results (Supplement Table 2). Until we are able to grow virus in culture to establish ELISA sensitivities, there is the risk of misinterpreting OD ratio results, either positive or negative, depending on what cutoff value is used.

Conclusions

This is the first comprehensive investigation of EEHV antibody seroprevalence in an Asian range country. Our study showed that 43.8% of young elephants were antibody seropositive, similar to the older age groups, which suggests that elephants of all ages are being exposed to this potentially deadly virus. Results highlight the need for additional research to determine the immunopathogenesis of an EEHV infection, especially to elucidate why antibody seroprevalence is higher in the North, and in elephants that are more extensively managed. Genomic analyses to identify potential genetic factors associated to pathogenesis also might help explain why most elephants survive, and some do not. It is particularly important to track elephants with EEHV antibody titers over longer periods of time (longitudinally), especially if they have been suspected of prior active infection.

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Abbreviations

EEHV: Elephant Endotheliotropic Herpesvirus; HD: Hemorrhagic Disease; PCR: Polymerase Chain Reaction; qPCR: Quantitative Polymerase Chain Reaction; gB: Glycoprotein B; OD: Optical Density; ELISA: Enzyme-Linked Immunosorbent Assay; OR: Odd's Ratio; CI: Confidence Interval; AIC: Akaike's Information Criterion; DLD: Department of Livestock Development.

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None

Availability of data and materials

The datasets generated and/or analyzed during the current study are not publicly available due to the fact that the data is part of ongoing research. However, the data are available from the corresponding author on reasonable request.

Authors' contributions

TA and VR developed the concept and design of the study. TA conducted the sample collection. TA and VR drafted the manuscript and were the corresponding authors. MN, KL and HV performed all the statistical analysis and were instrumental in interpretation of study results. JB, PD, PK and WS assisted in ELISA testing and in revising the manuscript for submission to a journal. All authors read and approved the final manuscript.

Ethics approval

This work was conducted as part of official duties of the Mobile Elephant Clinic project of the National Elephant Institute (NEI), Thailand to conduct routine health checks of captive elephants in Thailand. All procedures followed government regulations and guidelines, and those of the NEI.

Competing interests

The authors declare they have no competing interests

Consent for publication

Not applicable as the manuscript does not contain any data from any individual person.

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CHAPTER 7

Summarizing Discussion

The Asian elephant (Elephas maximus) has been an important part of Thai society for thousands of years. Thai people believe that elephants evolved with Lord Buddha and the Buddhist religion, and they are part of national history. Today, elephants are regaled in royal Thai society and a national symbol. They are also important economically and a key component of Thai tourism.

Elephants in Thailand are classified as wild or captive, and each of these are subject to their own set of regulations. Killing or capture of wild elephants has been prohibited since 1921 by the Wild Elephant Protection Act. Today, Asian wild elephants are categorized as a protected animals under the Wildlife conservation and Protection Act of 1992, whereas the Draught Animal Act of 1939 classified elephants as private property and confers no animal welfare protection. Governmental organizations work cooperatively to register, census, microchip, and DNA fingerprint of all captive elephants. Registration within 90 days of birth is now mandatory, to prevent the illegal capture of wild elephants for use in tourism and unregistered elephants without the microchip or DNA databases are confiscated and sent to a government facility, usually the National Elephant Institute (NEI) in Lampang.

During the last 2 decades, elephant camp standards were issued by the Department of Livestock Development (DLD) and additional standards by the Department of Tourism. The more recent Cruelty Prevention and Welfare of Animals Act (2014) has two main objectives: to prevent animal cruelty and to promote animal welfare, so far law enforcement is not in place and specific welfare criteria have yet to be defined.

Good health care is key to a good quality of life for captive elephants, which includes studying and understanding life-threatening diseases, such as TB and EEHV **(Chapter 2)**, next to many others affecting elephant health and welfare **(Chapter 1)**. Elephant TB is caused by infection with bacteria of the *Mycobacterium tuberculosis* complex (MTBC), mainly *M. tuberculosis* like in humans and *M. bovis*. EEHV-HD is particularly devastating to young elephant calves, it is an acute hemorrhagic disease and highly fatal. The goal of this dissertation was to conduct studies on these two diseases to monitor and control infection, improve diagnostic tools, and understand risk factors for infection. Understanding the epidemiology of these diseases is crucial to improve the management and care of captive elephants, which in the future will be critical to study disease transmission, from human to captive elephants and vice versa (TB), and between individual elephants within and between herds, as well as between wild and captive elephants.

Tuberculosis

Elephant tuberculosis: *M. tuberculosis* culture and transmission

Over the past three decades, increasing numbers of Asian elephants infected with *M. tuberculosis* have been reported around the world, in the U.S. (Michalak et al., 1998, Mikota et al.,2001, Payeur et al., 2002, Dumonceaux et al., 2011, Murphree et al., 2011, Feldman et al., 2013, Zlot et al., 2016 Simpson et al., 2017; Miller et al., 2018), Europe (Lewerin et al., 2005), Australia (Stephen et al., 2013; Volgelnest et al., 2015) and Asia (Angkawanish et al., 2010,

Ong et al., 2013, Paudel et al., 2014, Yakubu et al., 2016, Zachariah et al., 2017). Nucleotide sequence polymorphism in the *gyrB* gene of the mycobacteria isolates confirmed the identity of *M. tuberculosis* in four elephants at the NEI (Angkawanish et al., 2010). *M. tuberculosis* can be classified into ancestral and modern strains based on *M. tuberculosis* specific deletion (TbD_1) (Brocsh et al.,2002), and our analysis showed that *M. tuberculosis* isolated from one elephant had a *gyrB* gene sequence identical to strains of the ancient TbD_1 positive strain **(Chapter 3)**. The other three were infected with strains identical to *M. tuberculosis* ATCC 27294, the modern type that is widely spread in humans, is highly pathogenic and more virulent than the ancient strain. In US, genotyped isolates of infected elephants and their caretakers from facilities are identical (Murphree et al., 2011). Other MTBC species shown to infect elephants include *M. bovis* (Greenwald et al.,2009) and *M. caprae*, in a Bornean elephant, (Yoshida et al., 2018) caused tuberculosis. In contrast *M. avium,* commonly found in trunk wash samples, (Payeur et al., 2002, Angkawanish et al., 2010), is potentially related to contamination of soil and feed and does not cause disease (Yong et al., 2011). As described in humans (Kaufmann, 2002), MTBC are intracellular pathogens that reside in macrophages and evoke cell mediated immunity (CMI), including lymphocytes producing Th_1 cytokines, macrophages, and dendritic cells, finally resulting in formation of granulomas, that limit the spread of the tubercle bacilli. During progression of the disease, a switch from Th_1 to Th_2 type responses may occur, which gives rise to increasing antibody titers, while CMI wanes. Control of replication and spread of the mycobacteria is lost, which may lead to shedding and transmission of the mycobacteria (Landolfi et al., 2015). Alternatively like in humans TB tuberculosis may be in a latent state, with limited progression of disease (Water et al., 2014), potentially implying low or absent shedding of bacteria Little is known about latency and reactivation statuses of the disease in elephants, hence this needs further study.

