Epidemiology and molecular characterization of *Mycobacterium tuberculosis* including a drug-resistant strain associated with mortality of Asian elephants in Nepal 2019-2022.

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Abstract

Tuberculosis (TB) is an emerging threat to the survival of elephants in Nepal. We investigated the lung tissue samples from nine elephants that died from 2019 to 2022 in Nepal using culture, conventional PCR, and loop-mediated isothermal amplification (LAMP) and then

performed genotyping of five PCR-positive isolates to understand the possible transmission dynamics of *Mycobacterium tuberculosis* (*Mtb*). Results showed that two-thirds (6/9) of elephants were confirmed to be infected from *Mtb* by LAMP, 5/9 by PCR, and 4/9 by culture. Genotyping of *Mtb* isolates showed that elephants were infected with the Indo-Oceanic and Beijing lineages including an isoniazid-resistant Beijing lineage. MIRU-VNTR-based phylogeny, *gyrA,* and *katG* sequencing showed the possibility of ongoing transmission of Indo-Oceanic lineages and likely transmission of the drug-resistant Beijing lineage from human to elephant. Implementation of comprehensive surveillance and preventive measures are urgently needed to address this zoonotic disease and protect elephants from TB in Nepal.

Keywords: Elephant, Tuberculosis, Nepal, Genotyping, Transmission dynamics.

1. Introduction

Asian elephants (*Elephas maximus*), with an estimated population of only 48,000 to 51,000 [1] in the wild are an endangered species due to a drastic decline in population and habitat loss [2]. In Nepal, the current population of elephants is approximately 200 in captivity and 227 in the wild [3,4]. Besides habitat encroachment and fragmentation, human-elephant conflict, and poaching [3,5], infectious diseases such as tuberculosis (TB) are also becoming a threat to elephants [6–9].

TB in Asian elephants is mainly caused by *Mycobacterium tuberculosis* (*Mtb*) (6). Though many elephants do not show clinical signs, some may exhibit chronic weight loss, weakness, exercise intolerance, and anorexia [6]. TB in Asian elephants has been reported in several elephant range countries including Nepal, India, Thailand, Sri Lanka, and Malaysia, and

captive facilities outside Asia, such as in Switzerland, Sweden, and the USA [6,7,9–17]. TB has also been reported in wild elephants in India and Sri Lanka [13,15,18], suggesting an emerging conservation threat to this endangered species. Furthermore, earlier studies have documented the possibility of elephant-to-elephant [17] and elephant-to-human [19] transmission of *Mtb.*

Previous studies have shown that 13% to 22% of the captive elephants in Nepal were seropositive for TB when tested using the Elephant TB STAT-PAK® or DPP VetTB® (Chembio Inc., USA) assays [7,9]. *Mtb* was found to be the causative agent of TB in captive elephants of Nepal and the source of infection was suggested to be from humans who had close contact with elephants [6,20]. Different lineages of *Mtb*, such as Indo-Oceanic, Beijing, and CAS-Delhi have been isolated from Nepal elephants, with the Indo-Oceanic lineage as the dominant lineage [6,20,21].

The threat posed by TB to the elephant population led the Nepal government to approve the Elephant Tuberculosis Control and Management Action Plan (2011-2015), which recommended regular surveillance and treatment of the disease [22]. Despite this plan, which was never fully implemented, captive elephants in Nepal have continued to die from suspected or confirmed TB. Since *Mtb*-associated TB is endemic in this population, we conducted this study to investigate recent elephant deaths in Nepal to determine whether TB was a factor in these deaths.

