Studies on genotype and diagnosis of tuberculosis in Asian elephants

(Elephas maximus) of Nepal

（ネパールのアジアゾウにおける結核の遺伝子型別および診断に関する研究）

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CONTENTS

ABBREVIATIONS

ABC	Avidin-Biotin Complex
BNP	Bardia National Park
CAS	Central Asian Strain
CFP-10	Culture filtrate protein 10
CNP	Chitwan National Park
DR	Direct repeat
DVR	Direct variant repeat
EAI	East African-Indian
EDTA	Ethylenediaminetetraacetic acid
EIFN-γ	Elephant interferon-gamma
ELISA	Enzyme-linked immunosorbent assay
ESAT-6	Early secretory antigenic 6 kDa
GENETUP	German Nepal Tuberculosis Project
gyrA	Gene encoding DNA gyrase A subunit
gyrB	Gene encoding DNA gyrase B subunit
IFN- γ	Interferon-gamma

IGRA	Interferon-gamma release assay
InhA	Enoyl-acyl carrier protein reductase
katG	Gene encoding Catalase-peroxidase
KTWR	Koshi Tappu Wildlife Reserve
L-J	Lowenstein-Jensen
LOD	Lower limit of detection
LSP	Large sequence polymorphism
MLVA	Multi-locus variable number of tandem repeat analysis
MTC	*Mycobacterium tuberculosis* complex
PBS	Phosphate buffered saline
PBST	Phosphate buffered saline tween-20
PHA	Phytohaemagglutinin
PMA/I	Phorbolmyristateaceteate/ ionomycin
PWM	Pokweed mitogen
rEIFN-γ	Recombinant elephant interferon-γ
rpoB	Gene encoding RNA polymerase B-subunit
rrs	Gene encoding 16S rRNA

SIT	Spoligo-international type
SNP	Single nucleotide polymorphism
SpolDB4	The fourth international spoligotyping database
TB	Tuberculosis
Th_1	Type 1 T helper cells
TMB	Tetramethylbenzidine
VNTR	Variable number of tandem repeats
WHO	World Health Organization
WT	Wild type

PREFACE

Tuberculosis (TB) in elephants is an emerging disease primarily caused by *Mycobacterium tuberculosis*. Although infection with *M. bovis* and non-tuberculous Mycobacteria (NTM) species has been documented (16, 22, 24, 25, 27, 44) the majority of reported cases in captive elephants have been caused by *M. tuberculosis*. Many elephants infected with TB do not manifest clinical signs; however, some may have chronic weight loss, anorexia, and weakness. Exercise tolerance may be seen in working elephants. In some cases, the elephants may show symptoms only in the terminal stage of disease or are diagnosed-postmortem (22, 25). Culture of trunk wash sample is regarded as a gold standard for the diagnosis of TB in elephants; however, this technique has many limitations (17). Very few molecular studies have been done in *M. tuberculosis* isolates from the elephants from the range countries to date (2).

TB was first identified in the Nepalese captive elephant population in 2002. Since captive elephants are in close contact with humans for a protracted period, it is likely that elephants contracted TB from humans at some point in time as TB has not been reported in wild elephants except for one case in an ex-captive African elephant (33).

Cell mediated immune response is elicited by the body at an early stage of infection for the protection. Interferon-γ (IFN-γ) is one of the major cytokines secreted mainly by Th_1 cells and Natural Killer cells. Asian elephants are highly susceptible to the infection by intracellular pathogens like *Mycobacterium tuberculosis* (24) and elephant endotheliotropic herpesviruses (38). IFN-γ causes the activation of macrophage that will kill the obligate intracellular microbes mediated by up regulation of reactive oxygen intermediates and toxic NO (4. IFN-γ plays an

important role in the immuno-pathogenesis of TB (7). Bovigam (Prionics AG, Switzerland) was the first commercial IFN-γ release assay (IGRA) developed for the diagnosis of TB in bovine species. Quantiferon Gold-in-Tube (Cellestis Inc., Australia) was later developed as an IGRA for TB diagnosis in humans. Recently, IGRA has also been developed in wildlife species including deer (42), lion (18) and rhinoceros (29) for the diagnosis of TB.

The present thesis consists of three chapters; in chapter I, I have performed the molecular characterization of *Mycobacterium tuberculosis* isolates from three elephants that died with the extensive lung lesions. I conducted the spoligotyping, multi-locus variable number of tandem repeat analysis (MLVA) and TbD1 detection in three isolates. I have also compared the *M. tuberculosis* isolates from seven patients in Nepal that had the same spoligotypes with the elephant isolates in this study. In chapter II, I have conducted the genotyping of *M. tuberculosis* isolates from two elephants that died of suspected TB lesions in the lungs. The spoligotyping and large sequence polymorphism (LSP) was performed on these two isolates to detect the *M. tuberculosis* lineage. We first time reported the mixed infection of *M. tuberculosis* in Asian elephants of Nepal with two different lineages present in Elephant D and E. In chapter III, I have described the development and evaluation of the Asian elephant specific IFN-γ release assay (IGRA) to see its potential as a tool for the early diagnosis of TB in Asian elephants.

CHAPTER I

Molecular characterization of *Mycobacterium tuberculosis* isolates from elephants of Nepal

Introduction

Nepal has a population of more than 200 captive elephants that are used for patrolling the protected areas, in eco-tourism and for wildlife research projects (37). The government of Nepal has endorsed the Nepal Elephant Tuberculosis Control and Management Action Plan (2011-2015) that detail guidelines for the management of TB including the diagnosis and treatment of TB in elephants of Nepal (32). Nepal is a country with a high burden of TB in humans (43). Exposure to infected elephants has resulted in transmission of TB to humans as evidenced by tuberculin skin test conversions (30, 34, 39)Stephens *et al.*, 2013; Murphee *et al.*, 2011; Oh *et al.*, 2002) or active disease (20). To clarify the transmission route, an epidemiological study including precise typing of isolated bacteria is needed. However, to date, few genotyping studies have been done on TB isolates from elephants (2). In the current study, we performed genotyping on three *M. tuberculosis* isolates obtained from three captive elephants and compared them with seven human isolates in Nepal.

Materials and Methods

Study isolates.

Elephant isolates

M. tuberculosis isolates from three elephants were included in the study. All three elephants were owned by the Government of Nepal and kept in two protected areas. Elephants A and C were located at Chitwan National Park (CNP), and Elephant B was located at Koshi Tappu Wildlife Reserve (KTWR) (Figure I-1). These elephants were used to patrol the protected areas for wildlife management and conservation purposes. The elephants were housed in open-air, roofed stables adjacent to other elephants. The elephants at each facility foraged and worked together for most time of the day, often coming in contact with domestic and wild animals such as rhinos and various deer species. Each captive elephant is taken care by three handlers and these handlers spend a long-time together with their elephants.

Elephant A was an adult female about 65 years old. She was brought to CNP from Motipur area of Sarlahi district near to the Indian border (Figure I-1) when she was about 34 years. She was suspected to be suffering from TB and was in permanent segregation for almost two years before she died. Several trunk wash cultures collected from her failed to yield a positive isolate. Her body condition deteriorated significantly in the last six months before she collapsed and died in August 2009.

Elephant B was a female aged approximately 60 years old. She was brought to KTWR from a town Sitamarhi northern India (Figure I-1) when she was about 30 years old. This town is located near to Sarlahi, a district where the Elephant A was previously kept. She had never been

tested for TB before she died in September 2009. For the last 2-3 months before she collapsed, she did not sleep well and lost weight resulting in poor body condition.

Elephant C was a male elephant aged approximately 31 years old. He was born in KTWR and was together with Elephant B for four years before he was transferred to CNP at the age of 7. He lost weight and began coughing 6 months before he collapsed in September 2012.

Human isolates

M. tuberculosis isolates from seven patients in Nepal having the same spoligotypes with the elephant isolates were selected for this study. All of them were picked up from the isolates banked at German Nepal Tuberculosis Project (GENETUP), Nepal which were collected from 2007 to 2010. One person was from Chitwan near CNP, four were from Kathmandu, one from Butwal and one from Birgunj (Figure I-1). One person each from Birgunj and Hetauda had migrated to Kathmandu. DNA was extracted and the genetic analyses were performed in these isolates as described elsewhere (36).