In three of the elephants, in our study **(Chapter 3)** gross and microscopic pathology showed TB like lesions, confirmed by *M. tuberculosis* culture. Trunk wash cultures had been negative before, and in a retro-serological study, the earliest detection of antibodies was 23 months before TB infection was confirmed. The fourth animal clearly showed active TB, because the gold standard diagnostic method trunk wash sampling and culture was positive. Sensitivity was very low only two out of 60 trunk wash samples resulted in positive cultures. Annual screening of staff (n=223) by chest radiography was so far negative. TB screening of humans in contact with *M. tuberculosis* infected elephants by tuberculin skin test (TST), Interferon gamma (IFN-γ) release assay (e.g. the QFT test) is important, particularly in countries where people are not given the Bacillus Calmette Guerin (BCG) vaccine, (Oh et al., 2002; Simpson et al., 2017). As an example, in the U.S., one person was infected and developed active TB after being exposed to elephants at a facility during a TB outbreak in elephants. A cohort study conducted in elephant caretakers and other employees, indicated that especially close and prolonged contact are risk factors for *M. tuberculosis* transmission from elephants to humans (Zlot et al., 2016).

As a consequence, early detection of *M. tuberculosis* infection in elephants is key and can rely on assessment of CMI responses, like IFN-γ production in an interferon gamma release assay IGRA **(Chapter 4)** and/or Humoral immune responses in serological assays **(Chapter 5)**.

Prevalence of *M. tuberculosis* infection: Interferon Gamma Release Assay (IGRA) and serological assays

Although bacterial culture is considered the gold standard for diagnosis of TB in elephants, it is insensitive with a high probability of false negatives. Thus, we developed an elephant IGRA **(Chapter 4)**, similar to that used for the diagnosis of *M. tuberculosis* infection in humans. This diagnostic test determines cell mediated immune responses for early detection and measures interferon gamma (IFN-γ) release after *in vitro* stimulation with mitogens PWM, PMA/I), MTBC antigens (ESAT-6/CFP10, PPDB) PPDA. The assay has a lower detection limit of 1 pg/ml and is capable of distinguishing between non-infected and infected elephants, whether suspect animals can be identified as infected needs further study. To increase sensitivity of IFN-γ detection, instead of using whole blood stimulation, isolated peripheral blood mononuclear cells (PBMC) including T-lymphocytes can be stimulated. IFN-γ produced can be assessed using the elephant IFN-γ specific ELISA or by an enzyme-linked immunospot (ELISPOT) a method quantifying stimulation by enumeration of IFN-γ producing T-cells a clearly laboratory based. Increased sensitivity and specificity, can also be achieved by the use of alternative recombinant MTBC antigens, apart from ESAT-6 and CFP10; for example MPB83. To establish the assay as early detection tool for monitoring disease, larger numbers of elephant samples should be tested, including those from as yet untested and exposed (suspect) animals, as well as known non-infected, and know infected elephants as controls. Preferably also longitudinal to further validate the diagnostic value of the technique. At present a large survey is being conducted in Europe where blood samples from all elephants that are translocated between Zoo's is subjected to an Elephant IGRA. On the longer run this should result in validation of the assay for TB diagnosis (Guidelines for TB management in captive elephants in the European Elephant TAG, 2018). Because IFN-γ production based assays require fresh whole blood and nearby laboratory facilities, they may be cumbersome when conducting large countrywide surveys. For that reasons we also investigated the potential of using serological assays for discriminating between infected (seropositive) and non-infected (seronegative) elephants. For that study, ESAT-6, CFP10 and MPB83 specific ELISAs as well as the Elephant TB Stat-Pak assay were used, and single and parallel interpretation considered, especially in view of differential (in specificity) immune reactivity in course of disease progression.

In our study **(Chapter 5)** Latent Class Analysis (LCA) was used to predict the three serological TB statuses, of the 708 Asian elephants tested. Population prevalence of *positive, inconclusive* and *negative* statuses were 17.3%, 48.7% and 34% respectively (Table 2 and 3), were slightly different from individual based percentages (Table 4). After dichotomizing S/P ratios of the individual tests based on the cut off values determined in the ROC analysis comparing *positive* versus *not positive* status (inconclusive combined with negative statuses), 71.7 % (ESAT-6) to 87% (MPB83) of the elephants predicted to have a positive serological TB status were found positive in the individual ELISAs. In the elephants predicted to be of inconclusive serological TB status 13.5% (CFP10) to 48.3% (ESAT-6) were still positive in individual ELISAs. This indicates that potentially 15-65 % of the elephants has been exposed to or infected with *M. tuberculosis.* This may of course be latent and without shedding, but based on human studies around 10% of latent TB may become active TB (Kaufmann, 2002). Most elephants in this study resided in tourist camps, and about 15-20% were managed in an

extensive care system that allows foraging and free roaming in forested habitats and interactions with wild elephants is possible. So, transmission of TB between captive and wild elephant is a threat in need of further study. Although these assays have not been thoroughly validated yet, results showed promise for their use as diagnostic tests for assessing the *M. tuberculosis* infection status, as well as assessing which demographic factors are potential risk factors for the disease.

Risk factors for a positive serological TB status as an indicator of infection with *M. tuberculosis*

In **Chapter 5** we examined association of demographic and environmental factors with LCA predicted TB statuses (Verma-Kumar et al., 2012), and found that elephants in good health and good working conditions like in the larger camps are at a lower risk than animals in smaller camps. Old age implied a higher risk than young age to have a positive serological TB status. When associations with "regions" were assessed, elephants in the west and south regions, which may connect with different elephant populations, such as foraging captive elephants, wild elephants in protected areas, and where a high incidence of human TB is present, were at highest risk, compared to the other areas. The factors management system (intensive or extensive) nor Feed type were significantly related to serological TB status. Considerations about variables that were not included in the model analysis, elephant contact statuses with (non) exposed and infected elephants tend to be related to demographic variables in the present TB status analysis, particularly in view of the long period of gradual progression of tuberculosis. However, to confirm TB status in live elephant might be difficult and complicated. To starting with history of TB exposed could be an option to study about elephant TB risk factor analysis. Moreover, additional studies including longitudinal samples of exposed individuals should be follow, before and after confirmation of the infection status. Finally, to determining risk factors for elephant TB would benefit from using a combination of CMI and humoral immune response assays to monitor enable to detect immune responsiveness from the earliest time of exposure until infection is confirmed and thereafter.

Evidence of high EEHV antibody seroprevalence and spatial variations

Infection with EEHV may result in acute and highly fatal hemorrhagic disease (EEHV-HD), mainly in young Asian elephants. Little is known about pathogenesis and transmission. The study presented in this thesis **(Chapter 6)** aimed at determining the sero-prevalence of EEHV in a large number of Thai elephants (18,7% of the captive population) and to assess risk factors potentially associated with infection. In a capture ELISA the EEHV gB protein of EEHV-1A (van den Doel et al., 2015), the most predominant strain among Thai elephants as assessed by PCR so far (Boonprasert et al.,2019). Of the elephants in our survey, 42.3% (43,8% of young elephants) were found seropositive, representing one fourth of the elephant population in Thailand. This indicates that EEHV-1A is widely present in the captive elephant population, as a latent infection. Because EEHV-1B is closely related to EEHV1A, it may be detected based on cross reactivity in the ELISA. A clear weakness of the ELISA is difficulty in interpretation that may have resulted in an under-estimate of the proportion of actually infected elephants. A survey, using qPCR to detect the viral load in trunk wash fluid and eye secretion of captive free ranging elephants in India showed that EEHV was present in 35% of the elephants tested

(Stanton et al., 2014). Although EEHV1, EEHV3/4 as well as EEHV5 were found clinical symptoms were not observed. Analyses of risk factors was performed using univariable and multivariable logistic regression models and "Regions" was found to be the most important risk factor for infection and it was shown that EEHV infection occurs more frequently in the "North" **(Chapter 6)**. One explanation is that "region" is closely related to "elephant management", which differs across regions. Most elephants in the north interact more during the work day and even after work at night compared to other regions, and so may have more opportunities to transmit disease. Therefore, more epidemiological investigation, particular in a high seropositive area, like Northern Thailand, would reveal more on the transmission of the disease, and pathogenesis of this fatal virus. Moreover, other related factors e.g. transportation, weaning age of the calves, environmental change, nutrition, calf management etc. should be studied in association with the serological outcome to see more risk factors.

