Genetic characterization of *Mycobacterium orygis* isolates from animals of South Asia

（南アジアの動物から分離された *Mycobacterium orygis* の遺伝的特徴）

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LIST OF PAPERS

The contents of this thesis have been published in advance as journal articles and a book chapter as followings:

Peer-reviewed journals

1. **Thapa J**, Nakajima C, Maharjan B, Poudel A, Suzuki Y. Molecular characterization of *Mycobacterium orygis* isolates from wild animals of Nepal. Jpn J Vet Res 63(3), 151-158, 2015.
2. **Thapa J***, Paudel S*, Sadaula A, Shah Y, Maharjan B, Kaufman GE, McCauley D, Gairhe KP, Tsubota T, Suzuki Y, Nakajima C. 2016. *Mycobacterium orygis*–associated tuberculosis in free-ranging rhinoceros, Nepal, 2015. Emerg Infect Dis 22 (3), 570-572, 2016.
3. Rahim Z*, **Thapa J*,** Fukushima Y, Suzuki H, van der Zanden, Adri GM, Gordon SV, Suzuki Y, Nakajima C. Tuberculosis caused by *Mycobacterium orygis* in dairy cattle and captured monkeys in Bangladesh: a new scenario of tuberculosis in South Asia. Transbound Emerg Dis In press.

Book chapter

- **Thapa J**, Nakajima C, Gairhe KP, Maharjan B, Paudel S, Shah Y, Mikota S, Kaufman GE, McCauley D, Tsubota T, Gordon SV, Suzuki Y. Wildlife Tuberculosis: An Emerging Threat for Conservation in South Asia. In: Global Exposition of Wildlife Management, Lameed GA. ed. InTech. pp. 73-90, 2017.

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ABBREVIATION

CITES	Convention on international trade in endangered species
CNP	Chitwan National Park
DNPWC	Department of national park and wildlife conservation
DR	Direct repeat
GENETUP	German Nepal tuberculosis project
IUCN	International union for conservation of nature
L-J	Löwenstein-Jensen
MLST	Multi locus sequence typing
MLVA	Multi locus variable number of tandem repeat analysis
MST	Minimum Spanning Tree
MTBC	*Mycobacterium tuberculosis* complex
PNB	Para-nitrobenzoic acid
RD	Region of differences
SIT	Spoligo-international type
SNP	Single nucleotide polymorphism
TB	Tuberculosis
UPGMA	Unweighted pair group method with arithmetic mean

PREFACE

Mycobacterium orygis, previously described as the oryx bacillus or the antelope clade, is a new member and a subspecies of the *M. tuberculosis* complex (MTBC) (Figure 1) that causes tuberculosis (TB) in animals and humans (32). It was first reported in antelopes (oryx and waterbuck) at a zoo in the Netherlands, where the authors reported it as *M. bovis* but described its unusual genetic feature of having a high copy number of the IS*6110* insertion sequence. In the same study, infection with a similar strain of MTBC to a human was suspected, and possibility of transmission between human and animal was argued (33). The clear molecular genetic distinction between this subspecies and other members of the MTBC was reported in 2005 (16). While the subspecies name of this MTBC member was proposed as *M. orygis* to convey the fact that it was first characterized from oryx (31) or as an antelope clade to convey the group of animals from which it was most frequently isolated (2, 10, 16, 23), recently it has been isolated from many other animals and humans (Table 1). Thus, the isolation of *M. orygis* from a wide range of host species challenges previous concepts of it being an exclusively antelope clade and its host range.

My initial objective of the PhD study was to understand dynamics of zoonotic tuberculosis among human, wildlife and livestock interface in Nepal. I performed TB surveillances in livestock of Nepal in 2013-2015 by observing TB suspected lesions in slaughtered buffaloes and by conducting tuberculin test in old cows and later performing postmortem investigation on the dead cows. However, I was not able to obtain any positive MTBC isolates. For human interface study, I targeted extrapulmonary TB suspected samples from human TB cases as zoonotic TB is associated with extrapulmonary TB (7). Out of 247 extrapulmonary TB suspected samples, 14 MTBC isolates which were later determined to be *M. tuberculosis,* a human adapted MTBC were obtained. However, I was successful in isolating 3 *M. orygis* isolates from different wild animals in Nepal. Similarly, through our collaborative

work in Bangladesh, I could analyze additional 20 *M. orygis* isolates. I noticed that most of the reported *M. orygis* isolates were from animals and humans of South Asia (Table 1), So, for my PhD thesis research, I decided to focus on understanding the genetic characteristics and distribution of *M. orygis* isolates in the region.

This thesis is consisted of 2 chapters. In the chapter I, I have discussed about isolation and genotyping of 3 *M. orygis* isolates from different wild animals namely spotted deer, blue bull and free-ranging rhinoceros in Nepal. The purpose of the study was to genetically characterize these *M. orygis* isolates to understand their molecular epidemiology. In the chapter II, I have discussed about isolation and genotyping of 20 *M. orygis* isolates from dairy cattle and rhesus monkey in Bangladesh. The purpose of the study was to genetically characterize these *M. orygis* isolates to understand their molecular epidemiology. Thus, by overall genetic characterization of *M. orygis* isolates from Nepal and Bangladesh and complementing my findings with the results of previously reported *M. orygis* isolates, I have elucidated molecular epidemiology and distribution of *M. orygis* in Nepal, Bangladesh and South Asia.

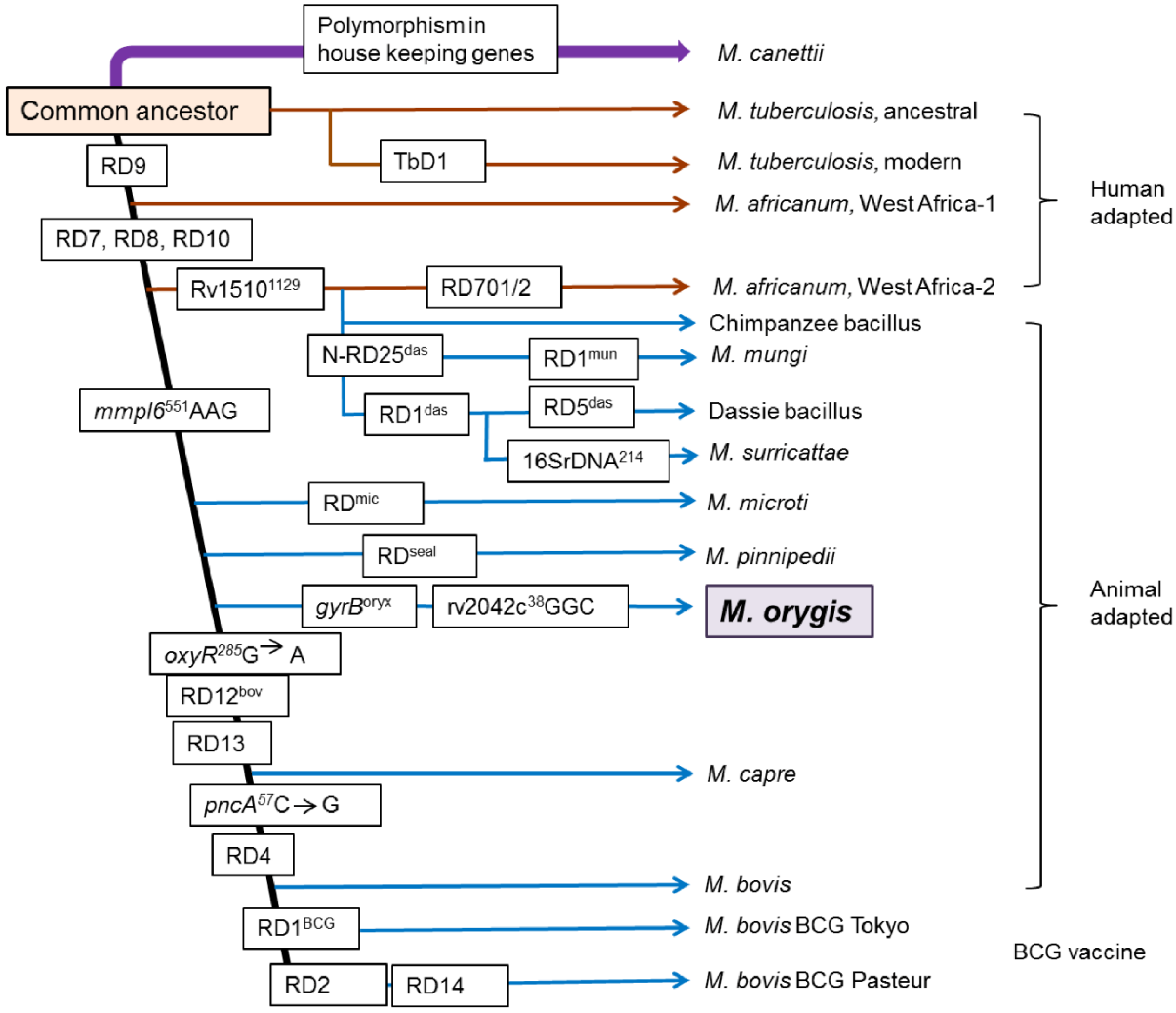


Figure 1. Updated phylogeny of *Mycobacterium tuberculosis* complex (MTBC) (30). The phylogeny is based on presence or absence of region of differences and single nucleotide polymorphisms.

