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

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SUMMARY

Mycobacterium tuberculosis was cultured from the lung tissues of 3 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 3 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 2 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.

Key words: Asian elephants, *Mycobacterium tuberculosis*, Multi-Locus Variable Number Tandem Repeat Analysis (MLVA), Spoligotyping

INTRODUCTION

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,[1,2,3,4,5] 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.[1,2] Postmortem lesions typically include granulomatous nodules in the lungs and bronchial lymph nodes sometimes with caseous foci. In the advanced stage of the disease, extensive caseocalcareous and cavitating lesions may be observed throughout the entire lung with enlarged bronchial and thoracic lymph nodes.[1]

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.[6] TB was first identified in the Nepalese captive elephant population in 2002. 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.[7] Nepal is a country with a high burden of TB in humans.[8] Since captive elephants are in close contact with humans, 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.[9] Exposure to infected elephants has resulted in transmission of TB to humans as evidenced by tuberculin skin test conversions[10,11,12] or active disease.[13] 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.[14,15] In the current study, we performed genotyping on three *M. tuberculosis* isolates obtained from 3 captive elephants and compared them with human isolates in Nepal.

MATERIALS AND METHODS

Study isolates

Elephant isolates:

M. tuberculosis isolates from 3 elephants were included in the study. All 3 elephants were owned by the Government of Nepal and kept in 2 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 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 3 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 1) when she was about 34 years. She was suspected to be suffering from TB and was in permanent segregation for almost 2 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 6 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 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 4 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 7 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, 4 were from Kathmandu, 1 from Butwal and 1 from Birgunj (Figure 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.[16]

Necropsy

All 3 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 [17] 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.[18] 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 8 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°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[19] and was confirmed by a *gyrB* sequence analysis.[20] The spoligotype was determined as previously described.[21] 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).[22] 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.[20] Multi-locus variable number of tandem repeat analysis (MLVA)[23] was performed as described [24] 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.[16,24] 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 testing

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.[19,20] 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[22,25] 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.[22] 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 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 2)[26]. In TbD1 detection PCR, all 3 samples were positive and determined as ancestral type of *M. tuberculosis*.[20] 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[16] (Table 1, Figure 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 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.[27]

The diagnosis of TB by culture is considered the gold standard; however, it has very poor sensitivity, especially for ante-mortem diagnosis in elephants.[28,29,30] 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.[15] In another study, only 58% of elephants with confirmed TB infection at necropsy had positive isolations from trunk wash samples.[28] All of the trunk wash samples of Elephant A were negative on culture in the current study.

Our findings demonstrated that these 3 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.[22,25] This lineage is predominantly observed in Indo-Oceanic areas like south India or the Philippines;[31,32] however, its prevalence in Nepal is relatively low. In the recent report,[25] the prevalence of Indo-Oceanic lineage was 11.5% in Nepal and our observation results were also very similar (unpublished data). Spoligotypes of 2 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.[21]

One of the isolate Elp-A had a new synonymous SNP in its *gyrA* sequence, T231C, and the same SNP was found in 2 human isolates from Kathmandu, the capital city of Nepal. Both of the human isolates had spoligotype SIT138 categorized as EAI5,[22] which is the most frequently observed EAI type in this country.[25] 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 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.[20] 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 1, Figure 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 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*.[32] 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*.[15] 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 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 1 and

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 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 [31] 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.

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Figure legends

Figure 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 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 given according to their position in the DR region (ref. 26). *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 rearrangement position are shown in the balloons. DVR50, 67 and 68 had the same mutations as ref. 26 (shown in shadowed rectangles).

Figure 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.