Although exposure to other elephants was not an important variable in the model, a number of reports shows that elephants could intermittently shed the virus in trunk secretion (Ackerman et al., 2017; Azab et al., 2018, Bauer et al. 2018, Bennette et al., 2015; Hardman et al., 2012). There is evidences showing that the possible contact and transmission of the virus through normal communication behavior of elephants, i.c. using the trunk to touch and to put in each other's mouth (Schulte, 2006). In our study (Chapter 6) among 994 elephants, only one elephant showed symptoms of EEHV-HD and was positive in conventional PCR a few weeks before sampling while Sripiboon (2016) found viral shedding via trunk wash and eyes secretions. Comparison of secretion samples and serum samples of 23 elephants by PCR showed that in five elephants the virus was present, two of them being reactive in the serological test as well (Sripiboon, 2016). This suggests that in some infected elephants, antibody levels may be undetectable, but they may not be shedding the virus. Hence, to determine the elephant's EEHV infection status, it is important to include exposed, non-infected and infected animals in the assessment and to use a combination of diagnostic tools such as PCR and serology tests. Furthermore, understanding of pathogenesis as well as design of strategies for diagnosis and prevention of infection with EEHV would benefit from longitudinal follow up of infected elephants.

Conclusion

Tuberculosis is an ancient disease that affects a wide range of wild and captivity species including wild and captive elephants and humans. Due to changes in host immune responsiveness, the disease may progress, clinical symptoms develop, and shedding of mycobacteria may occur. Assays assessing immune responses are instrumental to disease diagnosis, designing strategies for prevention and control, and also for managing risk factors. EEHV-HD presents as a disease caused by members of a *deltaherpesvirus* that evolved separately from other herpesviruses in ancestors of modern elephants around 35-40 million years ago (Zong et al., 2014). EEHV is particularly fatal for elephants 1 and 8 years of age, leading to severe hemorrhagic disease. Although EEHV has been recognized in elephants for over two decades, little is known about the role of immune responsiveness in health and disease. Both diseases have serious impacts on elephant health and population sustainability and are inadequately diagnosed and controlled. Communication, collaboration and proactive

testing is required and should involve all stakeholders in the elephant community to control the spread of these devasting diseases, and ensure the survival of a Thai national symbol, *Elephas maximus.*

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CHAPTER 8

Summary

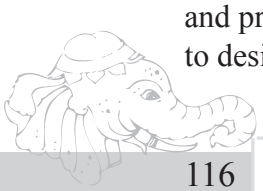
The Asian elephant *(Elephas maximus)* is a mega-herbivore qualified as endangered species (CITES, Appendix 1). Around 40,000-50,000 wild Asian elephants live in the 13 Elephant range countries in Asia, whereas another 13,000 elephants are maintained under human care. Besides approximately 1,700 Asian elephants are kept under human care in the rest of the world.

Apart from decreasing territory sizes and scattering of elephant habitats, these have to be shared with livestock, wildlife and humans. The latter potentially causes human-elephant conflicts. Moreover, the Asian elephant's health, well-being and population sustainability are affected by several life-threatening diseases **(Chapter 1)**. Tuberculosis (TB), with potential zoonotic impact, and EEHV-HD, live threatening especially for young elephants, are amongst the most serious ones **(Chapter 2)**. Besides, they have the potential to severely affect the economy, particularly in relation to tourism.

The goal of this dissertation was to conduct studies on these two diseases to strengthen monitoring and control of infections, improve diagnostic tools, and understand risk factors for infection.

Infections of elephants with *M. tuberculosis* and EEHV have been reported worldwide, both in wild and captive elephants. Since in Thailand, frequent contact between elephants and *M. tuberculosis* infected human individuals, as well as between wild and captive elephants is likely, there is a need for tools to enable early and accurate diagnosis. Out of four elephants, showing clinical signs of TB, included in our initial study **(Chapter 3)**, three reacted positive in a serological test (TB STAT Pak, Chembio). In serum of one of these, antibodies were found retrospectively, 23 months prior to bacterial culture from trunk wash samples. The fourth animal, that showed severe clinical signs of TB was only tested for antibodies 7 days before death, but was found negative. For all four elephants *M. tb* infection was confirmed by gross pathology and PCR on tissue samples. Of 60 trunk wash samples of one elephant only 2 confirmed positive by trunk wash bacterial culture. This elephant was kept in a restricted area after abrogated treatment due to severe clinical signs, that comprised lethargy, anorexia and ataxia. Moreover, blood chemistry indicated liver and renal problems. Sequence analysis of *M. tuberculosis* isolates from the four animals classified them as ancient strains (n=1) based on presence of a *M. tuberculosis* specific deletion (TbD_1) and modern strains (n=3) such Beijing, Haarlem and African *M. tuberculosis* cluster, identical to *M. tuberculosis* ATCC 2794. Mycobacterial interspersed, repetitive unit variable-number tandem-repeat (MIRU-VTR) typing of the ETR-A gene showed that the three representatives of the modern strains differed. Hence, there must have been different sources of infection for each of the four TB elephants included in the present study.

Although there is little information about elephant immune responses in TB, based on knowledge from diagnostic practices and pathogenesis studies in humans, non-human primates and ruminants we set out to study **(Chapter 4)** production of interferon gamma (IFN-γ). Detection of this representative of a range of cytokines produced upon stimulation with *M. tuberculosis* derived antigens, is used for diagnosis at the early stage of infection with M. tb. For this purpose, we cloned and expressed recombinant Elephant IFN-γ (rEpIFN-γ) and produced monoclonal antibodies (MoAbs) specific for elephant IFN-γ. These were used to design and validate a capture enzyme linked immunosorbent assay (ELISA). The titration



curve of rEpIFN-γ used in the capture ELISA showed detection levels ranging between 1 and 10,000 pg/ml. The assay was found to be able to detect native elephant IFN-γ of both African and Asian elephant whole blood cultures.