Table 1. Reported cases of *Mycobacterium orygis* from different host species and geographical location (30).

Host (number of isolates)	Geographical location	Wild/Captive/Domestic	References
Antelope (2) Water buck (3)	Netherland		
Oryx (1)		Captive	
Antelope (1)	South Africa		(32)
Deer (1)	United Kingdon		
Human (10)	South Asia	Not applicable	
Human (1)	South East Asia		
African buffalo (1)	South Africa	Captive	(8)
Cattle (1)	New Zealand	Domestic	
Human (1)	New Zealand/Indian Immigrant	Not applicable	(5)
Blue bull (1) Spotted deer (1)	Nepal	Captive	(28)
Rhinoceros (1)		Wild	(29)
Monkey (2)	Bangladesh	Captive/ wild captured	(22)
Cattle (18)		Domestic	

CHAPTER I

Molecular characterization of *Mycobacterium orygis* isolates from wild animals of Nepal

Introduction

Nepal is one of the high burden countries for human tuberculosis (TB) with an incidence 156/100,000 and mortality of 15/100,000 population (36). Despite few studies of TB in livestock, *M. bovis* has been isolated from milking cattle and buffalo in Nepal (11). Wildlife TB received an attention in Nepal after some of the captive elephants died of TB which led to national wide elephant TB surveillance program (15). *M. tuberculosis* was found to be causative agent of TB in captive elephants in Nepal and same strain of *M. tuberculosis* was found to be circulated in people of Nepal (19). During my professional career in Nepal, I had observed TB was one of the causes of deaths in a captive wild animal facility in Nepal. However, I was not able to diagnose the species of TB bacterium. Based on these background information, I pursued to continue to study TB in wild animals of Nepal as the situation was not fully understood.

In chapter I, I report the identification of *M. orygis* isolates from TB-infected lesions of a spotted deer (*Axis axis*) and a blue bull (*Boselaphus tragocamelus*) at a captive wild animal facility in Nepal and from a free ranging one-horned rhinoceros (*Rhinoceros unicornis*) at Chitwan National Park (CNP). I have performed comprehensive genetic analysis of these isolates to understand their molecular epidemiology.

Materials and methods

M. orygis isolates collection

M. orygis isolates from a deer and a blue bull in a captive facility in Kathmandu and a free-ranging rhinoceros in CNP were examined in this study. The deer and blue bull had died on February 4, 2013 and February 18, 2014 respectively. On necropsy, TB suspected lesions were observed on those animals. The samples of suspected TB lesions were collected from the lungs and the extrapulmonary granulomatous lesions in the deer (Figure 2) where as in case of the blue bull, samples were collected only from the lung lesion. No detailed postmortems were conducted in both cases due to the potential risk of spread of the organism to the environment. Thus, comprehensive descriptions of TB lesions from other organs were not available.

The rhinoceros *M. orygis* isolate was collected from the buffer zone area of the western sector of CNP near Amaltari. On February 16, 2015, the officials of CNP observed a sick female rhinoceros. The rhinoceros was dull, depressed and was not feeding. On the following day, it was found dead in the same area (Figure 3). By the necropsy, several granulomatous lesions were observed in the lungs and considered to be compatible with TB infection (Figure 2). The tissue sample from suspected TB lesions in the lungs was collected for further analysis.

Microscopy and culture

Tissue samples were processed as described by the European Society of Microbiology (9). Briefly, samples were aseptically sliced into small pieces, mixed with 4% sulphuric acid, and incubated for 20 minutes at room temperature. Thereafter the samples were mixed with 4% sodium hydroxide using bromothymol blue indicator and centrifuged at 3,000 × *g* for 20 minutes. The resulting supernatant was discarded and the samples were washed once with sterile distilled water. Samples were again centrifuged at 3,000 × *g* for 20 minutes, and the supernatant was discarded and sediment was used for further analysis. Fluorescence

microscopy analysis was performed. Briefly, a smear was prepared from the sediment, stained with auramin solution, washed with 20% sulphuric acid, counter stained with ink blue and finally observed by fluorescent microscope. For culturing the samples, an aliquot from the sediment was inoculated into Löwenstein-Jensen (L-J) growth media. The inoculant was grown for 8 weeks.

DNA extraction

Colonies of the deer and rhinoceros isolate were collected with an inoculum loop, suspended in 300 µL of distilled water and heated for 20 min at 95^{o}C in a water bath. Heat-killed samples were stored at -30 °C until further use. DNA from the deer isolate was extracted by alternating freezing (-80°C) and boiling (100°C) for five minutes. The procedure was repeated three times. Colonies of the blue bull isolate were suspended in 300 µL of distilled water and heated for 20 min at 95^{o}C. Afterwards, cells in the suspension were disrupted by incubation for 15 min in an ultrasonic bath to disrupt cells followed by centrifugation at 13,000 × *g* for 5 min. The supernatant containing the bacterial DNA was used for further analysis.

Genetic analysis

A standard TB genotyping analysis was conducted to genetically characterize the isolates (Figure 4). The region of difference (RD) analysis was performed to determine three genetic regions, *cfp32*, RD9 and RD12, as previously described with some modification (17). Since the multiplex PCR of those genetic regions did not yield a good result, an individual PCR for each genetic region was conducted. Spoligotyping was performed as previously described (12), and the result was interpreted using the international spoligotyping database (SpolDB4) to determine spoligo-international types (SIT) (3). The multi locus variable number of tandem repeat analysis (MLVA) typing was carried out on 22 loci following a basic protocol as

previously described (26). The MLVA pattern obtained was compared with the previously reported data (8, 32) to construct an unweighted pair group method with arithmetic mean (UPGMA) dendrogram using MIRU-VNTR*plus* (www.miru-vntrplus.org). The multi locus sequence typing (MLST) analysis was conducted on *gyrB, mmpL6,* TbD1 and *PPE55* loci, and on Rv2042c gene, as previously described (10, 32).

Results

Microscopy and culture

The result from the fluorescence microscopy analysis carried out on tissue smears were positive for TB (data not shown). The culture from tissues yielded smooth and moist colonies (Figure 4).

Genetic analysis

Spoligotyping of tuberculosis isolates from all deer, blue bull and rhinoceros isolates showed the SIT number 587 in the SpolDB4 database and was determined to be *M. orygis*, similar to the result from a previous study (32). On spoligotyping on three isolate, the spacer 3 showed a very faint reactivity that was difficult to confirm by visual inspection (Figure 6). The presence of spacer 3 was confirmed by partial sequencing of the DR region as previously described (31). On sequencing, the spacer 3 had two-point mutations (Table 2).

The RD analysis showed that the isolates had *cfp32,* but deleted RD9 and RD12. The results of the MLST analysis of the *gyrB, PPEE5, mmpL6,* Rv2042c genes and the TbD1 genetic region corresponded to *M. orygis* (Table 3). Thus, the bacterial species of the isolate was determined as *M. orygis*. The MLVA type of deer and blue bull isolates was the same and similar to that of most reported isolates (8, 32) and had the same position as that of most reported isolates in the UPGMA dendogram (Figure 7). The rhinoceros *M. orygis* isolate fell in a unique position in the dendogram, with a difference identified in only one locus (locus

424) when I compared it with the largest cluster of reported *M. orygis* isolates, including the deer and blue bull isolates (Figure 7).

Discussion

Genetic analysis of the isolates by spoligotyping, region of difference and MLST showed that they belonged to *M. orygis*. The absence of clear band of spacer 3 in spoligotyping perhaps was due to the two-point mutations found in the spacer (Table 2). Although all 3 *M. orygis* isolates from these animals had the same spoligotyping pattern (SIT587), further molecular characterization by multi locus variable number of tandem repeat analysis (MLVA) revealed that both the deer and blue bull isolate had the same MLVA type while the rhinoceros isolate was a different type differing at 1 MLVA locus indicating different strain type of *M. orygis* in Nepal.

We were aware of TB infections in the wild animals in the captive facility, and had assumed it to be caused by *M. bovis*, however, after molecular characterization, the TB isolates were confirmed to be *M. orygis*. Although the deer and the blue bull were placed in different enclosures, around 200 meters apart, these animals may have contact opportunities when they were placed together for a short time during repair and maintenance of enclosures. Also, the possibility of aerosol transmission and contamination via food, utensils and other unknown sources should not be ruled out. Thus, the result indicates that there may be ongoing transmission of *M. orygis* in the captivity facility.

When I isolated *M. orygis* from deer and blue bull from a captive wild animal facility, I had postulated that the origin of TB might be from other animals in the CNP where they originated. The discovery of a different strain type of *M. orygis* in a free-ranging rhinoceros in the park supported my hypothesis. Other reports of *M. orygis* from different animals and humans in South Asia including 2 cattle and a monkey in Bangladesh (32) and an Indian

immigrant in New Zealand (5) further supports for its wide distribution in South Asia, and attests to the One Health significance of this organism.