Necropsy.

All three postmortem examinations were carried out at the sites where each elephant collapsed. All personnel involved in the procedure used personal protective equipment including N-95 masks. The abdomen was opened first, and the gastro-intestinal tract and other visceral organs including liver and spleen were observed. The thoracic cavity was approached through the diaphragm per recommendations (26) and the caudal lobe of the lung was observed. Because suspected TB lesions were seen, the thoracic cavity was not further exposed due to the risk of spreading the organism in the environment. Representative lung lesions were collected in sterile screw-top tubes for laboratory analysis.

Culture.

The lung tissue samples were processed according to guidelines of European Society for Mycobacteriology (10). In brief, the lung tissue was aseptically cut into small pieces using a surgical blade, mixed with 4% sulphuric acid, and incubated in a sterile falcon tube for 20 min at room temperature. Then the sample was neutralized with 4% sodium hydroxide using bromo-thymol blue indicator and centrifuged at 3000g for 20 min. The supernatant was discarded and then sample was washed once with sterile distilled water, followed by centrifugation at 3000g for 20 min. The supernatant was discarded and the inoculation was done from the deposit into L-J media. The tubes were examined for growth weekly for eight weeks.

DNA extraction.

The DNA extraction was done for molecular studies using the GenoType® DNA isolation kit (Hain Lifescience GMBH, Nehren, Germany) from the colony that grew on the culture media. The colonies on the culture media were scraped and suspended in 300 µL of molecular biology grade water in a sterile Twist Top 1.7 ml conical vial and heated for 20 min at 95^{o}C in water bath. Then the sample was incubated for 15 min in an ultrasonic bath for cellular disruption, followed by centrifugation at 13,000g for 5 min. Finally, the supernatant was taken containing the bacterial DNA.

Drug susceptibility test.

Drug susceptibility test was performed on the mycobacterial isolates from all the elephants by the proportional method on L-J solid media with critical concentration of 0.2 µg/mL of isoniazid, 40 µg/mL of rifampin, 2 µg/mL of ethambutol and 4 µg/mL of streptomycin on all 3 isolates.

Genetic analyses.

Bacterial species was identified by a multiplex PCR targeting *cfp32*, RD9 and RD12 (31) and was confirmed by a *gyrB* sequence analysis (14). The spoligotype was determined as previously described (15). Briefly, the direct-repeat (DR) region was amplified with a primer pair and the PCR products were hybridized to a set of 43 oligonucleotide probes corresponding to each spacer, which were covalently bound to the membrane. The spoligo-international type (SIT) was determined by comparing spoligotypes with the international spoligotyping database (SpolDB4) (3). DR region rearrangement was confirmed by a PCR and sequencing with following primers, IS-LiP-TB3': CAACGCCAGAGACCAGCCGCCGGCTGAG, spacer37R: GACTGTGGACGAGTTCGCGCTC and DR region-R: TCACCGTCAACGCCGCCATCATGCTC. TbD1 detection was carried out by PCR as previously described (14). Multi-locus variable number of tandem repeat analysis (MLVA) (40) was performed as described (35) with following 18 chosen loci, which showed higher variability among EAI isolates; VNTR424, ETR-C, MIRU4, MIRU40, MIRU10, VNTR1955, QUB11a, QUB11b, ETR-A, VNTR2401, ETR-B, MIEU26, MIRU31, QUB3232, QUB3336, VNTR3690, QUB26 and MIRU39. A dendogram was drawn by UPGMA with BioNumerics ver. 6.0. Genetic regions thought to be associating with drug resistance, i.e., partial *rpoB, katG, inhA* promoter region, *gyrA* and *rrs* sequences, were sequenced and analyzed as described (35, 36). Sequences that had mutations were compared with the public database using NCBI blast search system (http://blast.ncbi.nlm.nih.gov/Blast.cgi). Seven human derived isolates having the same spoligotype were also subjected to the same analyses.

Results

The necropsy results of Elephant-A showed that she had liquefied caeseous lesions in lungs. The post-mortem findings of Elephant-B showed that the right lung had tuberculous-like lesions. Similarly, the necropsy findings of Elephant-C showed that the left lung at its dorso-posterior section had abscesses containing white pus. Upon excision, the mediastinal lymph node contained yellowish caseated material.

Culture.

There was growth of *M. tuberculosis* complex from the representative lung lesion samples from elephants A, B and C.

Drug susceptibility test.

The isolates from the elephants A, B and C were susceptible to isoniazid, rifampin, ethambutol and streptomycin.

Species determination and genetic analyses.

Bacterial species was determined as *M. tuberculosis* by a multiplex PCR and was confirmed by *gyrB* sequencing (14, 31). In *gyrB* sequence, all the elephant isolates had a single nucleotide polymorphism (SNP) from G to C at the position 990 that leads an amino acid substitution of Met 330 Ile. This mutation was revealed as lineage specific in strains belonging to EAI or Indo-Oceanic lineage (3, 19) by NCBI blast search. Elephant C isolate (Elp-C) had a spoligotype belonging to the Indo-Oceanic lineage (EAI5, SIT138) while the other 2 had different new spoligotypes that were not found in the SpolDB4 database (3) . Elephant A isolate (Elp-A) showed only 2 spacers, spacer 38 and 39, positive. In elephant B isolate (Elp-B), the spacer 1 to 28 and 35 to 39 were positive and the pattern is 1 spacer, spacer 33, differed from

spoligotype SIT 138 belonging to EAI5 clade (Table I-1). Both of the DR region rearrangements, which were the cause of the spoligotype alteration, were confirmed by sequencings. In Elp-A, *IS*6110 was inserted at the position of spacer 37, and in Elp-B, the spacer 33 was deleted presumably by a homologous recombination (Figure I-2) (41). In TbD1 detection PCR, all 3 samples were positive and determined as ancestral type of *M. tuberculosis* (14). The *gyrA* sequence of Elp-A had a synonymous SNP from T to C at the position of 231, while Elp-B and C had a wild type sequence. This *gyrA* SNP was not found in the public database, however, the same SNP was detected in two human samples, having spoligotype SIT138, collected in Nepal (36) (Table I-1, Figure I-3). Other drug resistance determination region sequences, *rpoB, katG, inhA* promoter region and *rrs,* were wild type in all the samples. In MLVA, Elp-B and Elp-C made a cluster with 1 locus difference. Elp-A formed a cluster with human isolates having the same *gyrA* SNP, T231C (Figure I-3).

Discussion

M. tuberculosis infections in 3 Asian elephants with extensive TB lesions in the lungs are described. The clinical signs shown by these 3 elephants varied although the body condition of all elephants was deteriorating. All 3 elephants had similar lesions in the lungs during necropsy. As in humans, TB in elephants appears to primarily affect the lungs (23).

The diagnosis of TB by culture is considered the gold standard; however, it has very poor sensitivity, especially for ante-mortem diagnosis in elephants (9, 17, 21). A study in Thailand reported that *M. tuberculosis* was isolated from only 2 out of 60 trunk wash samples from 3 elephants with positive postmortem culture isolations (2). In another study, only 58% of elephants with confirmed TB infection at necropsy had positive isolations from trunk wash

samples (9). All of the trunk wash samples of Elephant A were negative on culture in the current study.

Our findings demonstrated that these three elephants were infected with *M. tuberculosis*. For the first time, *M. tuberculosis* was isolated from elephants of Nepal from the tissue samples. The drug susceptibility test showed that all elephant isolates were susceptible to first-line TB drugs. As those elephants had not received any anti-TB drugs, this result was plausible.