All previous studies on the molecular epidemiology of *Mtb* among captive elephants of Nepal involved isolation of bacteria from samples collected during necropsy from TB suspects [6,21]; however, this method has many limitations, including access to a specialized laboratory, slow turn-around time, and logistic issues of storage and transportation of samples [23–25]. Additionally, TB in elephants can be a paucibacillary disease [26]. In this study, we analyzed

postmortem lung tissue samples from nine elephants, conducted culture, conventional PCR, and LAMP-based molecular detection for TB, and genotyped *Mtb* isolates obtained from five elephants. We aimed to determine if this combination of methods could enhance our ability to diagnose TB in elephants and provide insights regarding transmission dynamics. [27,28].

2. Methods

2.1 Workflow

The methodology workflow is summarized in Figure 1, outlining the sequential research procedures in this study.

2.2 Study elephants and sample collection

A total of nine elephants that died during the study period (2019-2022) were assessed for TB and the GPS locations of the deceased elephants were plotted (Figure 2). Most of the elephant deaths occurred at elephant stables either in the buffer zone or inside the core area of the Chitwan National Park (CNP). We recorded the sex and year of death of each elephant. The exact age of the elephants was not known although all were adults.

Eight elephants were owned by the government wildlife authority and were used for conservation purposes such as patrolling protected areas or for tourism where they have close interaction with humans, other elephants, and other wildlife species. Each government elephant is cared for by three elephant handlers. Most of the captive elephants are stationed at the Elephant Breeding Centre and Elephant Stables in Sauraha, in the eastern section of CNP which is also a hotspot for tourist activities that can include human-elephant interaction. One elephant was privately owned and mainly involved in tourist activities like elephant-back safaris or

elephant-bathing. These types of tourist activities provide opportunities for elephant-human interactions as well as elephant-elephant interactions.

2.3 Serological testing

Longitudinal serological testing for TB in elephants in Nepal has been conducted by one of the authors (Mikota, unpublished) using the DPP VetTB Assay or the Elephant STAT-PAK test (Chembio Diagnostic Inc, Medford, NY, USA Inc., USA).

Elephants 1, 2, 3, 5, 6, and 8 were serologically reactive on one or more tests; elephants 4,7, and 9 were serologically non-reactive. Elephant 3 was the only elephant that was treated for TB, and she became serologically non-reactive post-treatment.

2.4 Necropsy

Post-mortem examinations were performed on all nine elephants and TB suspect lesions were collected as recommended [6,29] and stored at -20°C until further analyses. According to the postmortem protocol, only lung tissue samples with suspected TB lesions, such as granulomas, were collected. Samples were collected through a window in the diaphragm rather than completely opening the chest cavity, to prevent the aerosolization of bacteria into the environment and to protect the personnel involved in the necropsy.

The presence or absence of TB-suspect lung lesions was recorded. Representative lung samples with granulomas were collected for further examination.

2.5 Sample processing and culture

The tissue samples were processed for culture according to the guidelines of the European Society of Microbiology [6,30] in the laboratory of the Nepal Anti-Tuberculosis Association, German Nepal TB Project (NATA-GENETUP), Kathmandu. The resulting sediment after decontamination was used for DNA extraction and culture. 100 µl of the sediment was inoculated into L-J media and incubated at 37°C for 8 weeks.

2.6 DNA extraction of the samples

We extracted DNA using three methods. Firstly, DNA was directly extracted from all nine preserved samples. Approximately 25 mg of the tissue sample was minced into small pieces using a sterile surgical blade and DNA extraction was performed using the Qiagen DNeasy Blood & tissue kit (Qiagen, Venlo, Netherlands) following the manufacturer's protocol with slight modification [31]. Secondly, 200 µl of the concentrated sediment deposit prepared for culture examination of all nine samples was used for DNA extraction using the same protocol as above [31]. In the third method, DNA was extracted from the positive colonies after culturing the tissue samples. Four elephant tissue samples yielded pure positive colonies, which were used for DNA extraction. DNA was extracted from four positive colonies using the GenoLyse® VER 1.0 extraction kit (Hain Lifescience GmbH, Nehren, Germany), according to the manufacturer's protocol [32].