This study suggests *M. orygis* as an emerging threat to the conservation of wild animals in Nepal. The greater one-horned rhinoceros is the largest species of rhinoceros that is listed in Appendix I of the Convention on International Trade in Endangered Species (CITES), categorized as vulnerable by the International Union for Conservation of Nature (IUCN) Red List, and listed as a protected species by the Government of Nepal (6, 27, 36). The present day free-ranging population of rhinoceroses in Nepal and India has increased from only 600 individuals in 1975 to 3,555 individuals by mid-2015 (36). In Nepal, the population of rhinoceroses is 645 individuals, of which 605 individuals live in CNP in a relatively narrow area of riverine grassland (18, 25). From a conservation point of view, having a chronic and devastating disease like TB in this vulnerable and isolated population, that is already threatened from habitat destruction and poaching, is a matter of great concern for the animal's long term survival. Also, CNP is listed by the United Nations Educational, Scientific and Cultural Organization (UNESCO) as a World Heritage site because of its rich biodiversity and being home to globally protected animals such as Bengal tigers, Asian elephant and greater one-horned rhinoceroses. The finding of TB in rhinoceroses in the park could also signify TB as a threat in other animals, including a strong possibility of unknown maintenance hosts of *M. orygis* in and around the national park.

In this study, only three isolates from a deer, a blue bull and a free-ranging rhinoceros were studied. A more comprehensive study with a larger sample size and in-depth genetic characterization at whole genome level would help better the molecular characterization of *M. orygis* in Nepal. Ascertaining the prevalence of *M. orygis* in other wild animals of Nepal will be also important. Future surveillance should be conducted in livestock and people living

around CNP should be conducted to ascertain transmission dynamics and prevent potential zoonosis.

Summary

M. orygis associated TB was found to be the cause of death of a spotted deer, a blue bull and a free-ranging rhinoceros. The TB suspected samples were subjected with standard mycobacterial laboratory procedure of microscopy and culture. The obtained culture isolates were genetically characterized with standard TB genotyping techniques of spoligotyping, MLVA analysis and MLST. All the 3 *M. orygis* isolates had identical spoligptye pattern of SIT 587. Identical MLVA pattern of *M. orygis* isolates from spotted deer and blue bull suggested possible transmission of TB. The different strain type of *M. orygis* in rhinoceros suggested possibility of different strains of *M. orygis* circulating in CNP. The CNP is a world heritage site and home to many endangered animals like rhinoceros, tigers and elephants, so my findings, strongly suggests *M. orygis* associated TB as an emerging threat to the conservation of wild animals in Nepal.

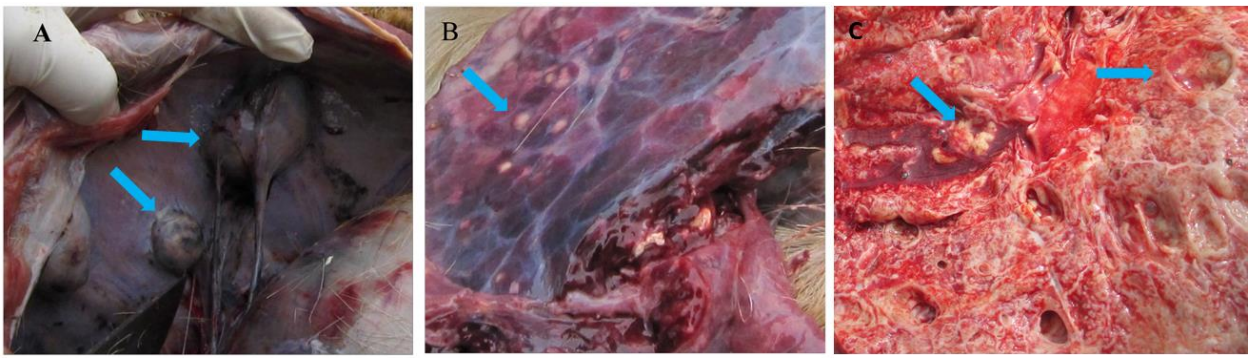


Figure 2. Tuberculosis suspected lesions in a dead spotted deer and rhinoceros during postmortem (28-29). A) Extrapulmonary from a spotted deer, B) lungs from a spotted deer and C) lungs from a rhinoceros. From spotted deer, extrapulmonary tuberculosis lesions were of various sizes and capsulated with extensive liquefaction and pulmonary tuberculosis lesions were of varying sizes from a single focal granuloma of 1 to 2 cm to extensive granulomatic lesion affecting a larger area of lung tissue. In rhinoceros, the lesions in the lungs were extensively distributed, were well encapsulated, and contained caseating granuloma.

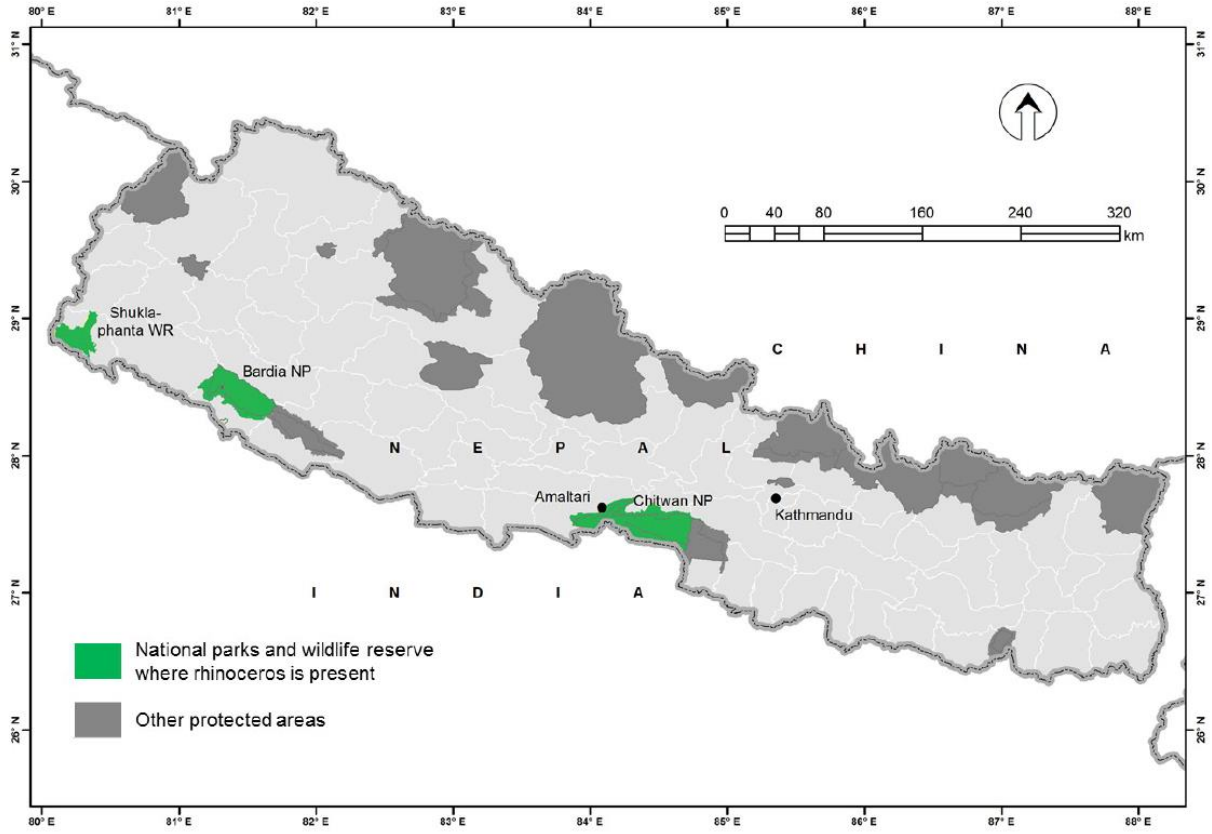


Figure 3. Location of places from where *M. orygis* isolates were collected from different animals,(29). Amaltari in CNP is a place from where rhinoceros was found dead and Kathmandu is place of captive facility from where *M. orygis* was reported from wild animals.

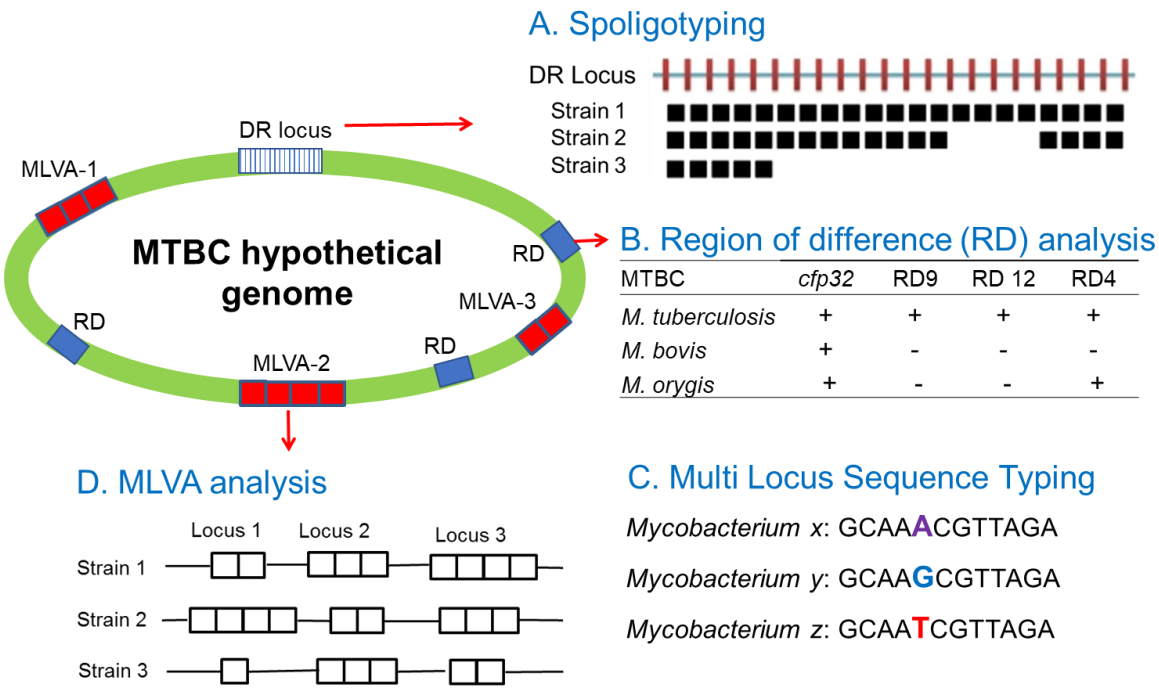


Figure 4. Schematic illustration of MTBC hypothetical genome and its genetic markers used for standard TB genotyping analysis. A) Spoligotyping is a PCR based method to detect 43 unique spacer sequence in direct repeat locus. B) Region of difference (RD) analysis detects presence or absence genetic regions in MTBC genome. C) Multi locus sequence typing (MLST) method sequences specific genes or genetic regions to observe polymorphism on sequence alleles. D) Mycobacterial interspersed variable number of tandem repeat (MLVA) analysis identifies different number of tandem repeats in locus scattered throughout the genome of MTBC.