Genetic analyses of those isolates, i.e., spoligotyping, lineage specific deletion and mutation analyses, showed that all belonged to the ancestral type of *M. tuberculosis*, so-called EAI or Indo-Oceanic lineage (3, 19). This lineage is predominantly observed in Indo-Oceanic areas like south India or the Philippines (11, 13); however, its prevalence in Nepal is relatively low. In the recent report (19), the prevalence of Indo-Oceanic lineage was 11.5% in Nepal and our observation results were also very similar (unpublished data). Spoligotypes of two elephant isolates were different from known EAI patterns; however, those patterns are producible from EAI by massive spacer deletions by an *IS*6110 insertion or a homologous recombination, which is occasionally observed in this region consisted of repetitive sequences (15). One of the isolate Elp-A had a new synonymous SNP in its *gyrA* sequence, T231C, and the same SNP was found in two human isolates from Kathmandu, the capital city of Nepal. Both of the human isolates had spoligotype SIT138 categorized as EAI5 (3), which is the most frequently observed EAI type in this country (19). This SNP seems to have occurred on a specific lineage of the clade, since other EAI5-SIT138 isolates obtained in Nepal did not have the SNP (Figure I-3). SNP information accurately reflects the evolutionary relationship between *M. tuberculosis* isolates when compared with other typing methods depending on repetitive genetic structures like spoligotyping or MLVA (14). Having the same SNP suggests that those isolates are closely related and have the

same origin. Elp-A isolate is obviously a progeny of this T231C mutated strain, in which massive spacer deletions in the DR region occurred (Table I-1, Figure I-3). Thus, elephant A was infected with a *M. tuberculosis* strain that seemed to be a local lineage that evolved domestically, and we suspect that the elephant was infected from a native elephant handler.

Elephant B was also infected with a strain, which seemed to be a derivative of EAI5-SIT138 lineage and Elephant C was infected with an EAI5-SIT138. The reason why all the elephants were infected with EAI lineage was unclear as the elephants were kept in 2 distanced locations (Figure I-1) and the prevalence of this lineage in Nepal is relatively low.

The EAI lineage is an ancestral type of *M. tuberculosis* that is closer to the animal type lineage, which shows preference to other animals rather than human, including species like *M. bovis* or *M. microti* (13). It can be speculated that this lineage might show higher adaptability to elephants than other lineages. However, in a previous study in Thailand, only 1 elephant out of 4 was infected with an ancestral type *M. tuberculosis* (2). Thus, the reason may be simply the prevalence of this lineage among people in the animal habitat areas was higher than in the city area in Nepal. The locations, where human isolates having the same spoligotype SIT138 were obtained, are shown in Figure I-1 (black filled circle). Those, other than Kathmandu, are located near the Nepal-Indian border from middle of the country to the east, which includes areas where the captive elephants were located. The majority of the human samples were from Kathmandu; however, most of the residents of Kathmandu had come from other areas as seen in sample number h8 from Hetauda, locating between Kathmandu and Birganj, and h277 from Birgunj (Figures I-1 and I-3). From Birgunj residents, we have obtained 6 isolates and 4 out of them were EAI lineage (unpublished data). Thus, EAI lineage prevalence in this area seems to be high and

infection of the elephants might be a reflection of the prevalence of local *M. tuberculosis* strains in humans.

Elp-A and Elp-C isolates had totally different genetic characteristics. Thus their infection origins should be different although they had been kept together for about 20 years in CNP. Elephant A might have been infected with TB in previous town before she developed active TB later in her life while she was in CNP. On the other hand, Elp-B and Elp-C had very similar VNTR pattern, and they made a cluster (Figure I-3). These two elephants were together for four years in KTWR, so they might have been infected from the same source. Elephant B might also have been infected with TB while in India and had it for more than 20 years before getting the active TB. Due to the open border between India and Nepal, there is movement of people from one country to another. This might have provided opportunities for Nepalese people and elephants to be exposed to Indo-oceanic lineage of *M. tuberculosis*, which is more common lineage in India (11) than Nepal. However, the possibility of TB transmission from elephant B to C seemed to be low, since the spacer number in the spoligotype in Elp-B isolate was smaller than Elp-C (lacking spacer 33), and also, they had not shown any symptoms until their terminal stage. They might have been infected with the bacteria from their handlers; however it is unclear whether from the same person or from different persons having closely related strains. Comprehensive TB screening of personnel who work directly with elephants will help to solve the transmission route and prevent the spread of TB in future.

This study has revealed the important basic information about TB in elephants of Nepal and has identified the novel polymorphisms which may be very useful in monitoring the transmission of TB in these animals. Our findings emphasize the immediate need of screening of the personnel who work directly with the elephants and to treat the infected handlers for the

prevention of transmission of this disease to the elephants. Since little information has been published on TB genotypes in elephants, further investigation is needed to better understand the epidemiology of this disease in elephants and the relationship to TB in humans.

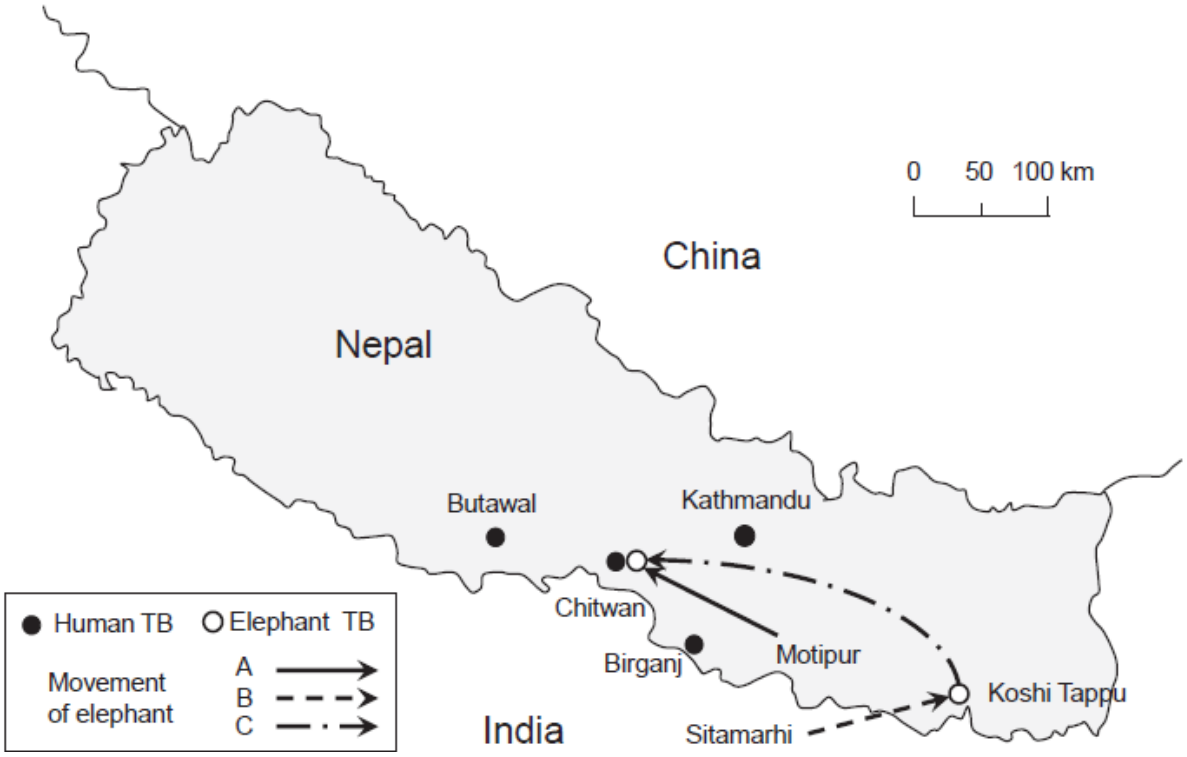


Figure I-1. Movement of elephants and the distribution of elephant and human TB isolates in Nepal. Chitwan and Koshi Tappu are locations of the protected areas where the elephants were kept. Elephant A was stationed at a small town, Motipur, in Southern Nepal near to the Indian border before she was transferred to Chitwan. Elephant B was previously kept in an Indian town, Sitamarhi, near to the Nepalese border and transferred to Koshi Tappu. And elephant C was kept at Koshi Tappu and transferred to Chitwan.