In vitro whole blood cultures containing elephant T-cells, were exposed to positive and negative control stimulants as well as MTBC specific antigens, H37Rv *M. tuberculosis* strains, early secreted antigen target 6 (ESAT-6), culture filtrate protein 10 (CFP-10), surface glycosylated lipoprotein (MPB-83) and classical Purified Protein Derivatives, both *M. bovis* (PPD-B) and *M. avium* (PPD-A) derived. To finalise the Interferon Gamma Release Assay (IGRA) cytokine presence in the culture supernatants was measured by a IFN-γ capture ELISA **(Chapter 4)**. Among Asian elephants, of different TB status, detection of IFN-γ upon in vitro stimulation of whole blood samples with ESAT6/CFP10, PPDB and PPDA, was negative for samples from non-infected elephants, as well as those from an elephant suspected of TB. Whole blood cell stimulation of a *M. tb* infected elephant with antigens ESAT6/CFP10 PPDB and PPDA resulted in detection of interferon gamma in all cultures, though to a lesser extent when stimulated with PPDA as a control for non MTBC environmental mycobacteria contact. To complete the diagnostic spectrum, but also since due to inadequate sampling circumstances the IGRA designed could not yet be used in Thailand, we also determined MTBC specific antibodies, supposed to be produced in later stages of infection, and potentially indicating progression of disease towards the clinical stage. The *M. tuberculosis* complex specific antigens, ESAT-6, CFP-10, and MPB83 ELISAs and the TB Stat Pak assay were used to define the serological TB statuses and determine and quantify the impact of potential demographic risk factors associated with *M. tuberculosis* infection **(Chapter 5)**. ELISA results of serum samples of 708 elephants were subjected to Latent Class Analysis (LCA) to predict their serological TB statuses. Depending on LCA approaches, in casu population versus individual elephant based, relative numbers (percentages) for the different statuses were “positive” 15-17.3%, “inconclusive” 48.7-50% and “negative” 34-35% of the animals. Correlation between test performances of individual assays was high between ELISAs but low between ELISAs and the TB Stat Pak assay. Demographic risk factors including sex, body condition score (BCS), age, working period, feed type, management system, camp size and region were assessed based on cut off values, determined by ROC analysis. Old age elephants were more likely to be of a positive serological TB status, than younger animals. Elephants working 7 hours per day and those in good condition (BCS 7-11) were less likely to be of the positive serological TB status. In addition, fewer animals in the large camp size (31-50 elephants) were found to be positive in ELISA tests, compared to elephants in the other camp sizes. In this study, the North region had the lowest percentages of elephants predicted to be of positive serological TB status. The West region and to a lesser extend the other regions showed clearly higher percentages. Even though assays used in the present study have not been validated yet, results obtained showed promise as diagnostic tests, especially when used in combination, both in identification of *M. tuberculosis* infected and non-infected elephants as well as in assessment of demographic factors as potential risk factors.

Haemorrhagic Disease caused by Elephant Endotheliotropic Herpes Virus (EEHV-HD) **(Chapter 6)** is mostly lethal, it is believed that elephants that survive primary infection, potentially due to still present maternal antibodies, are likely to be protected against secondary infection. To assess the prevalence of EEHV infection in Elephants in Thailand we performed a cross sectional serological survey, using an EEHV1 glycoprotein B (gB) specific antibody

ELISA. Of 994 elephants included in the study 42.3% were positive and 57.7%negative, while none were inconclusive, based on the criterion that a serum sample is considered positive if at least one serum dilution tested positive. Associations between seropositivity and potential risk factors for EEHV infection including: elephant age (<11 years, 11-50 years, >50 years), sex, camp cluster size (<10, 10-50, >50 elephants), management type (intensive and extensive), sampling period (wet and dry) and location of camp (central, east, north, northeast, south, west) were assessed. Univariable regression analysis identified that i) elephants in extensive management showed higher seroprevalence than those under more intense management and ii) elephants in central, northeast and east regions had lower odds to be seropositive as compared to those in the north region (49.4% seroprevalence). In the final multivariable regression model the variable "region" was represented.

In conclusion, the goal of the studies reported in this PhD thesis was to conduct studies on TB and EEHV-HD diagnostic tools to monitor and control both infections, and to assess risk factors. Clear proof has been found for the presence of tuberculosis caused by multiple strains of *M. tuberculosis* in the Thai elephant populations. To prevent, or at least reduce, transmission amongst elephants and man an efficient diagnostic regimen needs to be in place. The IGRA developed, has adequate sensitivity to detect relevant IFN-γ levels, but its use is hampered by relative difficult quality conditions. Hence establishment of IGRA for TB diagnosis at peripheral laboratories needs to be considered. Serological testing as presently used suffers from high percentages of animals classified as inconclusive, hence it is in urgent need of improvement. Combination of serological assays preferentially including IGRA will be the better way of diagnosis, and risk factors more accurately assessment in support of management. Even though the present EEHV ELISA is rather cumbersome, it showed a high prevalence of EEHV, that will be a continuous threat to Asian elephants in Thailand and beyond. In general, but specifically in the north region, showing high prevalence, elephant contact management should be considered, particularly for young calves. In addition to behavioural observation and monitoring activities presence of EEHV could be determined by frequent serological and molecular detection in serum and secretion samples. Ultimately a vaccine needs to be developed.

Finally, publication of this thesis will increase professional and public awareness of the threats of TB and EEHV infection in Elephants in Thailand.

Samenvatting

De Aziatische olifant *(Elephas maximus)* is een mega-herbivoor die met uitsterven bedreigd wordt (CITES, Appendix 1). Naar schatting 40.000-50.000 wilde Aziatische olifanten leven in de zg. "Elephant range countries" in Azië en daarnaast zijn er ongeveer 13.000 gedomesticeerde olifanten. In de rest van de wereld zijn er ongeveer 1700 Aziatische olifanten in gevangenschap.

Naast afname in omvang van territoria en versnippering van leefgebieden, moeten deze in toenemende mate gedeeld worden met andere wilde dieren, productiedieren en mensen, waardoor mens-olifant conflicten kunnen ontstaan. Ook worden gezondheid, welzijn en stabiliteit van de olifantenpopulatie beïnvloedt door een aantal levensbedreigende ziekten **(Hoofdstuk 1),** daaronder vooral tuberculose (TB), dat potentieel zoönotisch is en EEHV-HD, met name een bedreiging voor jonge olifanten **(Hoofdstuk 2)**. Deze kunnen ook ernstige economische consequenties hebben, zeker in relatie tot toerisme.

Het doel van het onderzoek beschreven in dit proefschrift, was het bestuderen van deze twee ziektes om monitoring en controle te versterken door verbeterde diagnostische technieken en het verwerven van inzicht in risico factoren voor de infecties. Infectie van olifanten met *M. tuberculosis* en EEHV worden wereldwijd waargenomen, zowel in wilde olifanten als in dieren in gevangenschap. Aangezien in Thailand veelvuldig contact tussen gedomesticeerde olifanten en mensen geïnfecteerd met *M. tuberculosis* en wilde olifanten voord de hand ligt, zijn testen voor vroege en accurate diagnose noodzakelijk. Van vier olifanten die klinische verschijnselen van TB vertoonden **(Hoofdstuk 3)** waren er drie positief in een serologische test (TB STAT Pak, Chembio). Van een van deze dieren werd retrospectief vastgesteld dat het 23 maanden voordat bacteriën gekweekt werden uit slurf spoelvloeistof seropositief was. Het vierde dier dat ernstige klinische verschijnselen van TB vertoonde, kon alleen 7 dagen voor sterfte getest worden, maar was seronegatief. Van alle vier dieren werd infectie bevestigd in klinische pathologisch onderzoek en in PCR op extracten van weefsel biopten. Uit de wasvloeistof van 60 slurfspoelingen van één olifant, kon slechts in twee gevallen *M. tuberculosis (M. tb)* worden gekweekt. Deze olifant werd in isolatie gehouden nadat behandeling moest worden afgebroken vanwege ernstige klinische verschijnselen, lethargie, anorexie, ataxie en resultaten van klinisch chemisch onderzoek die wezen op lever- en nierproblemen.