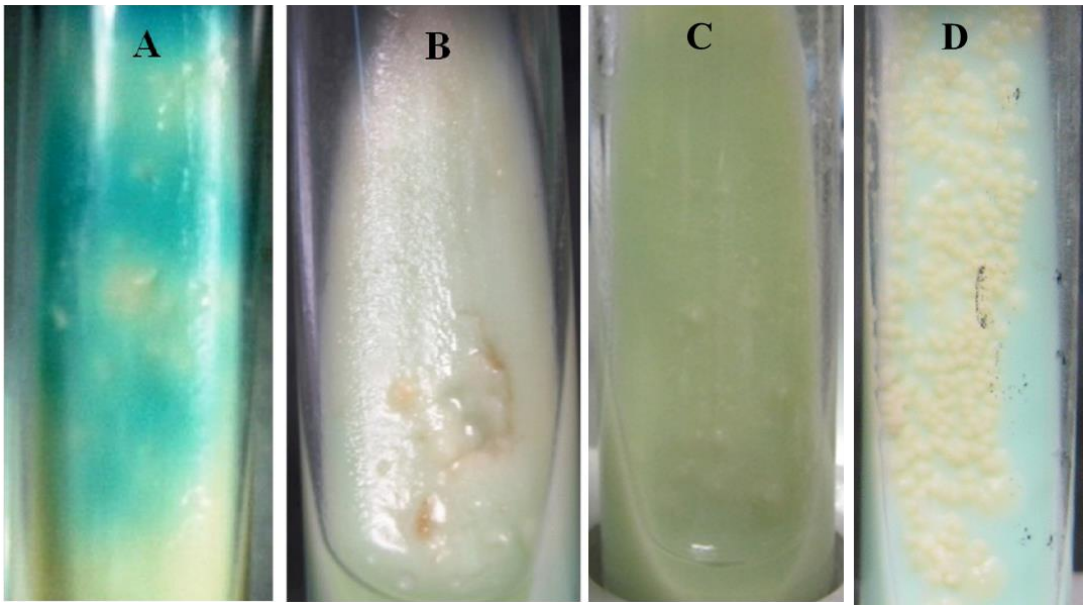


Figure 5. Culture of the deer isolate (A), the blue bull isolate (B), the rhinoceros isolate (C) and the *M. tuberculosis* isolate from a human (D) (28). The *M. tuberculosis* isolate from a human patient was randomly selected from the culture stock of *M. tuberculosis* at German Nepal Tuberculosis Project (GENETUP), Nepal to compare the culture morphology with the isolated *M. orygis*. All the cultures are on L-J medium in the same laboratory but at different times. The colonies of deer, blue bull and rhinoceros isolates were smooth and moist, whereas the culture of the *M. tuberculosis* isolate from human was rough and relatively dry.

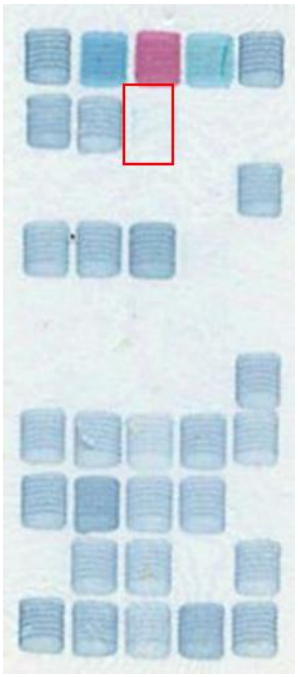


Figure 6. Photograph of hybridization pattern of spoligotying method. The red box denotes the very faint band in spacer 3 spot.

Table 2. Comparative analysis of the sequences of spacer 3

Species	Spacer 3 sequence
M. tuberculosis complex[a]	TCGCAAGCGCCGTGCTTCCAGTGATC**G**CCTT**C**TA
M. orygis of this study	TCGCAAGCGCCGTGCTTCCAGTGATC**A**CCTT**G**TA

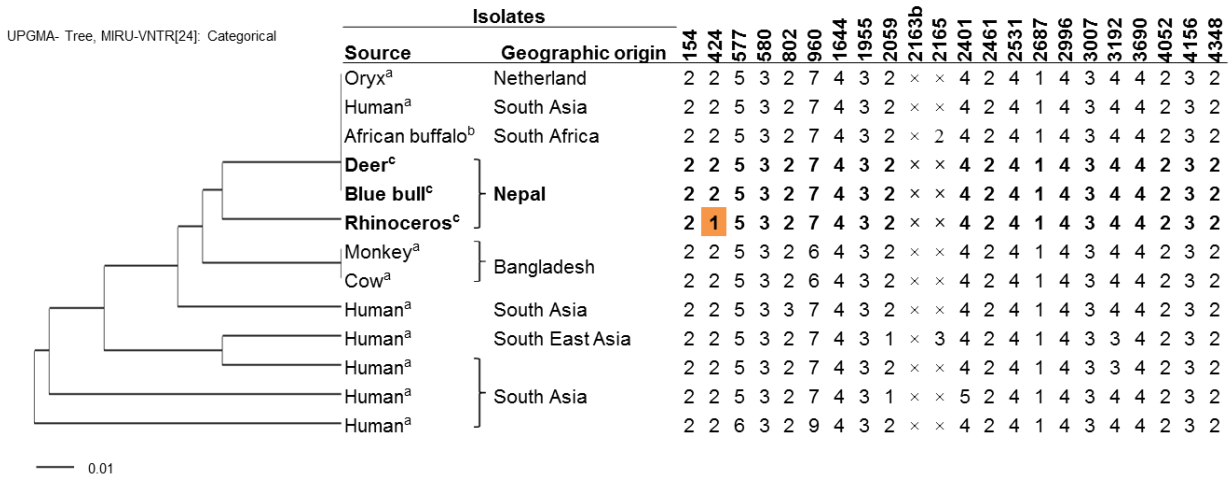
[a]Sequence information as reported in previous study (12, 31).

Bold and underline denotes SNPs.

Table 3. Summary of *M. orygis* differentiation from a selection of other MTBCs

MTBC	*cfp32* gene and RD analysis (17)			SNPs in following genes for MTBC species differentiation (10)						(32)
				gyrB		*mmpL6*	TbD1	*PPE55*		Rv2042c
	cfp32	RD9	RD12	1113	1450	551	171	2162	2163	38
M. tuberculosis H37Rv	P	P	P	G	G	C	C	T	C	T
M. bovis	P	N	N	G	T	G	C	T	C	T
M. orygis	P	N	N	A	T	G	G	G	T	G
Deer isolate	**P**	**N**	**N**	**A**	**T**	**G**	**G**	**G**	**T**	**G**
Blue bull isolate	**P**	**N**	**N**	**A**	**T**	**G**	**G**	**G**	**T**	**G**
Rhinoceros isolate	**P**	**N**	**N**	**A**	**T**	**G**	**G**	**G**	**T**	**G**

P: positive, the region is present; N: negative, the region is absent.