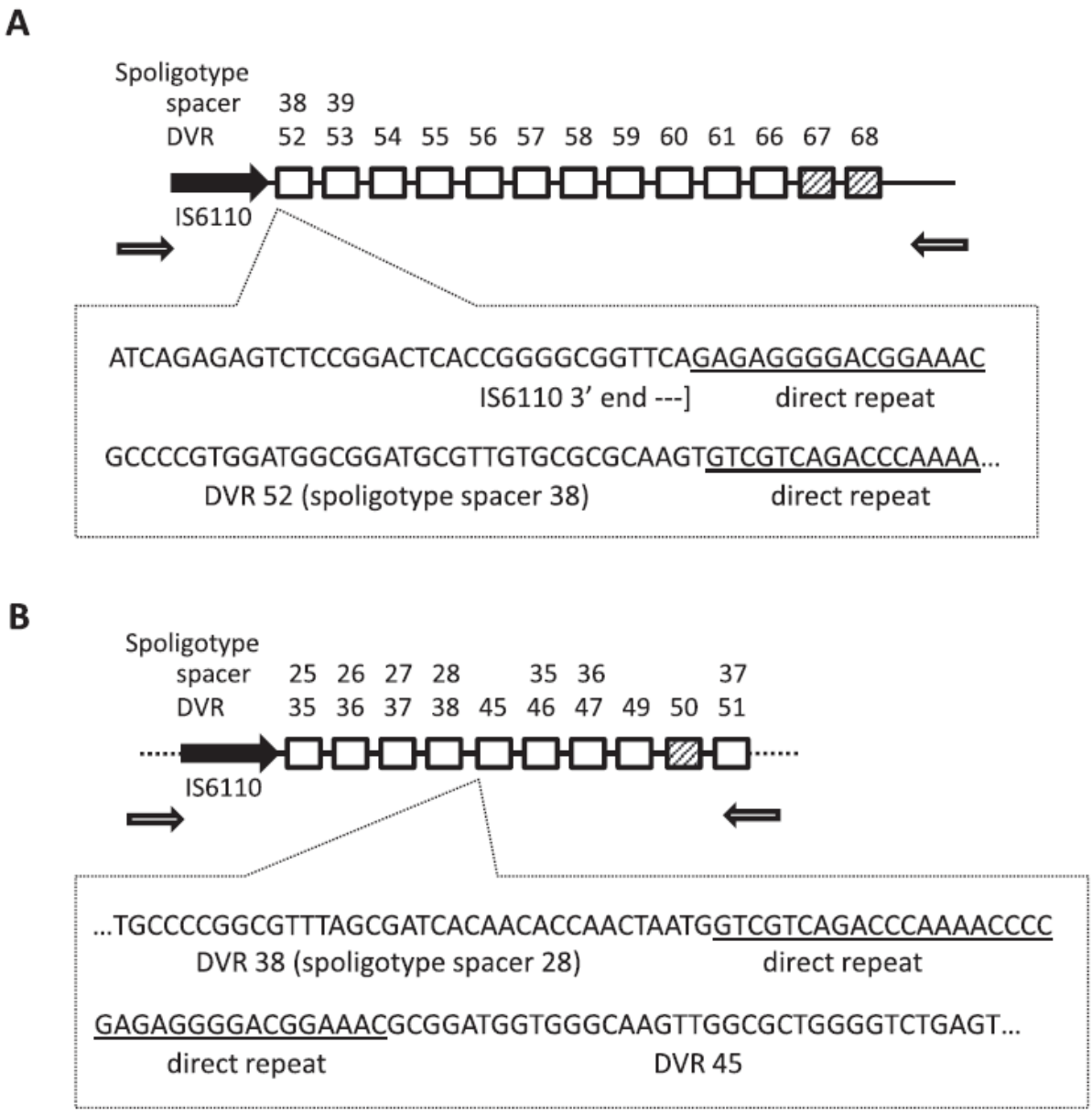
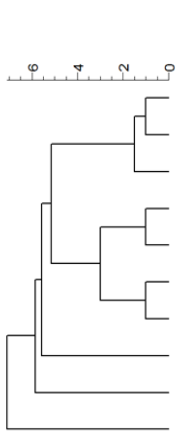


Figure I-2. Structural rearrangement in the DR region. A, Elp-A; B, Elp-B. DVR, direct variant repeat composed of a direct repeat and the adjacent spacer. Rectangles depict individual DVRs. Spacer numbers used in the spoligotyping are shown above the DVR numbers, which were give according to their position in the DR region (van Embden *et al.*, 2000). *IS*6110 and its orientation is shown as a black arrow. Small white arrows are showing the position and the orientation of the used primers; left, IS-LiP-TB3'; A-right, DR region-R; B-right, spacer37R. Nucleotide sequences of each re-arrangement position are shown in the ballons. DVR50, 67 and 68 had the same mutations as ref. 26 (shown in shadowed rectangles).



V424	ETRC	MIRU4	MIRU40	MIRU10	V1955	QUB11a	QUB11b	ETRA	V2401	ETRB	MIRU26	MIRU31	Q3232	Q3336	V3690	QUB26	MIRU39	Sample ID	Origin	Location	Genetic typing Spoligotype	*gyrA*
2	2	5	3	6	4	4	4	9	2	4	2	5	0	7	8	4	1	Elp-A	elephant	Chitwan (Motipur)	new	T231C
2	2	5	3	6	6	4	4	9	2	4	2	5	0	7	8	4	1	h-8	human	Kathmandu (Hetauda)	SIT138	T231C
2	2	5	3	5	6	4	4	9	2	4	2	5	0	7	8	4	1	h-3	human	Kathmandu	SIT138	T231C
2	2	4	3	6	6	8	6	9	2	4	2	5	0	7	3	4	1	Elp-B	elephant	Koshi Tappu (Sitamarhi)	new	WT
2	2	4	2	6	6	8	6	9	2	4	2	5	0	7	3	4	1	Elp-C	elephant	Chitwan (Koshi Tappu)	SIT138	WT
3	2	4	3	6	6	8	6	9	2	4	2	5	0	7	5	4	1	h-294	human	Butwal	SIT138	WT
3	2	5	3	6	6	8	6	9	2	4	2	5	0	7	5	4	1	h-296	human	Chitwan	SIT138	WT
2	2	5	3	6	6	8	5	9	2	3	2	5	0	10	7	4	1	h-277	human	Kathmandu (Birgunj)	SIT138	WT
2	2	5	3	6	8	2	7	4	2	4	2	5	0	7	5	4	1	h-200	human	Kathmandu	SIT138	WT
2	2	5	3	4	6	7	3	9	2	6	2	5	0	7	2	5	1	h-98	human	Birgunj	SIT138	WT

Figure I-3. Phylogenetic comparison of elephant and human derived *M. tuberculosis* isolates by MLVA. Dendrogram was drawn with the multi-locus VNTR analysis (MLVA) results of 18 loci. Place of former locations of human patients and elephants are shown in parenthesis in Sample Location.

Table I-1. Genotypic characteristics of elephant isolates compared with human isolates in Nepal

Sample	Spoligotype*					MPCR†	TbD1	*gyrA*‡	*gyrB*‡
	Binary code	SIT	Clade			C 9 12			
Elp-A	0000000000000000000000000000000000000001100000	New	new	+ + +	+	T231C	G990C
Elp-B	1111111111111111111111111111000000111110000	New	new	+ + +	+	WT	G990C
Elp-C	1111111111111111111111111111000010111110000	138	EAI5	+ + +	+	WT	G990C
h3§	1111111111111111111111111111000010111110000	138	EAI5	+ + +	ND	T231C	G990C
h98¶	1111111111111111111111111111000010111110000	138	EAI5	+ + +	ND	WT	G990C

*Spoligotype was determined according to Brudey *et al.* (2006). new: not found in the SpolDB4 list.

†Multiplex PCR results for the species determination, c: *cfp32*, 9: RD9, 12: RD12, +: detected, triple positive + + + : *M. tuberculosis*

‡Mutations in a partial sequence of *gyrA* and *gyrB*. *gyrA* T231C is synonymous while *gyrB* G990C is non-synonymous. WT: wild type sequence having no mutations.

§Two human isolates (h3 and h8) had same genetic features. ND: not done.

¶Five human isolates (h98, h200, h277, h294 and h296) had same genetic features.

Summary

Mycobacterium tuberculosis was cultured from the lung tissues of three captive elephants in Nepal that died with extensive lung lesions. Spoligotyping, TbD1 detection and multi-locus variable number of tandem repeat analysis (MLVA) results suggested three isolates belonged to a specific lineage of Indo-Oceanic clade, EAI5 SIT138. One of the elephant isolates had a new synonymoussingle nucleotide polymorphism (SNP) T231C in the *gyrA* sequence, and the same SNP was also found in human isolates in Nepal.MLVA results and transfer history of the elephants suggested that two of them might be infected with *M. tuberculosis* from the same source. These findings indicated the source of *M. tuberculosis* infection of those elephants were local residents, presumably their handlers.Further investigation including detailed genotyping of elephant and human isolates is needed to clarify the infection route and eventually prevent the transmission of tuberculosis to susceptible hosts.