Genotypering van de *M. tuberculosis* isolaten classificeerde een van de vier als "ancient" stam, vanwege de aanwezigheid van een specifieke deletie (TbD_1), respectievelijk als "modern" stammen, zoals Beijing, Haarlem en African, vrijwel identiek aan *M. tuberculosis* ATCC2794. Typeren van het ETR-A gen op basis van de "Mycobacterial interspersed, repetitive unit variable-number tandem-repeat" (MIRU-VTR) liet zien dat de drie "modern" stammen verschillend waren. Dit betekent, dat er vier verschillende bronnen van infectie waren voor de vier olifanten.

Alhoewel er weinig bekend is over de immuunrespons van olifanten in TB, werd op basis van kennis van diagnostische testen en pathogenese studies in mensen, niet-humane primaten en herkauwers een studie opgezet, gericht op het meten van de productie van interferon gamma (IFN-γ), als representatief voor de cytokines geproduceerd bij stimulatie van witte bloedcellen met *Mycobacterium Tuberculosis* Complex (MTBC) specifieke antigenen. Om deze, ook voor olifanten, in een vroeg stadium na infectie met *M. tuberculosis* te kunnen meten

werd de olifant specifieke "Interferon Gamma Release Assay", IGRA opgezet. Voor dit doel werd olifant (elephant) IFN-γ (rEpIFN-γ) gecloneerd en tot expressie gebracht en werden olifant IFN-γ specifiek monoclonale antilichamen geproduceerd. Deze werden gebruikt om een "capture" ELISA op te zetten en (technisch) te valideren. De detectie range voor rEpIFN-γ ligt tussen 1 and 10,000 pg/ml en in de assay kan natief IFN-γ van zowel Aziatische als Afrikaanse olifanten gemeten worden.

In *in vitro* volbloedkweken, werden olifanten T-cellen geconfronteerd met positieve en negatieve controle stimuli en MTBC specifieke antigenen, H37Rv *M. tuberculosis,* early secreted antigen target 6 (ESAT-6), culture filtrate protein 10 (CFP-10), surface glycosylated lipoprotein (MPB-83) en de klassieke Purified Protein Derivatives (PPD) van *M. bovis* (PPD-B) en *M. avium* (PPD-A) **(Hoofdstuk 4)**. De IGRA's met volbloed van niet geïnfecteerde Aziatische olifanten, gaven een negatief resultaat, hetgeen ook het geval was voor een "verdachte" olifant. Met bloed van een geïnfecteerde olifant kon productie na stimulatie met de MTBC specifieke antigenen en, maar in veel mindere mate, met PPD-A, als controle voor kruisreactiviteit met omgevingsmycobacteriën, gemeten worden.

Om het diagnostisch spectrum te completeren, maar ook vanwege de ongunstige omstandigheden voor bemonstering en transport van monsters, waardoor de IGRA in Thailand nog niet grootschalig kon worden gebruikt, werden ook MTBC specifieke antilichamen bepaald. Deze antilichamen zijn meestal in een later stadium van infectie meetbaar en kunnen wijzen op progressie naar het klinische stadium van de ziekte. De serologische TB status van de olifanten werd met behulp van ELISAs voor ESAT-6, CFP-10, en MPB83 en de TB Stat Pak assay bepaald en gebruikt om demografische risico factoren geassocieerd met *M. tuberculosis* infectie te bepalen en hun belang te kwantificeren **(Hoofdstuk 5)**.

De ELISA resultaten met serum van 708 olifanten werden onderworpen aan een "Latent Class Analysis" (LCA) om hun serologische TB status te voorspellen. Afhankelijk van de LCA benadering, populatie of individuele olifant gebaseerd, waren de relatieve aantallen (percentages) van de dieren 15-17.3% voor de status "positief", 48.7-50% voor de status "onbeslist" en 34-35% voor de status "negatief".

De correlatie tussen uitslagen van de verschillend individuele ELISAs wast hoog, die tussen de ELISAs en de TB Stat Pak assay laag. Demografische risico factoren, sekse, "body condition score" (BCS), leeftijd, werkduur, type voedsel, managementsysteem, kamp grootte en regio werden beoordeeld op basis van "cutoff" waarden bepaald in ROC-analyses. Oude olifanten hadden in de LCA voorspelling vaker een positieve serologische TB status dan jongere olifanten. Olifanten die 7 uur per dag werken en die met een goed BCS (7-11) hadden minder vaak een positieve serologische TB status. Daarnaast hadden minder dieren in grote kampen (31-50 olifanten) een serologisch positieve status, vergeleken met olifanten in de ander kamp grootten. In deze studie had de regio Noord het laagste percentage olifanten met een voorspelde positieve TB status. De regio West en in mindere mate de andere regio's hadden duidelijk hoger percentages. Alhoewel de assays, in de huidige studie gebruikt, nog niet gevalideerd zijn, waren ze, met name in combinatie, veelbelovend als diagnostische testen voor identificatie van geïnfecteerde en niet-geïnfecteerde dieren en voor beoordeling van demografische factoren als potentieel risico voor infectie.

De hemorrhagische ziekteverschijnselen veroorzaakt door het Elephant Endotheliotropic Herpes Virus (EEHV-HD) **(Hoofdstuk 6)** zijn meestal letaal. Aangenomen wordt dat jonge olifanten, die primaire infectie overleven, mogelijk door nog aanwezige maternale antilichamen, beschermd zijn tegen secundaire infectie

Om de prevalentie van EEHV infectie in olifanten in Thailand vast te stellen werd een cross-sectionele onderzoek verricht, waarbij gebruik gemaakt werd van een ELISA met als antigeen het EEHV1 glycoproteïne B (gB). Van de 994 olifanten die getest werden, was 42,3% seropositief, 57,7% seronegatief en waren er geen "inconclusive" uitslagen, gebaseerd op het criterium dat een olifant positief is wanneer een van de twee geteste serum verdunningen een positieve uitslag geeft.

Associaties tussen seropositiviteit en potentiële risicofactoren voor EEHV infectie werden onderzocht, te weten: leeftijd (<11 jaar, 11-50 jaar, >50 jaar), sekse, kamp(cluster) grootte, type management (intensief, extensief), bemonsteringsperiode (nat resp. droog seizoen), kamp locatie (centraal, oost, noord, noordoost, zuid, west). Univariabele regressie analyse toonde aan dat i) olifanten onder extensief management een hogere seroprevalentie lieten zien dan dieren onder meer intensief management en ii) olifanten in de regio's centraal, noordoost en oost minder risico hadden seropositief te zijn dan die in de regio noord (seroprevalentie 49,4%). In het uiteindelijke multivariabele regressie model was alleen de variabele "regio" nog vertegenwoordigd.

Samenvattend, het doel van de studies beschreven in dit proefschrift was onderzoek te doen naar diagnostische testen voor TB en EEHV-HD, om monitoring en controle van beide infecties te optimaliseren en risico factoren in beeld te brengen. Duidelijk bewijs werd geleverd voor het voorkomen van tuberculose veroorzaakt door *M. tuberculosis* in de Thaise olifantenpopulatie. Om transmissie van *M. tb* tussen olifanten en mensen te reduceren en op termijn te voorkomen, moet een efficiënt diagnostisch regime worden gehanteerd. De IGRA die ontwikkeld werd, is weliswaar geschikt voor detectie van relevante IFN-γ concentraties, maar het gebruik ervan wordt gehinderd door, naar Thaise omstandigheden, relatief moeilijke eisen ten aanzien van monstername en transport van monsters. Realiseren van de mogelijkheid de IGRA voor TB diagnostiek op perifere laboratoria uit te voeren, moet worden overwogen. De serologische testen die tot nu toe voorhanden zijn, laten hoge percentages zien van dieren die als "inconclusive" worden geclassificeerd en moeten dus hoogstnoodzakelijk verbeterd worden. Combinatie van serologische testen, liefst tezamen met de IGRA, is de beste diagnostische benadering en ook risico factoren kunnen op die wijze accurater beoordeeld worden, ter ondersteuning van managementmaatregelen.