Isolates																								
Source	Geographic origin	154	424	577	580	802	960	1644	1955	2059	2163b	2165	2401	2461	2531	2687	2996	3007	3192	3690	4052	4156	4348
Oryx[a]	Netherland	2	2	5	3	2	7	4	3	2	×	×	4	2	4	1	4	3	4	4	2	3	2
Human[a]	South Asia	2	2	5	3	2	7	4	3	2	×	×	4	2	4	1	4	3	4	4	2	3	2
African buffalo[b]	South Africa	2	2	5	3	2	7	4	3	2	×	2	4	2	4	1	4	3	4	4	2	3	2
Deer[c]	**Nepal**	**2**	**2**	**5**	**3**	**2**	**7**	**4**	**3**	**2**	×	×	**4**	**2**	**4**	**1**	**4**	**3**	**4**	**4**	**2**	**3**	**2**
Blue bull[c]		**2**	**2**	**5**	**3**	**2**	**7**	**4**	**3**	**2**	×	×	**4**	**2**	**4**	**1**	**4**	**3**	**4**	**4**	**2**	**3**	**2**
Rhinoceros[c]		**2**	**1**	**5**	**3**	**2**	**7**	**4**	**3**	**2**	×	×	**4**	**2**	**4**	**1**	**4**	**3**	**4**	**4**	**2**	**3**	**2**
Monkey[a]	Bangladesh	2	2	5	3	2	6	4	3	2	×	×	4	2	4	1	4	3	4	4	2	3	2
Cow[a]		2	2	5	3	2	6	4	3	2	×	×	4	2	4	1	4	3	4	4	2	3	2
Human[a]	South Asia	2	2	5	3	3	7	4	3	2	×	×	4	2	4	1	4	3	4	4	2	3	2
Human[a]	South East Asia	2	2	5	3	2	7	4	3	1	×	3	4	2	4	1	4	3	3	4	2	3	2
Human[a]	South Asia	2	2	5	3	2	7	4	3	2	×	×	4	2	4	1	4	3	3	4	2	3	2
Human[a]		2	2	5	3	2	7	4	3	1	×	×	5	2	4	1	4	3	4	4	2	3	2
Human[a]		2	2	6	3	2	9	4	3	2	×	×	4	2	4	1	4	3	4	4	2	3	2

Figure 7. Unweighed pair group method with arithmetic mean (UPGMA) dendrogram showing phylogeny of *M. orygis* isolates based on the MLVA results of 22 loci. The dendogram is drawn using MIRU-VNTR*plus* free software (www.miru-vntrplus.org). The order of MLVA loci is as follows, left to right: 154, 424, 577, 580, 802, 960, 1644, 1955, 2059, 2163b, 2165, 2401, 2461, 2531, 2687, 2996, 3007, 3192, 3690, 4052, 4156 and 4348. Highlighted MLVA copy number of locus 424 in rhinoceros isolate indicates a single difference in MLVA type from the largest cluster.

[a]Isolates from a previous study (32)

[b]Isolate from a previous study (8)

[c]Isolate from this study.

x: unamplifiable.

Chapter II

Tuberculosis caused by *Mycobacterium orygis* in dairy cattle and captured monkeys in Bangladesh

Introduction

My previous study on molecular epidemiology of *M. orygis* isolates in Nepal identified distribution of *M. orygis* in Nepal and potential wide distribution in South Asia. Although *M. orygis* was reported from 2 cattle and a rhesus monkey in Bangladesh in a previous study (32), detailed description of the isolates was not reported. Additionally, an earlier report of *M. africanum* from four dairy cows was erroneous, and with refined analysis, these isolates were shown to be *M. orygis*. At that time, it had been reported that those isolates were *M. africanum* because of similar spoligotype SIT587 and a Hein blot (Genotype MTBC assays, Hain lifescience GmbH, Nehren, Germany) result that showed the same single nucleotide polymorphism (SNP) in $gyrB^{1450}$ (G to T) as seen in with *M. africanum* (21). However, these reports indicated potential wide distribution of *M. orygis* associated TB in Bangladesh which required further clarification. In this chapter, I describe the comprehensive analysis of *M. orygis* isolates from 18 cattle and 2 rhesus monkeys in Bangladesh. I have performed molecular analysis on these isolates and have provided evidences for potential endemic distribution of *M. orygis* in animals in Bangladesh and potential endemicity in South Asia, thus revealing a new scenario of tuberculosis in South Asia.

Materials and methods

In 2008 February, a rhesus monkey died with pneumonia in a zoo in Dhaka, the capital of Bangladesh. The lung had tuberculous lesions and acid-fast bacilli were observed under a Ziehl-Neelsen stain. Mycobacterial colonies were obtained after several weeks' incubation of the treated sample on the L-J medium. In July 2008, another rhesus monkey died with tuberculosis (TB) in the same zoo, and again a mycobacterial isolate was obtained. Those monkeys had been captured in the wild forest near Dhaka and kept in the zoo for several years.

In 2009-2010, a total of 21 cattle were diagnosed with TB after postmortem in a dairy farm in Dhaka. Most of the lesions were observed in the lung; however, one of the cattle presented disseminated TB and tubercular lesions were observed in the spleen and the liver as well. From 19 out of 21 cattle samples, mycobacterial colonies were grown on the L-J media and 18 out of them were suspected as MTBC by the Para-nitrobenzoic acid (PNB) test.

Colonies from both the monkey and cattle isolates were suspended in distilled water and the genomic DNA was extracted by boiling. Isolates were subjected to the PCR to determine the presence or absence of different genetic regions: *cfp32* (aka Rv0577, a gene present in all MTBC); and regions of difference RD1, RD4, RD9, and RD12 that show variable presence/absence across the MTBC (10). Similarly, multi locus sequence typing (MLST) of *gyrB*, *PPE55*, *mmpL6*, *TbD1* and *Rv2042c* loci was performed as previously described (10, 32). Also, Spoligotyping was carried out to determine polymorphism in the direct repeat (DR) locus as described (12) and the spoligo-international type (SIT) was determined using the international spoligotyping database, SpolDB4 (3) and SITVIT WEB database (http://www.pasteur-guadeloupe.fr:8081/SITVIT_ONLINE/). Additionally, I performed multi locus variable number of tandem repeat analysis (MLVA) as described (26). The obtained MLVA typing results along with previously reported data (32) was

used to construct an unweighted pair group method with an arithmetic mean (UPGMA) dendrogram using MIRU-VNTR*plus* (www.miru-vntrplus.org) software.

Results

The *cfp32* gene was found to be present in all isolates, while the RD9 and RD12 loci were deleted. Additional analyses showed that the isolates were RD4 positive, while the *gyrB, mmpL6, PPE55, TbD1* and *Rv2042c* sequences (Table 4) indicated that the isolates were all *M. orygis*. The spoligotyping results showed that all isolates were SIT 587, a typical pattern of *M. orygis* (32). Hence, the molecular typing identified the isolates were all *M. orygis*. Further discrimination of the isolates using MLVA analysis divided the 20 isolates into three clusters (Figure 8). The biggest cluster, MLVA-type 1, was comprised of 15 cattle isolates and 2 monkey isolates. One cattle isolate had a difference at one MLVA locus (locus 4156) and was designated MLVA-type 2, whereas 2 cattle isolates were MLVA-type 3 with differences in 3 loci (loci 424, 960 and 3192).

Discussion

This study is the first of its kind to describe a larger number of *M. orygis* isolates from a single geographical location. While spoligotyping revealed all the isolates to have an identical spoligotype pattern (SIT 587), MLVA analysis allowed differentiation of the isolates into three clusters (Figure 8). As all the cattle were born in the farm, the fact that the captive monkey isolates and the cattle isolates belonged to the same cluster suggested the spread of this *M. orygis* strain in this area. Also, the existence of other strains in the same herd having 1 or 3 MLVA loci different suggested both a wide distribution of *M. orygis* and an endemic presence of this MTBC subspecies in this region of Bangladesh.

Another point worth highlighting is that all the animal cases were of lung TB, suggesting the major transmission route of this bacterium is also air borne, as is the case with some other MTBC. This study also supports the suggestion that TB caused by *M. orygis* might have been maintained in the cattle population in Bangladesh. Thus, these findings of TB by *M. orygis* in animals from Bangladesh and the level of strain variation suggest the endemic presence of *M. orygis* amongst wild and domestic animals in Bangladesh. As the farm had a history of introduction of cattle from the UK (Friesian breed) and Pakistan (Sahiwal breed), it is possible that the different MLVA strain types might have been introduced by imported cattle. However, in the UK, the majority of bovine tuberculosis are caused by *M. bovis* (24), while in Pakistan, which is of course also located in South Asia, the reported number of bovine TB cases is still low and the situation there is yet to be fully explored.

My result from chapter I on the distribution of *M. orygis* from wild animals in Nepal, other reports of many human and animal *M. orygis* isolates from South Asia (32) and an Indian immigrant to New Zealand (5), along with my study reported here, provides further evidence for the endemic distribution of *M. orygis* across South Asia. While there are several studies of bovine TB in Bangladesh based on serological diagnosis (13), to the best of my knowledge there are no confirmed diagnoses of *M. bovis* from animals in Bangladesh. Hence, the work presented in this thesis on *M. orygis* presents an important addition to the origin of TB in animals in South Asia. TB in animals in South Asia caused by *M. orygis* may pre-date *M. bovis* if the latter were introduced only with the importation of European cattle and then subsequently expanded. As was suggested by the phylogenetic analysis of the whole-genome SNPs of MTBC (1), *M. orygis* and *M. bovis*, together with *M. caprae*, share a common ancestor that branches off from the other members of the MTBC. With the migration of modern humans 'Out of Africa' and the subsequent

global dissemination of MTBC members, it is possible that distinct MTBC subspecies may have been seeded into geographical areas at different rates. Hence, it is possible that *M. orygis* may have established in South Asia prior to *M. bovis*. This is one explanation for my results that suggests the endemic presence of *M. orygis* in animals in South Asia without the obvious presence of *M. bovis*. In a similar vein, it is suggested that global dissemination of distinct *M. bovis* molecular types was linked to cattle trading between countries, showing how *M. bovis* presence in diverse countries is strongly driven by trade linkages (24). The finding of different strains of *M. orygis* in wild animals in Nepal, with no confirmed diagnosis of *M. bovis* in Bangladesh, may support my hypothesis.