CHAPTER II

Mixed infection of *Mycobacterium tuberculosis* lineages in Asian elephants of Nepal

Introduction

Nepal is a country with a higher burden of TB infection in the humans (43). Among the different types of *M. tuberculosis* lineages present in TB infected human patients in Nepal, CAS1_Delhi type was present in 40.6% followed by East-Asian lineage (Beijing genotype) in 32.2% and Indo-oceanic lineage in 11.5% (19). Tuberculosis is also increasingly seen in the captive elephants of Nepal. As the captive elephants remain in very close contact with their handlers for a long time which provides the suitable condition for transmission of this disease between the elephants and the handlers. The transmission of TB between elephants and humans has also been reported (20, 30).

The Chapter II documents the presence of mixed *M. tuberculosis* lineages in two other elephants that died of tuberculosis in Nepal. For this purpose, I performed the genotyping on *M. tuberculosis* isolates by spoligotyping and large sequence polymorphism (LSP) obtained from two Asian elephants of Nepal.

Materials and Methods

Elephant isolates.

M. tuberculosis isolates from two captive elephants were included in this study. Both elephants were owned by the Government of Nepal and were stationed in Chitwan National Park (CNP). These elephants were basically used in the patrolling of CNP and its buffer zone for the wildlife management and conservation of the national park. Both the elephants were kept in two different government elephant facilities within Chitwan National Park. Each of these elephants were taken care by three handlers and these handlers generally spend much longer time working very closely with their elephants.

Elephant D was a female working elephant from CNP. She was approximately 65-70 years old. She was purchased from Sarlahi district in southern Nepal and brought to CNP 26 years ago. She was stationed in Meghauli elephant facility in the western part of CNP. She was in complete rest for three years before she died in February, 2013.

Elephant E was a working male elephant from CNP. He was 32 years old. He was captive –borne in KoshiTappu Wildlife Reserve and was transferred to CNP when he was about six years old. For most of the time, he was stationed at Divyapuri elephant facility in the north western part of CNP. He was suspected of TB infection. Trunk wash samples collected a month before he died yielded no positive growth in L-J media. His body condition deteriorated greatly for few months before he died in March, 2013.

Necropsy.

Necropsies of both elephants were carried out at the sites where each elephant collapsed. All personnel involved in the procedure used personal protective equipment including N-95 masks. The abdomen was opened first, and the gastro-intestinal tract and other visceral organs including liver and spleen were observed. The thoracic cavity was approached through the diaphragm per recommendations (26) and the caudal lobe of the lung was observed. Because suspected TB lesions were seen, the thoracic cavity was not further exposed due to the risk of spreading the organism in the environment. The TB suspected lung lesions were collected in a sterile tube for the culture.

Culture and drug susceptibility testing.

The culture of the tissue sample was performed in L-J media at German Nepal Tuberculosis Project (GENETUP) in Kathmandu, Nepal. The processing of lung tissue for culture was done according to the established guidelines by the European Society of Mycobacteriology (10). The cultured tubes were observed weekly for eight weeks to see the growth of bacteria. The DNA was extracted from the isolates from two elephants using GenoType® DNA isolation kit (HainLifescience GMBH, Nerhen, Germany).

Drug susceptibility testing.

The phenotypic drug susceptibility test was performed on two isolates from the elephants by the proportional method on L-J solid media with critical concentration of 0.2 μg/mL of isoniazid, 40 μg/mL of rifampin, 2 μg/mL of ethambutol and 4 μg/mL of streptomycin.

Genetic analyses.

Genetic analyses were performed on two *M. tuberculosis* isolates by spoligotyping and LSP. Spoligotyping was performed as described previously (15). In short, the direct-repeat (DR) region was amplified with a primer pair and the PCR products were hybridized to a set of 43 oligonucleotide probes corresponding to each spacer that were covalently bound to the membrane. The spoligo-international type (SIT) was identified by comparing spoligotypes with the international spoligotyping database (SpolDB4) (3). The genetic regions associated with the drug resistance i.e. partial *rpoB*, *katG*, *inhA* promoter region and *gyrA* were sequenced and analyzed as described (35, 36).

LSP was performed on both the isolates using specific primers for respective lineages as described (8). Indo-Oceanic lineage and East-African-Indian lineage were analyzed with H73Rv for Elp-D isolate (Figure II-1). Similarly, East-Asian-Beijing Lineage was analyzed with positive control and BCG strain for Elp-E isolate.

Results

The necropsy results showed that both the elephants had tuberculous-like lesions in the lungs. Elephant D had extensive minute tuberculosis like lesions in both the lungs. Similarly, Elephant E had massive TB like lesions in both the lungs.

Culture.

There was growth of *M. tuberculosis* complex from the suspected lung lesions in the samples from elephants D and E in the L-J media.

Drug susceptibility test.

Both the isolates were susceptible to isoniazid, rifampin, ethambutol and streptomycin.

Genetic analyses.

Two elephant samples were revealed as a mixture of two strains by uneven Spoligotyping color development (suggesting mixture) and LSP (large sequence polymorphism) detection PCR results. One was a mixture of EAI and CAS1_Delhi (Figure II-1), and the other was a mixture of EAI and Beijing by the LSP analysis (8). In the *gyrA* sequence, both of the samples showed a mixed peak of T231C suggesting that the EAI is Nepal specific lineage that is same as Elep-A isolate. The drug resistance determination region sequences, *rpoB, katG, inhA* promoter region and *gyrA,* were wild type in all the samples.

Discussion

This study reports the mixed infection of *M tuberculosis* strains in Asian elephants in Nepal. Both elephants were stationed at two distant facilities in Chitwan National Park and there was minimal chance of contact between these two elephants. Thus, the possibility of TB transmission between these two elephants was very low. Both elephants had extensive TB lesions in the lungs during the necropsy. The phenotypic drug susceptibility test shows that both *M. tuberculosis* isolates from both elephants were susceptible to first line anti-tuberculosis drugs. Both the elephants were not treated for TB.

Spoligotyping has shown that Elp-D isolate had a new spoligotype whereas Elp-E had Indo-oceanic lineage. Among different lineages in human TB patients of Nepal, CAS1_Delhi type is present in 40.6% followed East-Asian lineage (Beijing genotype) in 32.2% and Indo-oceanic lineage in 11.5% of TB infected population (19). LSP results in Elp-D isolate

demonstrated that it was infected with two lineages viz. Indo-oceanic lineage and East-African-Indian (CAS-Delhi) lineage. Similarly, Elp-E isolate belonged to East-Asian (Beijing) lineage in LSP analysis in addition to Indo-oceanic lineage detected in spoligotyping. I have identified Indo-oceanic lineage in three elephants from Nepal in Chapter I. Both the elephant isolates in this study were infected with this lineage too. I can assume that Indo-oceanic lineage might have well adapted in Nepalese elephants than other lineage. Elephant E was infected with Beijing lineage in addition to Indo-Oceanic lineage. Beijing type is most frequently reported in mixed infection in humans (6, 12).

Elephant D and E died in different times of a year and the lung tissue samples from each elephant were processed in the laboratory for culture immediately after necropsy. So, there was very less chances of contamination between two samples. Mixed infection in both of these elephants might be due to double infection from human patients or elephants with similar lineages. There is high chance of infected elephant handlers transmitting TB to their elephants, as they are only the people who work very closely with the elephants for years.

This study has, for the first time, reported the mixed infection of *M. tuberculosis* in captive elephants of Nepal. More detailed study on the *M. tuberculosis* isolates from the elephants and their handlers will help to determine the source and route of infection of TB in this endangered animal which will eventually help to prevent the transmission of this disease between the elephants and other susceptible hosts.