2.7 Drug Susceptibility Test (DST)

Both genotypic and phenotypic methods were used for DST. The Genotype MTBDRplus VER 2.0-line probe assay (Hain Lifescience GmbH, Nehren, Germany) was performed using the DNA samples extracted from the culture-positive colonies, following the manufacturer's

protocol [33,34], for both first-line (rifampicin, isoniazid, streptomycin, and ethambutol) and second-line drugs (amikacin, clofazimine, levofloxacin, linezolid, and moxifloxacin). For phenotypic DST, positive L-J culture colonies were grown in Mycobacteria Growth Indicator Tube (BACTEC MGIT) liquid medium (Becton Dickinson, New Jersey, USA) as described in the World Health Organization technical manual [35]. To determine the drug resistance in the contaminated sample from Elephant 1, we used DNA that was directly extracted from unprocessed tissue samples to analyze drug resistance-associated mutations in the encoding region of *rpoB*, *katG*, and promoter region of *inhA*, using primers, PCR condition, and sequencing, as previously described [36].

2.8 Loop-mediated isothermal amplification (LAMP) and Conventional Polymerase chain reaction (PCR) for *Mtb* complex

We performed the LAMP (Eiken Chemical Company Ltd, Tokyo, Japan) using DNA extracted directly from the unprocessed lung tissue samples, as well as sediments of culture-processed tissue samples. We used a previously described LAMP system targeting the *Mtb* complex 16S rRNA gene [28,37]. Positive and negative controls were *Mycobacterium bovis* BCG Tokyo 172 genomic DNA and double distilled water (DDW), respectively. Positive results were indicated by turbidity greater than 0.1 (threshold value) in a real-time turbidimeter (LA-200, Teramecs Co. Ltd, Japan) [27,28], and visual observation of color change between positive and negative reaction by colori-fluorometric indicator (CFI) [27]. *Mtb* complex-specific PCR that targets *IS6110* [38] was performed to identify the presence or absence of *Mtb* complex DNA in the samples.

1702.9 Genotyping of *Mtb* isolates

171 We performed spoligotyping, multi-locus variable number of tandem repeat analysis
172 (MIRU-VNTR), large sequence polymorphism (LSP), PCR, and sequence typing on the elephant
173 samples that were culture positive (N=4) and the direct tissue sample (N=1). Spoligotyping was
174 performed as described [39]. Briefly, we amplified the direct repeat region with a pair of primers
175 and hybridized the resulting PCR products to a set of 43 oligonucleotide probes corresponding to
176 each distinct spacer sequence. The hybridized membrane was then visualized to determine the
177 spoligotype pattern. After entering these spoligotype patterns in the SITVITWEB database
178 (http://www.pasteur-guadeloupe.fr:8081/SITVIT_ONLINE), we obtained the spoligo-
179 international type (SIT) number and lineage of *Mtb*.

180 MIRU-VNTR was conducted using the following 24 loci: MIRU2, VN424, ETR-C,
181 MIRU4, MIRU40, MIRU10, MIRU16, VN1955, MIRU20, QUB11b, ETR-A, QUB3232,
182 VN2401, ETR-B, MIRU23, MIRU24, MIRU26, MIRU27, QUB11a, MIRU31, VN3690,
183 QUB26, VN4156, and MIRU39, as recommended [40]. The spoligotype and MIRU-VNTR
184 profile of each isolate from this study and other comparative studies [6] was used to construct a
185 UPGMA-based phylogenetic tree through the MIRU VNTR plus database
186 (www.miruntrplus.org) to analyze the transmission dynamics of *Mtb* strains in Asian elephants
187 of Nepal. A minimum spanning tree analysis was performed using MIRU-VNTR data with
188 BioNumerics version 6.0.