Alhoewel de huidige EEHV ELISA, duidelijke nadelen kent, bracht deze een hoge EEHV prevalentie (42,3%) aan het licht, die een blijvende bedreiging vormt voor de Aziatische olifanten in Thailand en omgeving. In de regio noord, waar de hoogste EEHV prevalentie werd waargenomen, zou, speciaal voor jonge olifanten, contact management moeten worden overwogen. Uiteraard in combinatie met monitoring van activiteiten, gedrag en frequente serologische en moleculaire diagnostiek voor EEHV viraemie. Uiteindelijk zal alleen een vaccin de oplossing van dit probleem kunnen brengen.

Tenslotte, publicatie van dit proefschrift zal professionele en publieke alertheid voor de gevaren van TB en EEHV infectie van Aziatisch olifanten doen toenemen.

บทสรุป

ช้างเอเชียเป็นสัตว์กินพืชขนาดใหญ่ ถูกจัดอยู่ในสัตว์ป่าใกล้สูญพันธุ์ อนุสัญญาว่าด้วยการค้าระหว่างประเทศซึ่งชนิดสัตว์ป่าและพืชป่าที่ใกล้สูญพันธุ์ กลุ่มที่ ๑ (CITES) มีถิ่นที่อยู่อาศัยใน ๑๓ ประเทศ ของทวีปเอเชีย ปัจจุบันมีช้างป่าราวๆ ๔๐,๐๐๐ - ๕๐,๐๐๐ ตัว และมีช้างเลี้ยงอยู่กับมนุษย์ประมาณ ๑๓,๐๐๐ เชือก ทั้งนี้มีช้างเอเชียอีกอย่างน้อย ๑,๗๐๐ เชือกที่เลี้ยงอยู่นอกถิ่น เช่น อเมริกา ยุโรป ออสเตรเลีย ญี่ปุ่น เป็นต้น ในปัจจุบันช้างประสบปัญหาจำนวนพื้นที่ป่าที่อยู่อาศัยลดน้อยลง ถูกบุกรุกถิ่นที่อยู่อาศัย ทำให้ช้างในป่าต้องใช้พื้นที่ร่วมกับสัตว์ป่าอื่นๆ ปศุสัตว์ และมนุษย์ ซึ่งมีแนวโน้มที่เกิดความขัดแย้งกันระหว่างคนกับช้างสูงมากขึ้น และยิ่งไปกว่านั้นยังเกิดปัญหาเกี่ยวกับสุขภาพ โรคติดต่อต่างๆ อันเนื่องมาจากความเป็นอยู่ที่ไม่เหมาะสมจนส่งผลต่อจำนวนประชากรช้างในที่สุด **(Chapter 1)** โรคติดเชื้อที่มีผลกระทบกับช้างได้แก่ วัณโรค ซึ่งเป็นโรคติดต่อจากสัตว์สู่คน และ โรคเฮอร์ปีส์ในช้าง ซึ่งตลอดระยะเวลาที่ผ่านมาคร่าชีวิตลูกช้างเป็นจำนวนมาก **(Chapter 2)** โดยโรคต่างๆ เหล่านี้ล้วนมีผลต่อเนื่องถึงเศรษฐกิจ การทำมาหากินของประชาชน คนเลี้ยงช้าง โดยเฉพาะอย่างยิ่งการท่องเที่ยว

วิทยานิพนธ์นี้มีจุดมุ่งหมายเพื่อการเฝ้าระวังและควบคุมโรคติดเชื้อ ทั้ง ๒ โรคนี้ โดยการปรับปรุงวิธีการตรวจวินิจฉัยโรคและศึกษาปัจจัยความเสี่ยงที่ก่อให้เกิดโรค ทั้งนี้เคยมีรายงานการเกิดโรควัณโรคและเฮอร์ปีส์จากทั่วโลก ในช้างเลี้ยงและช้างป่า ซึ่งในส่วนของประเทศไทยที่คนเลี้ยงช้างกับช้างมีความใกล้ชิดกันจึงมีโอกาสและความเสี่ยงที่จะเกิดการติดต่อกันระหว่างคนกับช้างโดยเฉพาะอย่างยิ่งเชื้อวัณโรค ด้วยเหตุนี้จึงจำเป็นอย่างยิ่งที่จะต้องพัฒนาการตรวจวินิจฉัยโรคที่สามารถตรวจหาเชื้อได้ตั้งแต่ระยะแรกของการติดเชื้อและมีความถูกต้องแม่นยำอีกด้วย ซึ่งจากการศึกษา **(Chapter 3)** พบว่ามีช้าง ๔ เชือกที่แสดงอาการของโรควัณโรค โดยพบว่าช้าง ๓ เชือก ให้ผลบวกต่อชุดตรวจซีรั่มวิทยา (TB STAT Pak) หนึ่งในนั้นตรวจพบว่าให้ผลบวกก่อนที่จะตรวจพบเชื้อก่อโรค (เพาะเชื้อจากตัวอย่างที่เก็บผ่านน้ำล้างงวง) ถึง ๒๓ เดือน หากแต่ในช้างเชือกที่ ๔ ซึ่งแสดงอาการป่วยวัณโรคอย่างชัดเจน กลับให้ผลลบต่อชุดตรวจดังกล่าว ซึ่งตรวจจากซีรั่มช้างเชือกนี้เพียง ๗ วันก่อนที่ช้างจะเสียชีวิตในเวลาต่อมา ช้างทั้งหมดนี้ได้รับการตรวจและเพาะเชื้อยืนยันผลทางชีวโมเลกุล (PCR) โดย ๓ เชือกเก็บตัวอย่างจากการผ่าซาก ส่วนอีก ๑ เชือกเก็บตัวอย่างจากการเก็บน้ำล้างงวง (ให้ผลบวก ๒ จาก ๖๐ ตัวอย่าง) ซึ่งช้างเชือกนี้ได้รับการดูแลจำกัดพื้นที่ภายหลังจากให้ยาปฏิชีวนะเพื่อรักษาแต่ไม่สัมฤทธิ์ผลต้องหยุดการให้ยาเนื่องจากช้างแสดงอาการปฏิเสธยา ซึม เหม่อลอย เบื่ออาหาร และเดินโซเซ รวมทั้งปัญหาการทำงานของตับและไตจากการตรวจทางเคมีชีววิทยา

ผลจากการตรวจวิเคราะห์ทางชีวโมเลกุลของเชื้อ *M. tuberculosis* ที่ตรวจได้จากช้างทั้ง ๔ เชือก สามารถจำแนกชนิดย่อยของเชื้อด้วยการดู *M. tuberculosis* specific deletion (TbD1) พบว่ามี ๑ เชือกเป็น ancient strains และมี ๓ เชือกเป็น modern strains ซึ่งมีความคล้ายคลึงกับ *M. tuberculosis* ATCC 2794 เช่น Beijing, Haarlem and African *M. tuberculosis* cluster เป็นต้น นอกจากนี้ยังพบว่าแหล่งของการติดเชื้อของช้างทั้ง ๓ เชือกที่เป็น modern strains นั้น มาจากแหล่งที่มาที่แตกต่างกัน จากการวิเคราะห์ typing ของ ETR-A gene ด้วยการใช้ Mycobacterial interspersed, repetitive unit variable-number tandem-repeat