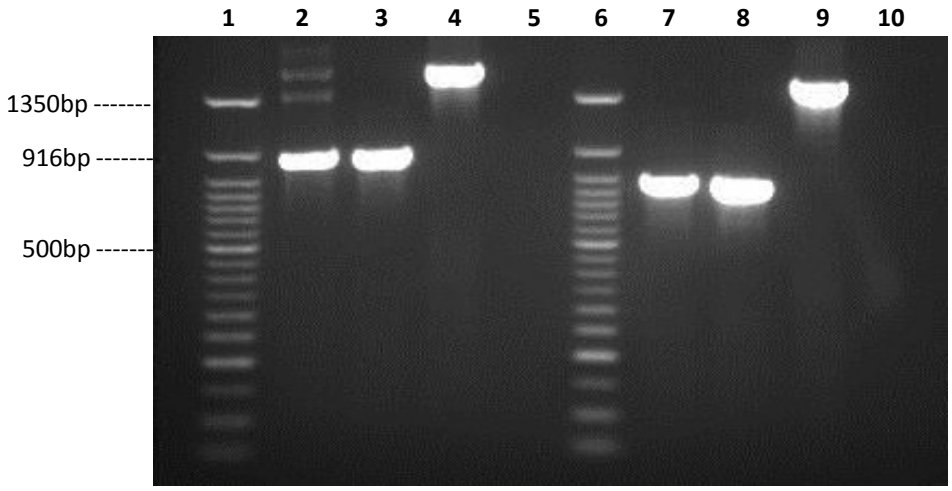


Figure II-1. LSP results of Elp-D isolate. Gel electrophoresis was run in isolate D using primers for Indo-oceanic lineage (lineage 1) and East-African-Indian lineage (lineage 3). Lanes: 1, 50bp DNA ladder; 2, elephant sample; 3, positive control for Indo-Oceanic lineage; 4, H36Rv (Euro-American lineage); 5, negative control; 6, 50bp DNA ladder; 7, elephant sample; 8, positive control for East-African-Indian lineage; 9, H37Rv (Euro-American lineage); 10, negative control. The expected PCR product of size of Indo-oceanic lineage is 888bp and that of East-African-Indian lineage is 743bp (Gagneux et al., 2006)

Summary

Tuberculosis in elephants is primarily caused by *Mycobacterium tuberculosis*, a human form of TB. *M. tuberculosis* was isolated from lung tissue samples of two elephants in Nepal. The genotyping of the isolates was performed by spoligotyping and large sequence polymorphism (LSP). The spoligotyping showed that Elp-D isolate had a new spoligotype that were not found in the SpolDB4 database while Elp-E isolate belonged to Indo-Oceanic lineage (EAI5, SIT1365). LSP results showed that Elp-D isolate belonged to two lineages of *M. tuberculosis* viz. Indo-oceanic lineage and East-African-Indian (CAS-Delhi) lineage while Elp-E isolate belonged to East-Asian (Beijing) lineage. Mixed infection in both of these elephants might be due to double infection from human patients or from infected elephants with similar lineages. Future study including the genotyping of elephant and human isolates will help to explain the epidemiology, source and route of TB transmission between the elephants and humans.

CHAPTER III

Development and evaluation of interferon-γ release assay (IGRA) in Asian elephants (*Elephas maximus*)

Introduction

Inteferon-γ (IFN-γ) is one of the major cytokine that causes the activation of macrophage that will kill the obligate intracellular microbes mediated by up regulation of reactive oxygen intermediates and toxic NO (4). IFN-γ plays an important role in the immuno-pathogenesis of tuberculosis (7). Currently, Inteferon-γ release assay (IGRA) has also been developed in some wildlife species including deer (42), lion (18) and rhinoceros (29) for the diagnosis of TB.

In Chapter III, I have described the development and evaluation of the Asian elephant specific IGRA. The purpose of this study is to develop the Asian elephant specific IGRA and see its potential as a potential diagnostic tool for infection with intracellular pathogens like *Mycobacterium* spps. in Asian elephants. The other aims of this study are to determine the lower limit of detection (LOD) of ELISA system and the selection of best mitogen as positive control stimulation for the future assay.

Material and Methods

Expression and purification of recombinant elephant interferon-γ (rEIFN-γ).

The elephant interferon-γ (EIFN-γ) gene (458 bp) was synthesized and cloned into *Nde* I and *Eco*R I sites of pET-17b vector (Merck KGaA, Darmstadt, Germany). *E. coli* SoluBL21(DE3) (Merck KGaA) harboring the EIFN-γ expression vector was grown in LB broth in the presence of 100 μg/ml of carbenicillin (Meiji Seika Pharma Co., Ltd., Tokyo, Japan) at 37°C. The recombinant protein expression was induced by the addition of isopropylthiogalactoside (IPTG) (Wako Pure Chemicals Industries, Ltd., Osaka, Japan) to a final concentration of 0.1 mM followed by incubation at 16°C for 22 h. The *E. coli* cells were resuspended in 40 mM Tris-HCl pH8, 0.15 M NaCl, 10% (w/v) sucrose containing EDTA-free complete (Roche Applied Science, Penzberg, Germany), disrupted using a sonicator, and the cell debris was removed by centrifugation at 140,000 X g for 120 min. All subsequent purification steps were performed at 4°C. The supernatant was mixed with TALON resin (Takara Bio Inc., Shiga, Japan), and the resin was washed with 20 mM TrisHCl pH 8, 0.15 M NaCl, 5 mM Immidazole (Wako Pure Chemicals Industries, Ltd.). The recombinant EIFN-γ was eluted from the resin with 20 mM Tris-HCl pH8, 0.15 M NaCl, 0.2 M Imidazole by a batch method and dialyzed against 20 mM Tris-HCl pH8.8, 0.1 M NaCl, 3% glycerol (v/v), 0.1 mM EDTA overnight and applied onto a 2.6 × 10 cm diethylaminoethanol fast flow (DEAE FF) column (GE Healthcare, Little Chalfont, United Kingdom). The protein was eluted with a 10 column volume (CV) of linear gradient (0.1 to 0.6 M NaCl). The eluted protein was concentrated, loaded onto a 1.6 x 60 cm Superdex 75 prep grade column (GE Healthcare) and eluted with 20 mM Tris-HCl pH8.8, 0.2 M NaCl, 3% glycerol (v/v), 0.1 mM EDTA. The eluted protein was dialyzed against

20 mM Tris-HCl pH 8.8, 0.15 M NaCl, 20% glycerol (v/v), 0.1 mM EDTA, concentrated, and stored at -83°C.

Production, purification and labelling of polyclonal antibodies.

The anti-EIFN-γ polyclonal antibodies were produced by immunization of recombinant rEIFN-γ in rabbits. Antibodies were raised in for 9 weeks, then the rabbits were bled and the serum was collected. Purification of antibodies was performed by affinity chromatography using protein A agarose (GE Healthcare) according to the manufacturer's protocol.

The purified antibodies were used as capture antibodies. For the purpose of producing detection antibodies, these antibodies were biotinylated using Biotin Labelling Kit-NH_2 (Dojindo Chemical Co., Ltd., Kumamoto, Japan) as per the manufacturer's instruction. Briefly, affinity-purified rabbit polyclonal antibody against rEIFN-γ with a recommended concentration between 50–200μg was mixed with recovery buffer in a filtration tube and centrifuged at 8,000 X g for 10 min. Then, reaction buffer and NH_2-reation biotin solution was mixed in the same tube and incubated for 10 min at 37°C. Unlabeled antibodies were washed by adding recovery buffer followed by centrifugation at 8,000 X g twice. Finally, 200 μl of recovery buffer was added to the tube and mixed thoroughly to recover the conjugate, then transferred to a sterile tube and stored at 4°C until use.

Determination of optimum conditions of coating and detection antibodies.