While the major host of *M. orygis* may be ruminants, including antelopes, increasing evidence of TB caused by *M. orygis* in other animals and humans has been accumulated. This suggests it might also have a wide host range like *M. bovis* and attest to the One Health significance of this organism. In the previous study with human TB mainly obtained in the Dhaka area of Bangladesh, all of the 300 isolates were identified as *M. tuberculosis* and no other MTBC species were detected (17). However, these 300 regional isolates might still be too small to ascertain the exact situation of zoonosis by *M. orygis* in human patients, and this question deserves further research.

To elucidate the ecology and epidemiology of *M. orygis* and to ascertain its impact as a zoonosis, detailed surveys that include MTBC species differentiation via genotyping should be carried out in both animal and human TB isolates especially in Bangladesh and other South Asian countries.

Summary

M. orygis was isolated from 18 cattle in a dairy farm and 2 captured rhesus monkeys in a zoo in Bangladesh. All the infected animals had tuberculosis lesions in their lungs, suggesting transmission and infection with *M. orygis* by an airborne route. The 20 isolates were analyzed using a range of conventional and molecular typing methods, and RD-deletion typing and sequencing of selected genes confirmed the isolates as *M. orygis*. The MLVA analysis allowed the isolates to be divided into three clusters based on the relatedness of their MLVA profiles. The 2 monkey isolates shared the same MLVA pattern with 15 of the cattle isolates, whereas the remaining three cattle isolates had different patterns, even though the latter animals had been kept in the same dairy farm. The diversity observed among isolates may suggest the bacteria have been established in this area for a long period. This study along with other recent findings that report the detection of *M. orygis* from animals as well as humans originating from South Asia potentially indicate endemic distribution of *M. orygis* in South Asia.

Table 4. Summary of *M. orygis* differentiation from a selection of other MTBCs

MTBCs	*cfp32* gene and RD analysis (10, 17)					SNPs in following genes for MTBC species differentiation (10)								Rv2042c (32)
						gyrB				*mmpL6*	TbD1	*PPE55*		
	cfp32	RD9	RD12	RD1	RD4	756	1113	1410	1450	551	171	2162	2163	38
M. tuberculosis H37Rv	P	P	P	P	P	G	G	C	G	C	C	T	C	T
M. bovis	P	N	N	P	N	A	G	T	T	G	C	T	C	T
M. bovis BCG	P	N	N	N	N	A	G	T	T	G	C	T	C	T
M. africanum	P	N	P	P	P	G	G	C	T	C	C	T	C	T
M. orygis	**P**	**N**	**N**	**P**	**P**	**G**	**A**	**C**	**T**	**G**	**G**	**G**	**T**	**G**
2 monkey isolates	**P**	**N**	**N**	**P**	**P**	**G**	**A**	**C**	**T**	**G**	**G**	**G**	**T**	**G**
18 cattle isolates	**P**	**N**	**N**	**P**	**P**	**G**	**A**	**C**	**T**	**G**	**G**	**G**	**T**	**G**

P: positive, the region is present; N: negative, the region is absent

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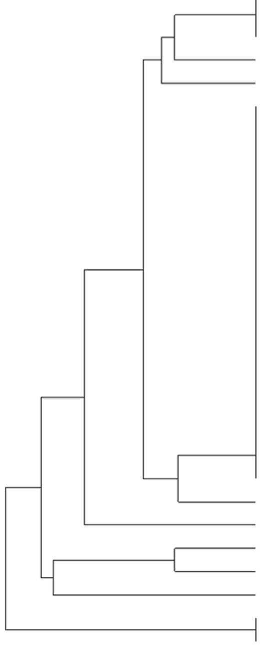

Source	Geographic origin	154	424	577	580	802	960	1644	1955	2059	2163b	2165	2401	2461	2531	2687	2996	3007	3192	3690	4052	4156	4348	
Oryx	Netherland	2	2	5	3	2	7	4	3	2	×	×	4	2	4	1	4	3	4	4	2	3	2	Common MLVA
Human	South Asia	2	2	5	3	2	7	4	3	2	×	×	4	2	4	1	4	3	4	4	2	3	2	
African buffalo	South Africa	2	2	5	3	2	7	4	3	2	×	2	4	2	4	1	4	3	4	4	2	3	2	
Deer	Nepal	2	2	5	3	2	7	4	3	2	×	×	4	2	4	1	4	3	4	4	2	3	2	
Blue bull		2	2	5	3	2	7	4	3	2	×	×	4	2	4	1	4	3	4	4	2	3	2	
Rhinoceros		2	1	5	3	2	7	4	3	2	×	×	4	2	4	1	4	3	4	4	2	3	2	
Human	South Asia	2	2	5	3	3	7	4	3	2	×	×	4	2	4	1	4	3	4	4	2	3	2	
Monkey	Bangladesh	2	2	5	3	2	6	4	3	2	×	×	4	2	4	1	4	3	4	4	2	3	2	MLVA-1
Monkey		2	2	5	3	2	6	4	3	2	×	×	4	2	4	1	4	3	4	4	2	3	2	
Cattle		2	2	5	3	2	6	4	3	2	×	4	4	2	4	1	4	3	4	4	2	3	2	
Cattle		2	2	5	3	2	6	4	3	2	×	4	4	2	4	1	4	3	4	4	2	3	2	
Cattle		2	2	5	3	2	6	4	3	2	×	4	4	2	4	1	4	3	4	4	2	3	2	
Cattle		2	2	5	3	2	6	4	3	2	×	4	4	2	4	1	4	3	4	4	2	3	2	
Cattle		2	2	5	3	2	6	4	3	2	×	×	4	2	4	1	4	3	4	4	2	3	2	
Cattle		2	2	5	3	2	6	4	3	2	×	×	4	2	4	1	4	3	4	4	2	3	2	
Cattle		2	2	5	3	2	6	4	3	2	×	×	4	2	4	1	4	3	4	4	2	3	2	
Cattle		2	2	5	3	2	6	4	3	2	×	4	4	2	4	1	4	3	4	4	2	3	2	
Cattle		2	2	5	3	2	6	4	3	2	×	×	4	2	4	1	4	3	4	4	2	3	2	
Cattle		2	2	5	3	2	6	4	3	2	×	4	4	2	4	1	4	3	4	4	2	3	2	
Cattle		2	2	5	3	2	6	4	3	2	×	4	4	2	4	1	4	3	4	4	2	3	2	
Cattle		2	2	5	3	2	6	4	3	2	×	×	4	2	4	1	4	3	4	4	2	3	2	
Cattle		2	2	5	3	2	6	4	3	2	×	4	4	2	4	1	4	3	4	4	2	3	2	
Cattle		2	2	5	3	2	6	4	3	2	×	4	4	2	4	1	4	3	4	4	2	3	2	
Cattle		2	2	5	3	2	6	4	3	2	×	×	4	2	4	1	4	3	4	4	2	3	2	
Cattle		2	2	5	3	2	6	4	3	2	×	4	4	2	4	1	4	3	4	4	2	**1**	2	→ MLVA-2
Human	South Asia	2	2	6	3	2	9	4	3	2	×	×	4	2	4	1	4	3	4	4	2	3	2	
Human	South East Asia	2	2	5	3	2	7	4	3	1	×	3	4	2	4	1	4	3	3	4	2	3	2	
Human	South Asia	2	2	5	3	2	7	4	3	2	×	×	4	2	4	1	4	3	3	4	2	3	2	
Human		2	2	5	3	2	7	4	3	1	×	×	5	2	4	1	4	3	4	4	2	3	2	
Cattle	Bangladesh	2	**1**	5	3	2	**8**	4	3	2	×	×	4	2	4	1	4	3	**3**	4	2	3	2	MLVA-3
Cattle		2	**1**	5	3	2	**8**	4	3	2	×	4	4	2	4	1	4	3	**3**	4	2	3	2	

Figure 8. UPGMA dendrogram of *M. orygis* isolates based on MLVA analysis. The order of 22 MLVA loci are as follows, left to right: 154, 424, 577, 580, 802, 960, 1644, 1955, 2059, 2163b, 2165, 2401, 2461, 2531, 2687, 2996, 3007, 3192, 3690, 4052, 4156 and 4348.

[a~d] Isolates of previous study; [a] (32), [b] (8)), [c-d] Chapter I (28-29)

[e] Isolates of this study.

x: unamplifiable.

CONCLUSION

This thesis studies genetic characteristics of recently recognized subspecies of MTBC, *M. orygis* isolated from different animals in Nepal and Bangladesh. I have tried to understand the molecular epidemiology of these *M. orygis* isolates. My study that consists of 2 chapters, describes my TB suspected sample collection strategies, microbiological processing of samples and genotyping of TB isolates by TB genotyping tools like spoligotyping, MLVA analysis, RD analysis and MLST.

In chapter I, I have described the molecular characterization of *M. orygis* isolates from wild animals of Nepal. I identified that *M. orygis* associated TB was the cause of death of a spotted deer, a blue bull and a free-ranging rhinoceros. All the 3 *M. orygis* isolates had identical spoligptye pattern of SIT 587. Identical MLVA pattern of *M. orygis* isolates from spotted deer and blue bull suggested possible transmission of TB. The different strain type of *M. orygis* in rhinoceros suggested possibility of different strains of *M. orygis* in CNP. This study scientifically demonstrates that *M. orygis* associated TB as an emerging threat to the conservation of wild animals in Nepal.