แม้ว่าจะยังมีความรู้ความเข้าใจเกี่ยวกับการทำงานของระบบภูมิคุ้มกันต่อเชื้อวัณโรคในช้างไม่มากนัก อย่างไรก็ตามเราสามารถใช้ฐานความรู้จากการศึกษาการตรวจวินิจฉัยและกระบวนการเกิดโรคในมนุษย์ไพรเมต และสัตว์เคี้ยวเอื้องได้ **(Chapter 4)** ซึ่งการตรวจระดับของไซโตไคที่ถูกกระตุ้นด้วยแอนติเจนของ *M. tuberculosis*

ได้แก่ interferon gamma (IFN-γ) สามารถใช้ในการตรวจวินิจฉัยการติดเชื้อวัณโรคในระยะแรกของการติดเชื้อได้ เราเริ่มต้นงานวิจัยจากการโคลน recombinant Elephant IFN-γ (rEpIFN-γ) และผลิตแอนติบอดี้ที่จำเพาะต่อ IFN-γ ของช้างขึ้นมา จากนั้นจึงออกแบบและตรวจวัดหาแอนติบอดี้ที่ดีที่สุดด้วยวิธีการตรวจ capture ELISA จากการทำไตเตรตพบว่าสามารถตรวจวัดระดับ rEpIFN-γ ได้ระหว่าง ๑-๑๐,๐๐๐ พิโคกรัม/มิลลิลิตร นอกจากนี้ยังสามารถตรวจ native elephant IFN-γ ที่อยู่ในเลือดช้างเอเชียและช้างแอฟริกาได้อีกด้วย

ในเลือดช้างที่นำมากระตุ้น ประกอบด้วย ที-เซลที่สัมผัสกับ positive control และ negative control ,แอนติเจนจำเพาะของ *M. tuberculosis* ได้แก่ ESAT-6, CFP-10, MPB-83 รวมทั้ง PPD-B และ PPD-A ซึ่ง native elephant IFN-γ ที่ได้จากการกระตุ้นจะอยู่ใน culture supernatants และตรวจวัดระดับด้วยวิธี capture ELISA **(Chapter 4)** จากการศึกษาในเลือดของกลุ่มช้างที่ปกติ (non infected) หลังจากที่กระตุ้นด้วย ESAT-6/CFP-10, PPD-B และ PPD-A ให้ผลลบเช่นเดียวกับกลุ่มช้างที่สงสัยว่าเคยสัมผัสเชื้อ (suspected) สำหรับกลุ่มช้างที่ติดเชื้อ (infected) ให้ผลบวกคือตรวจพบ elephant IFN-γ ในทุกแอนติเจนที่กระตุ้น รวมทั้ง PPD-A ที่ใช้เป็นตัวควบคุมลบสำหรับเชื้อที่อาจพบได้ในสิ่งแวดล้อม หากแต่มีระดับต่ำกว่าแอนติเจนอื่นๆ

เพื่อให้การตรวจวินิจฉัยวัณโรคในช้างครอบคลุมและนำไปใช้ได้ในทุกระยะของการติดเชื้อ เนื่องจากการตรวจวัดระดับ IFN-γ มีความยุ่งยากเกี่ยวกับการเก็บตัวอย่างและอาจยังไม่เหมาะสมสำหรับประเทศไทย เราจึงพัฒนาวิธีการตรวจวินิจฉัยวัณโรคช้างในช่วงระยะถัดมาของการติดเชื้อ ซึ่งสามารถติดตามพัฒนาการของโรค รวมทั้งอาการของโรคได้ ด้วยการใช้แอนติเจนที่จำเพาะต่อ *M. tuberculosis* ประกอบด้วย ESAT-6, CFP-10 และ MPB-83 ตรวจด้วยวิธี ELISA รวมทั้งนำข้อมูลที่ได้มาเปรียบเทียบกับวิธีการตรวจทางซีรั่มวิทยาอื่นๆ ได้แก่ TB Stat Pak เพื่อมาวิเคราะห์หาปัจจัยการเกิดโรค **(Chapter 5)** ผลการตรวจ ELISA จากซีรั่มช้าง ๗๐๘ เชือก นำไปวิเคราะห์ Latent Class Analysis (LCA) เพื่อประเมินสถานะการติดเชื้อซึ่งขึ้นอยู่กับเมื่อเทียบระหว่างประชากรต่อรายตัว พบว่ากลุ่มให้ผลบวกร้อยละ ๑๕-๑๗.๓, ผลลบร้อยละ ๓๔-๓๕ และให้ผลระหว่างบวก/ลบร้อยละ ๔๘.๗-๕๐

ค่าความสัมพันธ์ระหว่างแอนติเจนของ ELISA มีความสัมพันธ์กันสูงแต่ค่อนข้างต่ำเมื่อเทียบกับวิธี TB Stat Pak และ ในการหาปัจจัยเสี่ยงของการเกิดโรควัณโรคในช้าง ได้กำหนดตามพื้นฐานด้านประชากรศาสตร์ของช้างได้แก่ เพศ, ความสมบูรณ์ของร่างกาย, อายุ, ระยะเวลาการทำงาน, การให้อาหาร, ระบบการเลี้ยง, ขนาดของปาง และ ภูมิภาคที่ช้างอยู่ ทั้งนี้ได้กำหนดค่า cut off จาก การวิเคราะห์ ROC ผลการวิเคราะห์พบว่าช้างที่มีอายุมากมีโอกาสเสี่ยงมากกว่าช้างอายุน้อยในการแสดงผลบวกต่อวัณโรค, ช้างที่ทำงาน ๗ ชั่วโมงต่อวันและช้างที่มีความสมบูรณ์ของร่างกายที่ดี (๗-๑๑) มีความเสี่ยงน้อยกว่าที่จะแสดงผลบวกต่อวัณโรค, ช้างที่มีอยู่ในปางช้างขนาดประชากร ๓๑-๕๐ เชือกมีความเสี่ยงต่อโรคมากกว่าปางช้างอื่นๆ นอกจากนี้ยังพบว่าช้างในภาคตะวันตกมีความเสี่ยงต่อโรคสูงกว่าภูมิภาคอื่นๆในประเทศ โดยพบว่าช้างในภาคเหนือมีความเสี่ยงต่ำที่สุด

วิธีการตรวจวินิจฉัยวัณโรคในช้างทั้งสองวิธียังจำเป็นต้องพัฒนาเพื่อให้สามารถนำไปใช้ในทางปฏิบัติได้ ทั้งนี้การผสมผสานการตรวจทั้งสองอย่างเข้าด้วยจะช่วยให้การตรวจวินิจฉัยโรคมีประสิทธิภาพมากยิ่งขึ้น โดยเฉพาะอย่างยิ่งการตรวจเพื่อวินิจฉัยระหว่างช้างที่ติดเชื้อและไม่เชื้อเช่นเดียวกันกับการหาปัจจัยเสี่ยงที่เป็นแนวโน้มของการเกิดโรค