189 We performed Large Sequence Polymorphism (LSP) as previously described [21,39] to
190 determine the lineage of *Mtb* having new SIT numbers. Finally, we performed PCR targeting
191 *gyrA*, as described [6], to identify the Nepal-specific Indo-Oceanic lineage of *Mtb*. The *gyrA*
192 PCR products were sequenced using the BigDye Terminator v3.1 cycle sequencing kit (Life

193 Technologies Corp., CA, USA) on the 3500 Genetic Analyzer (Applied Biosystems). The
194 obtained sequences were analyzed using NCBI BLAST and BioEdit software (version 7.2.5.0).
195
196**3. Results**
1973.1 Profile of deceased elephants
198 All nine elephants that died between 2019 and 2022 were adults and 7/9 were female. Six
199 of the nine elephants had suspected TB lesions in the lungs at postmortem (Table 1).
200 Six elephants had a history of serological reactivity on the STAT-PAK and/or DPP tests. Among
201 them, one elephant (Elephant 3) was treated for TB based on a reactive STAT-PAK result and a
202 known exposure to another elephant that died of TB. Treatment was administered according to
203 the guidelines of the Nepal Elephant Tuberculosis Control and Management Action Plan (2011-
204 2015) and included a 60-dose course of isoniazid, rifampicin, and ethambutol followed by 300
205 doses of isoniazid and rifampicin (Table 1).
206
2073.2 Culture and DST
208 Four samples were culture-positive on L-J media (Table 2). Phenotypic drug susceptibility
209 testing revealed all four TB isolates were susceptible to first-line drugs (rifampicin, isoniazid,
210 streptomycin, and ethambutol) as well as to the second-line drugs (amikacin, clofazimine,
211 levofloxacin, linezolid, moxifloxacin 1 µg/ml, and moxifloxacin 0.25 µg/ml). In addition, the
212 four samples were confirmed to be drug-sensitive by genotypic DST by Genotype MTBDRplus
213 VER 2.0-line probe assay (Hain Lifescience, Nehren, Germany), which detects most significant
214 mutations associated with isoniazid and rifampicin. On sequence analysis of drug-resistant
215 associated genes of Elephant 1, we found a commonly described isoniazid resistance-associated

mutation in *katG* (Ser315Thr) [36]. There were no mutations in the *inhA* promoter region. However, sequence analysis of the rifampicin-resistant *rpoB* gene could not determine the mutation.

3.3 *Mtb* detection using LAMP and Conventional PCR

We detected *Mtb* complex using LAMP and conventional PCR targeting the16S rRNA gene and *IS6110* gene respectively of *Mtb* from the DNA, which was extracted directly from TB-suspect lung tissue samples, sediments of culture-processed tissue samples, and cultured colonies (Table 2 and Figure 3). The comparative results are summarized in Table 2. Among nine elephants, six were LAMP positive when tested using direct DNA extracted from tissue samples and five were LAMP positive using DNA extracted from sediments of culture-processed samples. We found five elephants positive in PCR using DNA extracted from either tissue samples. Elephant 9 was positive only on LAMP using DNA extracted from tissue samples (Table 2, Figure 3C, Figure 3D).

3.4 Genotyping of elephant *Mtb* isolates

Five PCR-positive isolates were genotyped by spoligotyping, LSP, and *gyrA* sequencing (Table 3). The spoligotyping result showed that three elephants were infected with new spoligotypes. Two elephants were infected with the Beijing lineage. The three new spoligotypes were confirmed to be of Indo-Oceanic lineage by LSP (Appendix Figure 2). In addition, all three strains of Indo-Oceanic lineage had Nepal-specific *gyrA* T231C single nucleotide polymorphism (SNP), as reported previously [6]. MIRU-VNTR analysis (Figure 4A) showed three distinct groups of *Mtb* strains. The first group was formed by the Beijing lineage strains of Elephants 1

and 5. There was a difference in at least three loci between these isolates. The second group was formed by Elephants 2 and 7 along with a previously reported elephant (Elp-A) and two human samples h-3, and h-8 from Nepal (9). There was a difference from 1 to 3 loci among these strains. The third group was formed by Elephant 8 with Elp-B and Elp-C as well as other human isolates (h-294, h-296, h-277, and h-98) from Nepal as previously reported (9). All strains in the second and third groups were of Indo-Oceanic lineage. This phylogenetic analysis is further clarified by minimum spanning tree analysis, which shows the relationship between elephant and human isolates (Figure 4B).