We performed a sandwich ELISA to determine the optimum conditions for coating and detection of antibodies. A flat-bottomed polystyrene microtiter plate (Nunc A/S, Roskilde, Denmark) was coated with variable dilutions of unlabeled anti-EIFN-γ polyclonal antibodies

(0.1, 0.3, 1, and 2 µg/ml) and incubated at 4°C overnight. The plate was washed three times with phosphate-buffered saline (PBS, pH 7.6) containing 0.05% Tween-20 (PBST) and incubated with a blocking solution containing 3% (w/v) bovine serum albumin (Roche Dx, Mannheim, Germany) for 1 h at 37°C and washed again as described earlier. Various dilutions of rEIFN-γ (1, 3, 10, 30, 100, 300 and 1,000 pg/ml) were added to the wells. After incubation for 1 h at 37ºC, the plate was washed three times with PBST and variable dilutions of biotin-labeled anti-EIFN-γ polyclonal antibodies (0.1, 0.3, 1, and 3µg/ml) were added to the wells and the plate was incubated for 1 h at 37°C. Three washes using PBST were performed, then 100µl of avidin-biotin complex stain (Thermo Fisher Scientific Inc.Waltham, MA, USA) was added and the plate was incubated at room temperature for 30 min. Finally, three additional PBST washes were performed, then 100µl of 3,3',5,5'-Tetramethylbenzidine (TMB) substrate (Sigma-Aldrich Inc., St. Louis, MO, USA) was added to each well and the plate was incubated at room temperature for 10 min. The reaction was stopped by adding 1M phosphoric acid (Wako Pure Chemicals Industries, Ltd.). The absorbance value of each well was measured at 450 nm using a microplate reader.

Selection of study subjects.

Forty captive Asian elephants were selected for the study from three protected areas of Nepal. Thirty-four elephants were females and 6 were males; ages ranged from 8–72 years. Eight elephants were from Bardia National Park, six from Parsa Wildlife Reserve and 26 were from Chitwan National Park. None of the elephants had positive *Mycobacteria* cultures at the time of blood collection.

Blood collection, stimulation and incubation.

A whole blood sample was collected from the auricular vein of each elephant into a Mitogen (Phytohaemagglutinin) (Quantiferon Gold in-Tubes; Cellestis Ltd. Australia) and a heparinized tube. One ml of blood was collected in the Mitogen tube and approximately 3 ml of blood was collected in the heparin tube. The tubes were shaken gently 10 times after blood collection. One ml blood from the heparin tube was put into each of two tubes containing PWM (Pokweed mitogen) and PMA/I (Phorbol myristate acetate and Ionomycin) (Sigma Aldrich, The Netherlands) respectively. These two tubes were also shaken gently 10 times after filling them with the heparinized blood. Different concentrations of PWM (5 µg/ml), PMA (100 ng/ml) and Ionomycin (2 µg/ml) were used. The incubation of the whole blood tubes was performed at 37°C for 16–24 h in the field before transporting the samples to the laboratory in Kathmandu for ELISA testing. The blood tubes were then centrifuged and the supernatant was harvested and subjected to a sandwich ELISA.

Sandwich ELISA.

Ninety six-well ELISA plates were coated with capture antibodies: 50 µl of anti-elephant IFN-γ rabbit polyclonal antibodies diluted with 1 x PBS at 1 µg/ml was placed in each well. The capture antibodies were incubated for 1 h at 37°C. The plates were washed manually three times at room temperature with PBST. The reaction was blocked with 50 µl per well of blocking buffer for 1 h at 37°C. The plates were washed with PBST three times then 50 µl of elephant plasma was placed in each appropriate well and incubated for 1 h at 37°C. The ELISA buffer was run as a negative control and the rEIFN-γ was run in duplicate in various concentrations (10; 30; 100; 300; 1000; 3000, and 10,000 pg/ml) as the positive control for each plate. The plates were again

washed three times with PBST. Biotin-labeled anti-elephant IFN-γ rabbit polyclonal antibodies (3 µg/ml) were diluted with ELISA buffer at 1:10,000 and 50 µl of this diluted antibody was placed into each well and incubated. The incubation was carried out for 1 h at 37°C. After incubation, the plates were washed three times with PBST then 100 µl of Avidin-Biotin Complex stain was added to each well and the plate was incubated at room temperature for 30 min. Washing was performed three times using PBST and 100 µl of TMB substrate was placed in each well for 10 min. The reaction was stopped using 100 µl stop solution and the optical density was measured at a wavelength of 450 nm. Optical density values were converted to IU/ml values and finally each IU/ml values was converted to pg/ml by multiplying each IU/ml value by 40pg/IU (5).

Results

The sandwich ELISA was performed as described above. The optimum combination was obtained at 1 µg/ml of capture antibodies and 3 µg/ml of detection antibodies. The lowest limit of detection (LOD) of rEIFN-γ in this optimized sandwich ELISA was 100 pg/ml (Figure III-1).

The sandwich ELISA system developed was able to detect rEIFN-γ as well as native interferon-γ from the elephants as elicited by stimulation using PMA/I, PWM and PHA (Figure III-2). Of the three mitogens, stimulation by PMA/I was highest followed by PWM and PHA (Figure III-2). Thus, PMA/I is the best mitogen to use as a positive control for an Asian elephant IGRA. The results also showed that the mean concentrations of PHA, PWM, and PMA/I was higher in the female than the male elephants. However, statistical analysis showed that there was no significant difference in the values of the three mitogens based on gender ($p > 0.05$) (Figure III-3).

Discussion

This study describes the expression and purification of rEIFN-γ, production of polyclonal antibodies specific for rEIFN-γ, and optimization of a sandwich ELISA. The LOD of the ELISA system was determined to be 100 pg/ml (Figure III-1). Similar detection levels have also been obtained in lions (18). Thus, the sensitivity of our sandwich ELISA was sufficient to detect native IFN-γ in elephants as elicited by mitogenic stimulation (Figure III-2).

IGRA performed after stimulation of elephant whole blood from 40 elephants showed that all the elephants were able to produce detectable levels of IFN- γ following stimulation with the three different mitogens. Among the mitogens, PMA/I was recognized as the best mitogen to use as a positive control for the stimulation of the elephant whole blood followed by PWM (Figure III-2). PMA/I has previously been identified as the best performing mitogen in Asian elephants (1) and rhinoceros (28).

Culture of trunk wash samples is considered the gold standard for TB diagnosis in elephants; however, this technique has several limitations including difficulty of sample collection, a long culture period, and poor sensitivity (17). The ELISA system developed in this study could be performed by incorporating ESAT-6/CFP-10 as a fusion protein for the whole blood stimulation for early diagnosis of TB in elephants in the future. If this assay is validated in elephants, it will offer several advantages over culture.

In conclusion, the development of an Asian elephant-specific IGRA that detects native IFN-γ in elephant whole blood provides promising results for application as a potential diagnostic tool for diseases such TB in Asian elephants. An IGRA study using a larger population of Asian elephants and including TB-specific antigens is the next step that will help to diagnose TB in the early stages of the disease.

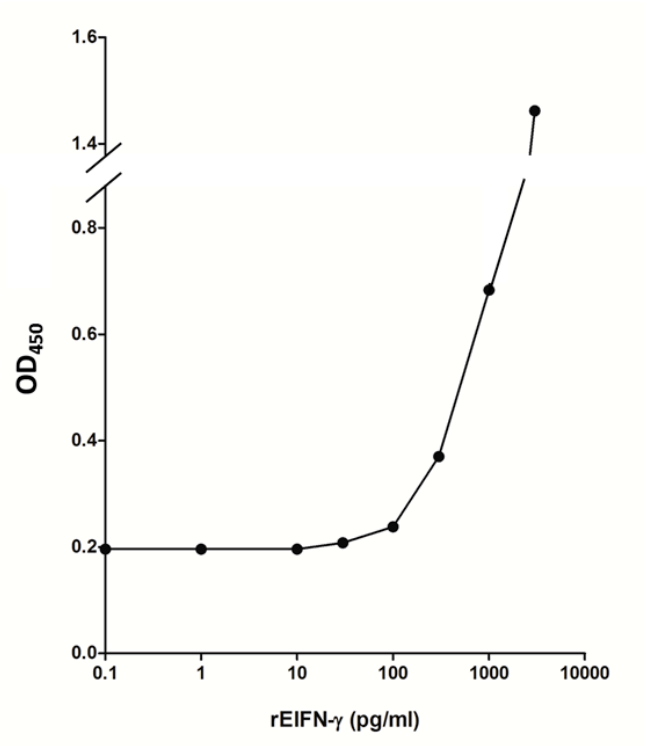


Figure III-1. Titration curve of recombinant elephant IFN- γ (rEIFN-γ) optimized by using coating antibodies (1 μg/ml) & detection antibodies (3 μg/ml).