In chapter II, I have identified *M. orygis* associated TB was the cause of deaths in 18 dairy cattle and 2 captured rhesus monkeys. TB suspected samples were collected from these animals and I performed standard laboratory and genotyping procedure to isolate and study genetic characteristics of all the collected *M. orygis* isolates. As was in chapter I, all the 20 *M. orygis* isolates had identical spoligotyping pattern SIT 587 but had 3 different MLVA patterns. The diversity observed among isolates may suggest the bacteria have been established in this area for a long period.

My study described in chapter I and II discusses molecular epidemiology of *M. orygis* isolates in different animals in Nepal and Bangladesh. I found that there were 5 MLVA strain types of *M. orygis* circulating in different animals in different geographic location (Figure 9).

Because of previous reports of different *M. orygis* isolates from animals and humans from South Asia (Figure 9), I report wide genetic diversity of *M. orygis* in South Asia region. I suggest that *M. orygis* may be endemically distributed in the region. I further hypothesize that *M. orygis* may have established in South Asia prior to *M. bovis* and believe that this hypothesis can be of the active areas of research to understand evolutionary history of MTBC. Finally, I believe that I will be able to understand more about TB bacteria by studying in detail about biology and epidemiology of *M. orygis*.

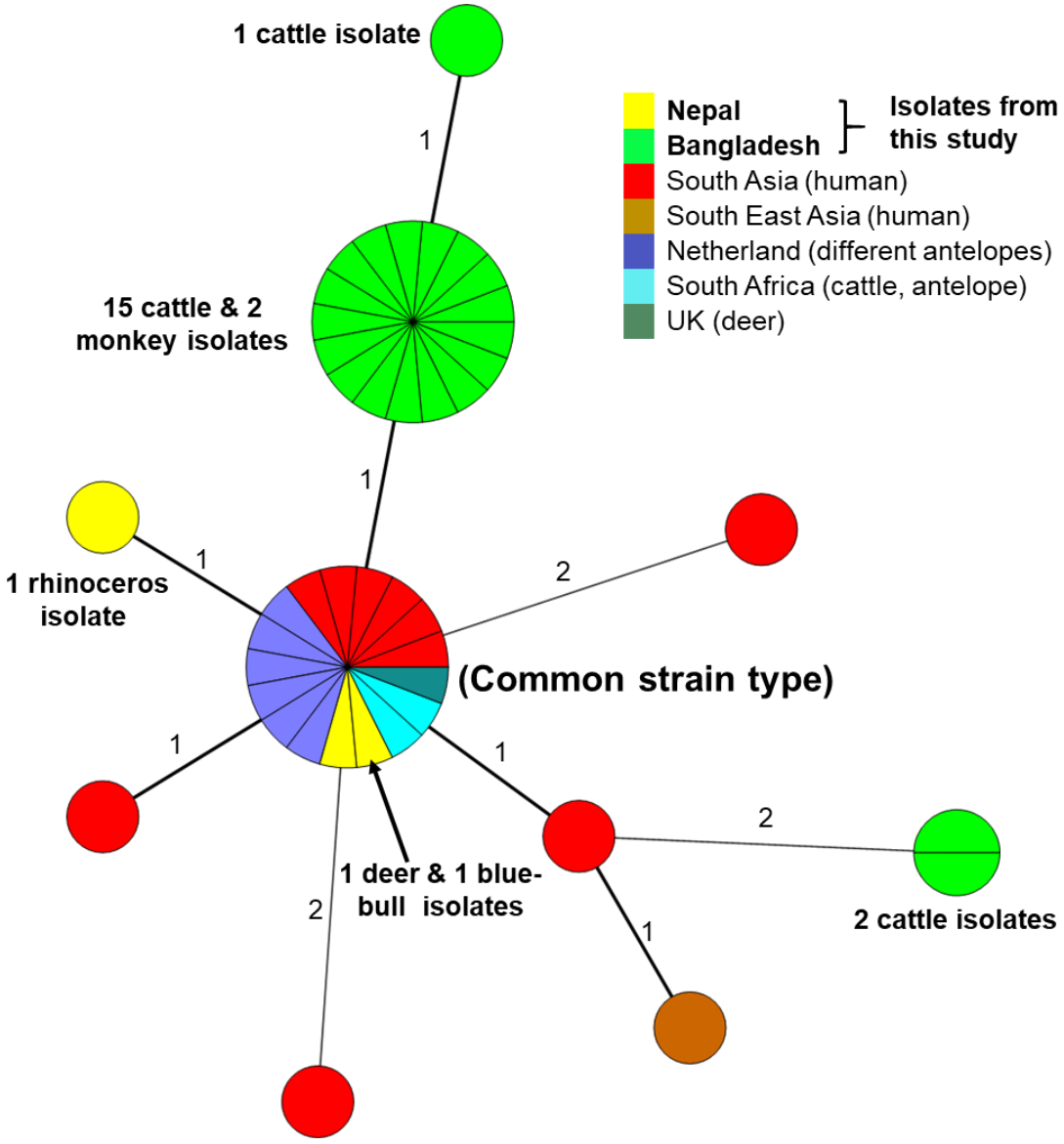

Figure 9. Minimum spanning tree (MST) of *M. orygis* isolates based on MLVA analysis of 20 MLVA loci: 154, 424, 577, 580, 802, 960, 1644, 1955, 2059, 2401, 2461, 2531, 2687, 2996, 3007, 3192, 3690, 4052, 4156 and 4348. The MST tree was constructed with BioNumerics ver. 6.6. The *M. orygis* isolates denoted in yellow and green color were obtained from this study and other isolates with different color are from previous studies (8, 32). The connecting lines indicate genetic distance and number on these lines indicate number of allelic difference in MLVA loci.

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REFERENCES

1. Bos KI, Harkins KM, Herbig A, Coscolla M, Weber M, Comas I, Forrest SA, Bryant JM, Harris SR, Schuenemann VJ, Campbell TJ, Majander K, Wilbur AK, Guichon RA, Steadman DLW, Cook DC, Niemann S, Behr MA, Zumarraga M, Bastida R, Huson D, Nieselt K, Young D, Parkhill J, Buikstra JE, Gagneux S, Stone AC, Krause J. Pre-Columbian mycobacterial genomes reveal seals as a source of New World human tuberculosis. Nature 514, 494-497, 2014.
2. Brosch R, Gordon SV, Marmiesse M, Brodin P, Buchrieser C, Eiglmeier K, Garnier T, Gutierrez C, Hewinson G, Kremer K, Parsons LM, Pym AS, Samper S, van Soolingen D, Cole ST. A new evolutionary scenario for the *Mycobacterium tuberculosis* complex. Proc Natl Acad Sci USA 19, 3684-3689, 2002.
3. Brudey K, Driscoll JR, Rigouts L, Prodinger WM, Gori A, Al-Hajoj SA, Allix C, Aristimuño L, Arora J, Baumanis V, Binder L, Cafrune P, Cataldi A, Cheong S, Diel R, Ellermeier C, Evans JT, Fauville-Dufaux M, Ferdinand S, Garcia de Viedma D, Garzelli C, Gazzola L, Gomes HM, Guttierez MC, Hawkey PM, van Helden PD, Kadival GV, Kreiswirth BN, Kremer K, Kubin M, Kulkarni SP, Liens B, Lillebaek T, Ho ML, Martin C, Martin C, Mokrousov I, Narvskaïa O, Ngeow YF, Naumann L, Niemann S, Parwati I, Rahim Z, Rasolofo-Razanamparany V, Rasolonavalona T, Rossetti ML, Rüsch-Gerdes S, Sajduda A, Samper S, Shemyakin IG, Singh UB, Somoskovi A, Skuce RA, van Soolingen D, Streicher EM, Suffys PN, Tortoli E, Tracevska T, Vincent V, Victor TC, Warren RM, Yap SF, Zaman K, Portaels F, Rastogi N, Sola C. *Mycobacterium tuberculosis* complex genetic diversity: mining the fourth international spoligotyping database (SpolDB4) for classification, population genetics and epidemiology. BMC Microbiol 6, 23, 2006.
4. Convention on International Trade in Endangered Species. Appendices. [Cited November 22 2015]. https://www.cites.org/eng/app/appendices.php

5. Dawson KL, Bell A, Kawakami RP, Coley K, Yates G, Collins DM. Transmission of *Mycobacteium orygis* (*M. tuberculosis* complex species) from a tuberculosis patient to a dairy cow in New Zealand. J Clin Microbiol 50, 3136-31388, 2012.
6. Department of National Park and Wildlife Conservatio. Protected species (mammals). [Cited Oct 30 2015]. http://www.dnpwc.gov.np/protected_species/species/mammals
7. Durr S, Muller B, Alonso S, Hattendorf J, Laisse CJM, van Helden PD, Zinsstag J. Differences in primary sites of infection between zoonotic and human tuberculosis: results from a worldwide systematic review. Plos Negl Trop Dis 7(8), e2399, 2013.
8. Gey van Pittius NC, Perrett KD, Michel AL, Keet DF, Hlokwe T, Streicher EM, Warren RM, van Helden PD. Infection of African buffalo (*Syncerus caffer*) by oryx bacillus, a rare member of the antelope clade of the *Mycobacterium tuberculosis* complex. J Wildl Dis 48, 849-857, 2012.
9. Groothuis DG, Yates MD. Decontamination, microscopy and isolation. In: Diagnostic and Public Health Mycobacteriology, 2nd ed., Groothuis DG, Yates MD. eds, Bureau of Hygiene and Tropical Diseases, European Society for Mycobacteriology, London, pp. 63, 1991.
10. Huard RC, Fabre M, de Hass P, Oliveir Lazzarini LC, van Soolingen D, Cousins D, Ho JL. Novel genetic polymorphisms that further delineate the phylogeny of the *Mycobacterium tuberculosis* complex. J Bacteriol 188, 4271-4287, 2006.
11. Jha VC, Morita Y, Dhakal M, Besnet B, Sato T, Nagai A, Kato M, Kozawa K, Yamamoto S, Kimura H. Isolation of *Mycobacterium* spp. from milking buffaloes and cattle in Nepal. J Vet Med Sci 69, 819-825, 2007.
12. Kamerbeek J, Schouls L, Kolk A, van Agterveld M, van Soolingen D, Kuijper S, Bunschoten A, Molhuizen H, Shaw R, Goyal M, van Embden J. Simultaneous detection

and strain differentiation of *Mycobacterium tuberculosis* for diagnosis and epidemiology. J Clin. Microbiol 35, 907-914, 1997.