4. Discussion

Previous molecular studies characterized *Mtb* isolates from three elephants that died of TB in 2009 - 2012 [6], from two dead elephants in 2013 [21], and from another two dead elephants [20], these studies had not assessed the impact of TB on mortality of elephants of Nepal. As 13% to 22% of elephants are TB seropositive at different times [7,9], TB is likely endemic in the elephant population of Nepal.

Most previous studies were able to detect *Mtb* from the culture of TB-suspected lesions [6,8,20,21] in Nepal as well as in the USA, Switzerland, India, and Sri Lanka [11,13,15,17,18]. In addition to diagnosis by culture, we explored the potential of molecular detection of *Mtb* complex directly from the TB-suspected tissue samples by conventional PCR and LAMP. While the culture of one PCR-positive sample was contaminated, both PCR and culture showed nearly similar results (Table 2). The slightly higher efficiency of LAMP might be due to its ability to detect a lower load of bacteria as previously reported [27,28,41]. This finding suggests the potential to use the LAMP method for the early detection of *Mtb* in elephants, improving

262 elephant TB surveillance in elephant range countries, including Nepal [27,28,42]. Elephant 9 was
263 negative on culture, PCR, and serology but positive in the LAMP test and necropsy only. This
264 might be due to the initial infection of TB with a lower density of bacteria in the lungs, but a
265 limited number of samples makes it difficult to validate. TB is often paucibacillary which may
266 explain the negative culture and PCR results. Another possibility might be a false positivity in
267 the LAMP test.
268 Elephants 1, 2, 5, and 8 that had a history of sero-reactivity were also positive on LAMP,
269 PCR, and culture. While Elephant 3 had a history of serological reactivity as indicated in Table
270 1, she had been treated with anti-TB drugs and serological tests post-treatment were non-reactive
271 (with one exception in 2011). That LAMP, PCR, and culture were negative at postmortem
272 suggests that anti-TB drugs might have been effective in this elephant. Elephant 7 was positive
273 on LAMP, PCR, and culture but was serologically non-reactive. This elephant died in 2019 but
274 her most recent serological test was in 2016 so we cannot know for sure if a subsequent test may
275 have been positive. We did not detect *Mtb* in one elephant (Elephant 6) that was TB sero-
276 reactive. Since our necropsy examination was limited to examination of lungs only, there is a
277 possibility that we may have missed TB-suspect lesions in this elephant, or the elephant may
278 have died of other causes. Elephant 4 was seronegative, and we did not detect *Mtb* in culture,
279 LAMP, and PCR suggesting that it might have died of other causes besides TB.
280 Genotyping of five PCR-positive isolates by spoligotyping, LSP, and *gyrA* Sequencing
281 revealed that three elephants had strains belonging to the Indo-Oceanic lineage with a *gyrA*
282 T231C mutation, like previous isolates. Additionally, two elephants had strains belonging to the
283 Beijing lineage; one strain was isoniazid-resistant [6,20,21] (Table 3).

From MIRU-VNTR analysis (Figure 4), we found two distinct groups of Indo-Oceanic strains where two cases (Elephants 2 and 7) form a group with elephant isolates, Elp-A and two human isolates, h-3, and h-8 [6] had a relatively close MIRU-VNTR profile among each other. Although Elephants 2 and 7 died in relatively distant places (Appendix Figure 1), these elephants likely had many opportunities for contact as elephants routinely move between different places in Nepal. Elephant 7 may have been infected by the same source as Elephant 2 while moving from Koshi Tappu Wildlife Reserve to CNP. The source may have been of human origin (e.g. from elephant handlers), as both elephant isolates had MIRU-VNTR patterns similar to those of human isolates.