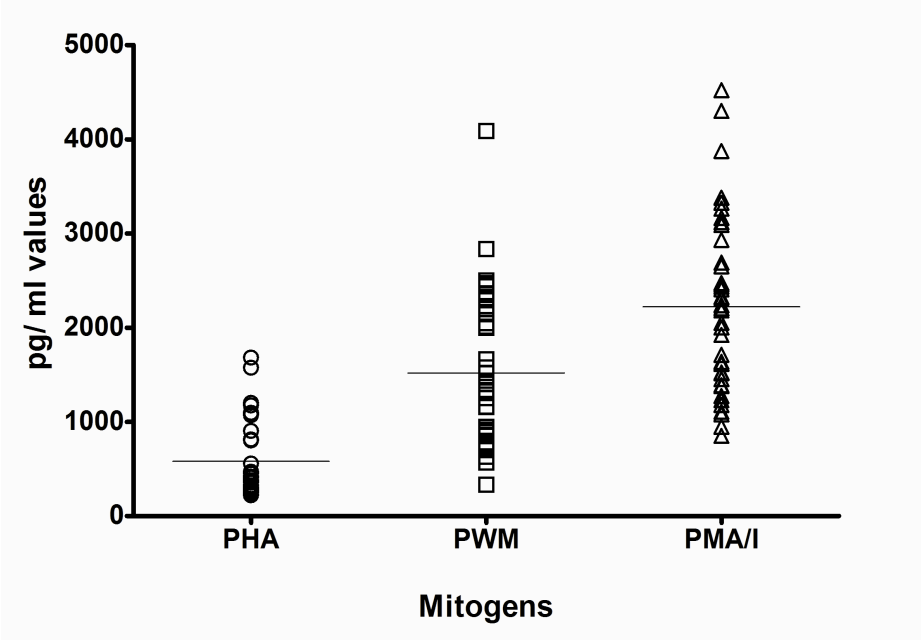


Figure III-2. Stimulation of elephant whole blood by three mitogens ie. phytohaemagglutinin (PHA), pokweed mitogen (PWM) and phorbolmyristateaceteate/ ionomycin (PMA/I)

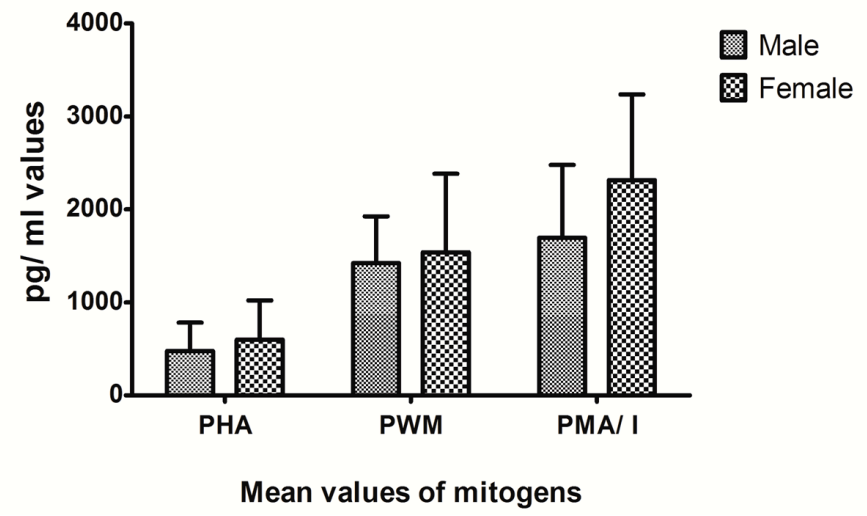


Figure III-3. Comparison of mitogen values among male and female elephants.

Summary

We developed an interferon-γ release assay (IGRA) specific for Asian elephants (*Elephas maximus*). Peripheral whole blood collected from forty captive elephants was stimulated with three different mitogens ie. phytohaemagglutinin (PHA), pokweed mitogen (PWM) and phorbol myristate aceteate/ionomycin (PMA/I). A sandwich ELISA that was able to recognize the rEIFN-γ as well as native interferon-γ from the elephants was performed using anti-elephant IFN-γ rabbit polyclonal antibodies as capture antibodies and biotinylated anti-elephant IFN-γ rabbit polyclonal antibodies as detection antibodies. PMA/I was the best mitogen as a positive elephant IGRA control. This preliminary study showed that this IGRA is a potential tool for the early diagnosis of tuberculosis in Asian elephants.

Conclusion

Tuberculosis is a re-emerging disease in the elephants; however, very less studies has been conducted on the molecular characterization of *M. tuberculosis* isolates form elephants in Nepal. In addition to the genotypes, mixed infection with two *M. tuberculosis* lineages has not yet been reported from Nepalese elephants. The early diagnosis of TB in elephants is important for the control of this disease spreading to the other susceptible hosts. The Asian elephant specific interferon gamma release assay (IGRA) might be a tool for the early diagnosis of TB in elephants.

In this regard, in chapter I, I performed the molecular characterization of the *M. tuberculosis* isolates from three captive elephants (Elephant A, B and C) of Nepal. I performed spoligotyping, TbD1 detection and multi-locus variable number of tandem repeat analysis (MLVA) which suggested three isolates belonged to a specific lineage of Indo-Oceanic clade, EAI5 SIT138. One of the elephant isolates had a new synonymous single nucleotide polymorphism (SNP) T231C in the *gyrA* sequence, and the same SNP was also found in human isolates in Nepal. MLVA results and transfer history of the elephants suggested that two of them might be infected with *M. tuberculosis* from the same source. These findings indicated the source of *M. tuberculosis* infection of those elephants were local residents, presumably their handlers. Further investigation including detailed genotyping of elephant and human isolates is needed to clarify the infection route and eventually prevent the transmission of tuberculosis to susceptible hosts.

In Chapter II, I conducted the genotyping of *M. tuberculosis* isolates from other two elephants (Elephant D and E) that died of suspected TB lesions in the lungs. The spoligotyping

and large sequence polymorphism (LSP) was performed on these two isolates to detect the *M. tuberculosis* lineage. The spoligotyping showed that Elephant D isolate had a new spoligotype that were not found in the SpolDB4 database while Elephant E isolate belonged to Indo-Oceanic lineage (EAI5, SIT1365). LSP results showed that Elephant D isolate belonged to two lineages of *M. tuberculosis* viz. Indo-oceanic lineage and East-African-Indian (CAS1_Delhi) lineage while Elephant E belonged to East-Asian (Beijing) lineage. Mixed infection in both of these elephants might be due to double infection from human patients or from infected elephants with similar lineages. Future study including the genotyping of elephant and human isolates will help to explain the epidemiology, source and route of TB transmission between the elephants and humans.

In Chapter III, I have described the development and evaluation of the Asian elephant (*Elephas maximus*) specific IGRA to see its potential as a tool for the early diagnosis of TB in Asian elephants. The peripheral whole blood collected from forty captive elephants was stimulated with three different mitogens ie. phytohaemagglutinin (PHA), pokweed mitogen (PWM) and phorbolmyristateaceteate/ ionomycin (PMA/I). Sandwich ELISA was performed using anti-elephant IFN-γ rabbit polyclonal antibodies as capture antibodies and biotinylated anti-elephant IFN-γ rabbit polyclonal antibodies as detection antibodies which was able to recognize the rEIFN-γ as well as native interferon-γ from the elephants. This study has shown that the lower limit of detection (LOD) was 100 pg/ml and PMA/I was the best mitogen as a positive control in elephant IGRA. ELISA system developed was able to detect the native IFN-γ from the Asian elephants. This preliminary study showed that it can be a potential tool for the early diagnosis of diseases like TB in Asian elephants.

I believe that the findings of my study on the genotyping and diagnosis of TB in Asian elephants of Nepal have expanded our understandings on the elephant TB and also provides the preliminary results on the possibility of using IGRA as a potential diagnostic tool for TB in Asian elephants. In addition, my findings puts emphasis on the regular screening of the elephants as well as their handlers for TB and treat the TB infected elephants and their handlers with the established treatment regimen which will eventually prevent the transmission of TB from elephants to other susceptible hosts.

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