โรคเฮอร์ปีส์ (EEHV) ในช้าง **(Chapter 6)** ส่วนใหญ่ช้างที่ติดเชื้อมักเสียชีวิต และเชื่อกันว่าช้างที่รอดชีวิตมักเกิดจากภูมิคุ้มกันจากแม่ที่ยังเหลืออยู่ ทำให้ป้องกันการติดเชื้อทุติยภูมิได้ การวิจัยในครั้งนี้เพื่อศึกษาหาความชุก (prevalence) ของโรค EEHV จากตัวอย่างซีรั่มช้างจำนวน ๙๙๔ เชือกในช่วงเวลาหนึ่งโดยใช้แอนติเจน

บทสรุป

EEHV1 glycoprotein B ในการตรวจด้วยวิธี ELISA พบว่า ให้ผลบวกร้อยละ ๔๒.๓ และ ผลลบร้อยละ ๕๗.๗ โดยกำหนดว่า ช้างที่ให้ผลบวก จะต้องมีผลตรวจ ELISA เป็นบวกอย่างน้อย ๑ ความเข้มข้นในความเข้มข้น ๑:๑๐๐ และ ๑:๒๐๐ ผลตรวจ ELISA ที่ได้ นำไปวิเคราะห์เพื่อหาปัจจัยเสี่ยงที่ก่อให้เกิดโรค EEHV ในช้าง ซึ่งปัจจัยที่ใช้ในการวิเคราะห์ประกอบด้วย อายุ (น้อยกว่า ๑๑ ปี, ๑๑-๕๐ ปี, มากกว่า ๕๐ ปี), เพศ, จำนวนช้างในแต่ละพื้นที่ ที่อยู่อาศัย (น้อยกว่า ๑๐ เชือก, ๑๐-๕๐ เชือก, มากกว่า ๕๐ เชือก), ระบบการเลี้ยง (เลี้ยงแบบปิด และ เลี้ยงแบบปล่อย), ช่วงเวลาเก็บตัวอย่าง (ฤดูฝน และ ฤดูแล้ง) และ ภูมิภาคที่ช้างอยู่ (ภาคกลาง, ตะวันออก, เหนือ, ตะวันออกเฉียงเหนือ, ใต้, ตะวันตก) ทุกปัจจัยนำมาวิเคราะห์ด้วยวิธี univariable regression model พบว่า มีเพียง ๒ ปัจจัยที่เป็นปัจจัยเสี่ยงคือ ระบบการเลี้ยงช้างแบบเปิดมีความเสี่ยงต่อโรค และ ช้างในภาคเหนือมีความเสี่ยงต่อการเกิดโรคสูงกว่าภูมิภาคอื่นๆ โดยภาคกลาง, ภาคตะวันออกเฉียงเหนือ และภาคตะวันออกมีความเสี่ยงน้อยที่สุด สำหรับการวิเคราะห์ใน multivariable regression model พบว่าภูมิภาคเป็นปัจจัยเสี่ยงต่อโรคเพียงปัจจัยเดียว และให้ผลเช่นเดียวกับการวิเคราะห์ univariable regression model

ในท้ายที่สุด เป้าหมายของการศึกษาในวิทยานิพนธ์นี้คือการศึกษาพัฒนาวิธีการตรวจวินิจฉัยโรควัณโรค และ เฮอร์ปีส์ในช้างเพื่อที่จะเฝ้าระวังและควบคุมโรคดังกล่าวให้ได้อย่างมีประสิทธิภาพ รวมทั้งศึกษาหาปัจจัยเสี่ยงที่มีผลต่อการเกิดโรค ซึ่งผลงานดังกล่าวได้แสดงให้เห็นว่าวัณโรคในช้างไทยเกิดจากเชื้อ *M. tuberculosis* หลากหลายสายพันธุ์ย่อย การป้องกัน หรือแม้แต่ลดการเกิดโรค การติดต่อกันระหว่างคนกับช้าง เครื่องมือที่ใช้ตรวจวินิจฉัยโรคที่มีประสิทธิภาพเป็นสิ่งสำคัญอย่างยิ่งยวด รวมไปถึงการตรวจระดับไซโตไค IFN-γ ที่ได้รับการพัฒนาให้มีความไวจนสามารถตรวจวินิจฉัยโรคได้ หากแต่การนำไปใช้ในทางปฏิบัติจำเป็นต้องปรับปรุง โดยเฉพาะอย่างยิ่งคุณภาพของตัวอย่าง ดังนั้นการยกระดับ พัฒนาห้องปฏิบัติการเพื่อวินิจฉัยวัณโรคช้างในระดับภูมิภาคของประเทศจึงมีความจำเป็นอย่างยิ่ง สำหรับการตรวจวินิจฉัยวัณโรคทางซีรั่มวิทยายังคงต้องพัฒนาปรับปรุงโดยเฉพาะอย่างยิ่งการวินิจฉัยแยกโรคระหว่างกลุ่มที่ให้ผลบวกกับกลุ่มที่ให้ผลก้ำกึ่งระหว่างบวกหรือลบ (inconclusive) ซึ่งมีความคาบเกี่ยวกันค่อนข้างสูง อย่างไรก็ตามการผสมผสานวิธีการตรวจวินิจฉัยวัณโรคในช้างระหว่างทางซีรั่มวิทยาและการตรวจวัดระดับ IFN-γ สามารถช่วยให้การวินิจฉัยมีความถูกต้องแม่นยำมากขึ้น รวมถึงการนำข้อมูลเกี่ยวกับปัจจัยเสี่ยงต่อการเกิดโรคไปใช้ในการจัดการด้วยอีกทางหนึ่ง

จากผลการศึกษาความชุกของโรค EEHV พบว่ามีอัตราค่อนข้างสูง ซึ่งถือว่าเป็นภัยคุกคามต่อประชากรช้างไทย โดยเฉพาะช้างทางภาคเหนือที่มีความเสี่ยงสูง จำเป็นต้องคำนึงถึงเรื่องการจัดการเกี่ยวกับการอยู่ด้วยกัน การสัมผัสกันของช้างในวัยเด็ก ดังนั้นการเฝ้าระวัง การติดตามการเกิดโรค ด้วยการตรวจทางซีรั่มวิทยา ชีวโมเลกุล และเก็บตัวอย่างสารคัดหลั่งต่างๆ เพื่อให้ทราบถึงกระบวนการเกิดโรคเป็นสิ่งที่พึงกระทำ รวมไปถึงการพัฒนาวัคซีนป้องกันโรคยังคงเป็นสิ่งที่ต้องดำเนินการต่อไป

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Curriculum vitae

Taweepoke Angkawanish was born on Saturday 10th of February 1973 in Nakornsawan, Thailand. He graduated as Doctor of Veterinary Medicine (DVM) from Chulalongkorn University (CU), Thailand in 1997. After graduation, he worked at the Fort Dodge Animal Health offices in Bangkok for 8 months. In February 1998, he began his work as a veterinary practitioner at the Thai Elephant Conservation Center, Forest Industry Organization, Lampang. In 2004, he received a new position as a chief of the elephant hospital at the National Elephant Institute, and ran mobile elephant clinics all over the country to provide health care services for domesticated elephants. In 2010, he had the opportunity to get a scholarship from the EU-Asia Link project between the National Elephant Institute, Lampang, Chiangmai and Kasetsart Universities, Thailand; Pinawala Elephant Orphanage and Peradeniya University, Sri Lanka; the Royal Veterinary College in England; and Utrecht University, the Netherlands, to perform his PhD studies, registered at Utrecht University. He started working in the Department of Infectious diseases and Immunology for a year and returned to Thailand to continue his studies. After his PhD thesis defense, he will continue to work at the National Elephant Institute, Forest Industry Organization, Lampang.