Another group comprised Elephant 8, elephants (Elp-B and Elp-C), and human strains. Elephant 8 had shared the location with Elp-B and Elp-C before going to the location of death and might have been infected with TB from those elephants or humans while traveling from one post to another. Both these groups suggest the wide distribution of this genotype in elephants and humans of Nepal. This elephant was in the eastern part of CNP, along with most of the other deceased elephants (Appendix Figure 1). Besides this, there is also a possibility of elephant-to-elephant transmission during rhino census operations, patrolling activities, and other activities where there is close interaction between elephants.

The difference of at least three loci between *Mtb* isolates of Elephants 1 and 5 suggests different sources of transmission for these two isolates. Elephant 1 may have been directly infected by a drug-resistant Beijing strain from close human contact infected with a drug-resistant *Mtb* isolate, as the Beijing lineage is the dominant lineage of multi-drug resistant (MDR) TB among humans in Nepal [43]. Another possibility is transmission from previously

306 treated elephants with *Mtb* (such as Elephant 3), where transmission might have occurred after
307 mutation. Elephant 5 might have been infected by an infected elephant or a human.
308 However, detailed contact tracing, along with whole-genome sequencing profiles of all the
309 elephant isolates and possible human isolates, are needed to confirm the transmission dynamics
310 of the disease. In addition, analysis of *Mtb* strains from TB-infected living elephants would
311 provide helpful information on the epidemiology and transmission dynamics of *Mtb* in elephants
312 of Nepal.
313 Most elephant deaths (7/9) and TB-associated mortality (4/6) occurred in the Sauraha sector
314 of the eastern part of CNP (Figure 2 and Appendix Figure 1). This sector of CNP is a tourist and
315 business hub of the national park and includes an elephant breeding center, where captive female
316 elephants are regularly mated by wild elephants, leading to the high possibility of intra- and
317 interspecies transmission of TB. The transmission of TB from captive to wild elephants could
318 have devastating consequences not only for the endangered wild elephant population but also for
319 many other endangered species such as rhinoceros and many other ungulates [44].
320

321**5. Conclusion**

322 In conclusion, our study underscores that TB continues to be associated with mortality in
323 elephants in Nepal. We advocate for early detection of *Mtb* using PCR and LAMP methods from
324 TB-suspect unprocessed tissue samples in Nepal. While the Indo-Oceanic lineage is the
325 dominant lineage of *Mtb*, there is an emergence of the Beijing lineage in Nepal elephants.
326 Human-to-elephant transmission of drug-resistant strains poses a threat to the control and
327 management of elephant TB, necessitating a holistic one-health approach for mitigation and
328 control. Urgent implementation of comprehensive surveillance and preventive measures is

crucial to safeguard both captive and wild elephants, along with human populations in regular contact.

Ethical approval

Not required.

Funding

This study received support from the Ministry of Education, Culture, Sports, Science and Technology (MEXT), Japan, and Japan Agency for Medical Research and Development (AMED) (Grant Numbers JP23jm011002, JP23wm0125008, JP233fa627005 to YS and JP21kk0120 to TT), Asahi Glass Foundation to TT, Mitsui & Co. Environmental Fund (R180029) to TT and The Rufford Foundation (31905-1).

Conflicts of interest statements

All authors have no conflict of interest.

Addresses of the institutes at which the work was performed

1. Biodiversity Conservation Centre, National Trust for Nature Conservation, Sauraha, Chitwan laboratory: Sample storage after postmortem examination, DNA extraction, Polymerase Chain Reaction (PCR), LAMP test.
2. German Nepal Tuberculosis Project (GENETUP), Kalimati, Kathmandu, Nepal: Culture of the tissue sample, Drug Susceptibility testing, DNA extraction from positive colonies, microscopic tests.