13. Mahmud MAA, Belal SMSH, Shoshe NZ. Prevalence of bovine tuberculosis in cattle in the selected Upazila of Sirajganj district in Bangladesh. Bangl J Vet Med 12, 141-145, 2014.
14. Malla B, Stucki D, Borrell S, Feldmann J, Maharjan B, Shrestha B, Fenner L, Gagneux S. First Insights into the Phylogenetic Diversity of *Mycobacterium tuberculosis* in Nepal. PLoS One 7, e52297, 2012.
15. Mikota SK, Gairhe K, Giri K, Hamilton K, Miller M, Paudel S, Lyashchenko K, Larsen RS, Payeur JB, Waters WR, Greenwald R, Dumonceaux G, Vincent B, Kaufman GE. Tuberculosis surveillance of elephants (*Elephas maximus*) in Nepal at the captive-wild interface. Eur J Wildl Res 61,221–229, 2015.
16. Mostowy S, Inwald J, Gordon S, Martin C, Warren R, Kremer K, Cousins D, Behr MA. Revisiting the evolution of *Mycobacterium bovis*. J Bacteriol 187, 6386-6395, 2005.
17. Nakajima C, Rahim Z, Fukushima Y, Sugawara I, van der Zanden AGM, Tamaru A, Suzuki Y. Identification of *Mycobacterium tuberculosis* clinical isolates in Bangladesh by a species distinguishable multiplex PCR. BMC Infect Dis 10, 118, 2010.
18. NTNC. National rhino count 2015: 645 individual rhinos in Nepal. [Cited Oct 30 2015]. http://www.ntnc.org.np/news/national-rhino-count-2015-645-individual-rhinos-nepal.
19. Paudel S, Mikota SK, Nakajima C, Gairhe KP, Maharjan B, Thapa J, Poudel A, Shimozuru M, Suzuki Y, Tsubota T. Molecular characterization of *Mycobacterium tuberculosis* isolates from elephants of Nepal. Tuberculosis 94, 287-292, 2014.
20. Poudel A, Nakajima C, Fukushima Y, Suzuki H, Pandey BD, Maharjan B, Suzuki Y. Molecular characterization of multidrug-resistant *Mycobacterium tuberculosis* isolated in Nepal. Antimicrob Agents Chemother 56, 2831-2836, 2012.

21. Rahim Z, Mollers M, te Koppele-Vije A, de Beer J, Zaman K, Matin MA, Kamal M, Raquib R, van Soolingen D, Baqi MA, Heilmann FGC, van der Zanden AGM. Characterization of *Mycobacterium africanum* subtype I among cows in a dairy farm in Bangladesh using spoligotyping. Southeast Asian J Trop Med Public Health 38, 706-713, 2007.
22. Rahim Z, Thapa J, Fukushima Y, Suzuki H, van der Zanden AGM, Gordon SV, Suzuki Y, Nakajima C. Tuberculosis caused by *Mycobacterium orygis* in dairy cattle and captured monkeys in Bangladesh: a new scenario of tuberculosis in South Asia. Transbound Emerg Dis In press.
23. Smith NH, Kremer K, Inwald J, Dale J, Driscoll JR, Gordon SV, van Soolingen D, Hewinson RG, Smith JM. Ecotypes of the *Mycobacterium tuberculosis* complex. J Theor Biol 239, 220-225, 2006.
24. Smith N H, Berg S, Dale J, Allen A, Rodriguez S, Romero B, Matos F, Ghebremichael S, Karoui C, Donati C, Machado Ada C, Mucavele C, Kazwala RR, Hilty M, Cadmus S, Ngandolo BN, Habtamu M, Oloya J, Muller A, Milian-Suazo F, Andrievskaia O, Projahn M, Barandiar S, Maccus A, Muller B, Zanini MS, Ikuta CY, Rodriguez CA, Pinheiro SR, Figueroa A, Cho SN, Mosavari N, Chuang PC, Jou R, Zinsstag J, van Soolingen D, Costello E, Aseffa A, Proa~no-Perez F, Portaels F, Rigouts L, Cataldi AA, Collins DM, Boschiroli ML, Hewinson RG, Ferreira Neto JS, Surujballi O, Tadyon K, Botelho A, Zarraga AM, Buller N, Skuce R, Michel A, Aranaz A, Gordon SV, Jeon BY, Kallenius G, Niemann S, Boniotti MB, van Helden PD, Harris B, Zumarraga MJ, Kremer K. European 1: a globally important clonal complex ofMycobacterium bovis. Infect Genet Evol 11, 1340–1351, 2011.
25. Subedi N, Jnawali SR, Dhakal M, Pradhan NMB, Lamichhane BR, Malla S, Amin R, Jhala YV. Population status, structure and distribution of the greater one-horned rhinoceros Rhinoceros unicornis in Nepal. Oryx 47, 352-360, 2013.

26. Supply P, Mazars E, Lesjean S, Vincent V, Gicquel B, Locht C. Variable human minisatellite-like regions in the *Mycobacterium tuberculosis* genome. Mol Microbiol 36, 762-771, 2000.

27. Talukdar BK, Emslie R, Bist SS, Choudhury A, Ellis S, Bonal BS, Malakar MC, Talukdar BN, Barua M. *Rhinoceros unicornis*. The IUCN Red List of Threatened Species 2008. e.T19496A8928657, 2008. http://dx.doi.org/10.2305/IUCN.UK.2008.RLTS.T19496A8928657.en.

28. Thapa J, Nakajima C, Maharjan B, Poudel A, Suzuki Y. Molecular characterization of *Mycobacterium orygis* isolates from wild animals of Nepal. Jpn J Vet Res 63(3), 151-158, 2015.

29. Thapa J, Paudel S, Sadaula A, Shah Y, Maharjan B, Kaufman GE, McCauley D, Gairhe KP, Tsubota T, Suzuki Y, Nakajima C. *Mycobacterium orygis*–associated tuberculosis in free-ranging rhinoceros, Nepal, 2015. Emerg Infect Dis 22 (3), 570-572, 2016.

30. Thapa J, Nakajima C, Gairhe KP, Maharjan B, Paudel S, Shah Y, Mikota S, Kaufman GE, McCauley D, Tsubota T, Gordon SV, Suzuki Y. 2017. Wildlife Tuberculosis: An Emerging Threat for Conservation in South Asia. In: Global Exposition of Wildlife Management, Lameed GA. ed. InTech, Crotia. pp. 73-90, 2017. Available from: https://www.intechopen.com/books/global-exposition-of-wildlife-management/wildlife-tuberculosis-an-emerging-threat-for-conservation-in-south-asia.

31. van Embden JDA, van Gorkom T, Kremer K, Jansen R, van der Zeijst BAM, Schouls LM. Genetic variation and evolutionary origin of the direct repeat locus *of Mycobacterium tuberculosis* complex bacteria. J Bacteriol 182, 2393-2401, 2000.

32. van Ingen J, Rahim Z, Mulder A, Boeree MJ, Simeone R, Brosch R, van Soolingen D. Characterization of *Mycobacterium orygis* as *M. tuberculosis* complex subspecies. Emerg Infect Dis 18, 653-655, 2012.
33. van Soolingen D, de Hass PE, Haagsma J, Eger T, Hermans PWM, Ritacco V, Alito A, van Embden JDA. Use of various genetic markers in differentiation of *Mycobacterium bovis* strains from animals and humans and for studying epidemiology of bovine tuberculosis. J. Clin. Microbiol 32, 2425-2433, 1994.
34. Wilbur AK, Engel GA, Rompis A, Putra IGAA, Lee BPYH, Aggimarangsee N, Chalise M, Shaw E, Oh G, Schillaci MA, Jones-Engel J. From the mouths of monkeys: detection of *Mycobacterium tuberculosis* complex DNA from buccal swabs of synanthropic Macaques. Am J Primatol 74, 676-686, 2012.
35. World Health Organization. 2016. Tuberculosis Profile of Nepal. [Cited May 2017]. https://extranet.who.int/sree/Reports?op=Replet&name=%2FWHO_HQ_Reports%2FG2%2FPROD%2FEXT%2FTBCountryProfile&ISO2=NP&LAN=EN&outtype=html
36. World Wildlife Fund. Greater one horned rhino. 2016. [Cited Oct 30 2015]. http://http://wwf.panda.org/what_we_do/endangered_species/rhinoceros/asian_rhinos/indian_rhinoceros/.