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359

360 **Credit authorship contribution statement**

361 **Arjun Pandit:** Writing original draft, Sample collection, data , laboratory work, data validation,
362 conceptualization; **Jeewan Thapa:** Manuscript revision, laboratory work, data validation,
363 conceptualization, supervision; **Amir Sadaula:** Manuscript revision, sample collection,
364 laboratory work; **Yasuhiko Suzuki:** Data validation, manuscript revision, Laboratory analysis
365 and overall supervision; **Chie Nakajima:** Data validation, manuscript revision and overall
366 supervision; **Susan K. Mikota:** Data validation, manuscript revision, supervision; **Naresh**
367 **Subedi:** Collaboration in the field for sample collection and laboratory work in NTNC
368 laboratory, manuscript revision, supervision; **Bijaya Kumar Shrestha:** Collaboration with
369 DNPWC for permission of research work, manuscript revision; **Michito Shimozuru:** Data
370 validation, manuscript revision, supervision; **Bhawana Shrestha:** Collaboration for laboratory
371 work in GENETUP laboratory, manuscript revision, supervision; **Bijendra Raya:** Laboratory
372 analysis, manuscript revision; **Sanjay Chaudhary:** Laboratory analysis, manuscript revision;
373 **Sarad Paudel:** Manuscript revision, supervision; **and Toshio Tsubota:** Overall supervision,
374 manuscript writing and manuscript revision, data validation.

Acknowledgments

I acknowledge the Department of National Parks and Wildlife Conservation (DNPWC), Nepal for permitting this research. I want to acknowledge Pradipa Silwal, Kiran Rijal, Baburam Lamichhane, Rachana Shah, and all the NTNC staff for their contribution. Similarly, I want to thank the GENETUP team and staff for their technical support.

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533 Table 1: Epidemiological profile of the elephants that died between 2019 and 2022 in Chitwan National Park, Nepal

Elephant ID	Sex	Year of elephant death	Reactivity on one or more serological tests for TB **	History of TB treatment	Gross TB lesions
1	F	2021	Yes	No treatment	Present
2	M	2020	Yes	No treatment	Present
3	F	2020	Yes	Treated from December 2008 to January 2010*	Absent
4	F	2022	No	No treatment	Absent
5	F	2019	Yes	No treatment	Present
6	F	2020	Yes	No treatment	Absent
7	F	2019	No	No treatment	Present
8	F	2020	Yes	No treatment	Present
9	M	2022	No	No treatment	Present

534

535 *Treated with a full course of TB drugs as recommended by Nepal Elephant Tuberculosis Control and Management Action Plan
536 2011-2015, with isoniazid, rifampicin, and ethambutol for 60 doses followed by 300 doses of isoniazid and rifampicin (23).
537 ** ElephantTB STAT-PAK Assay (Chembio Diagnostics Inc, Medford, NY, USA) and/or DPP VetTB Assay for Elephants (Chembio
538 Diagnostics Inc, Medford, NY, USA)

Table 2: Comparison of PCR, LAMP, and culture from postmortem elephant lung tissue

Elephant ID	DNA extracted directly from preserved raw tissue samples		DNA extracted from sediments of culture-processed tissue samples		Culture
	ϕLAMP	λ PCR	ϕLAMP	λ PCR	
1	+	+	+	+	-[$]
2	+	+	+	+	+
3	-	-	-	-	-
4	-	-	-	-	-
5	+	+	+	+	+
6	-	-	-	-	-
7	+	+	+	+	+
8	+	+	+	+	+
9	+	-	-	-	-

+ = Positive.

- = Negative.

[$] Considered negative because the culture was contaminated.

ϕ = Result of LAMP test targeting 16S rRNA gene of *Mtb*.

λ = Result of PCR targeting *IS6110* gene of *Mtb*.

549 Table 3: Genotypic characteristics of *Mtb* isolates from five elephants that died of tuberculosis in Nepal and comparison with those

550 from previous studies.

Elephant ID	**Spoligotype Octal number**	**SIT No.**	**Lineage type**	***gyrA***	**Remarks**
1	3771	1	Beijing		This study
2	7413700	New	Indo-Oceanic	T231C	This study
5	3771	1	Beijing		This study
7	7413700	New	Indo-Oceanic	T231C	This study
8	777477777413700	New	Indo-Oceanic	T231C	This study
Elephant A	703777747413771	New	Indo-Oceanic and Central Asian Strain (Mixed infection)	T231C	Previous study (22)
Elephant B	7413771	1365	Indo-Oceanic & Beijing (Mixed infection)	T231C	
Elp-A	300	New	Indo-Oceanic	T231C	Previous study (6)
Elp-B	777777777403700	New	Indo-Oceanic	T231C	
Elp-C	777777777413700	135	Indo-Oceanic	T231C	
PK			Indo-Oceanic		Previous study (21)
DG			Beijing		

551

552 **Figures**

553 **Figure 1.** Workflow in this study. This flowchart summarizes the various methods used for our
554 method in sequential order.
555 N = number of samples used in that study process.
556

557 **Figure 2**. Map of the study area. The map shows Chitwan National Park with GPS location
558 points where nine elephants died (ID 1 to 9). This map was created using QGIS software.
559 Two elephants, ID 1 & 8, share the same location overlapping their GPS points, and four
560 elephants, ID 2,3,4 & 6 share the same location overlapping their GPS points on the map.
561

562 **Figure 3**. Observation of loop-mediated isothermal amplification (LAMP) assay results by
563 turbidity and change in color. A & C. Positive amplification of *Mtb* DNA observed by increased
564 turbidity during LAMP reaction. B & D. Observation of LAMP assay result under LED light.
565 Positive reaction is indicated by light yellow color and negative reaction is indicated by orange
566 red color. A & B. DNA extracted from the concentrated sediments of culture-processed tissue
567 samples from nine elephants was used in A and B. Sample code for elephants 1 to 9 is as 1C to
568 9C; PC, positive control (*M. bovis* BCG Tokyo 172); NC, negative control (Double distilled
569 water). C & D. DNA extracted directly from raw tissue samples from nine elephants was used in
570 C and D. Sample code for elephants 1 to 9 is as 1D to 9D; PC, positive control (*M. bovis* BCG
571 Tokyo 172); NC, negative control (Double distilled water).
572

573 **Figure 4.** Phylogenetic comparison of *Mtb*. isolates from this study with other elephant and
574 human isolates. (A) UPGMA based phylogenetic analysis using MIRU-VNTR plus software

(https://www.miru-vntrplus.org/MIRU/index.faces) using MIRU-VNTR with 24 loci namely: MIRU2, VN424, ETR-C, MIRU4, MIRU40, MIRU10, MIRU16, VN1955, MIRU20, QUB11b, ETR-A, QUB3232, VN2401, ETR-B, MIRU23, MIRU24, MIRU26, MIRU27, QUB11a, MIRU31, VN3690, QUB26, VN4156, and MIRU39, respectively as well as Spoligotyping data. Elephants from our study are represented by ID 1,5,2,7, and 8. Elp-A, Elp-B, and Elp-C are three different elephant *Mtb* strains from a previous study [6]. Similarly, h-294, h-296, h-277, h-3, h-8, h-200, and h-98 are seven human isolates from a previous study [6]. The scale bar indicates genetic distance. DST indicates a Drug Sensitivity test where 'R' signifies resistance to Isoniazid and 'S' signifies sensitivity to all first-line and second-line drugs. (B) Minimum Spanning tree analysis based on MIRU-VNTR loci. Sample ID is indicated by the corresponding-colored node. Branch length between nodes is indicated by several differences in MIRU-VNTR loci.