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FULL PAPER

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

Jeewan Thapa[1)], Chie Nakajima[1, 2)], Bhagwan Maharjan[3)], Ajay Poudel[1)] and Yasuhiko Suzuki[1, 2*)]

[1)]Division of Bioresources, Hokkaido University Research Center for Zoonosis Control, Sapporo, Japan
[2)]Hokkaido University The Global Station for Zoonosis Control, Sapparo, Japan
[3)]Nepal Anti-Tuberculosis Association, German Nepal Tuberculosis Project, P.O. Box 1494, Kalimati, Kathmandu, Nepal

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Abstract

***Mycobacterium orygis*, a new member of the *Mycobacterium tuberculosis* complex, was isolated from a captive spotted deer (*Axis axis*) and a blue bull (*Boselaphus tragocamelus*) in Nepal. Analyses by spoligotyping, mycobacterial interspersed repetitive units-variable number of tandem repeats (MIRU-VNTR) typing, region of difference and single nucleotide polymorphism of genes *gyrB, mmpL6*, TbD1, *PPE55* and Rv2042c confirmed the isolates as *M. orygis*. Moreover, analyses by spoligotyping (SIT587) as well as MIRU-VNTR showed that the isolates shared a similar pattern with many reported isolates. From previous and the present studies, it can be inferred that South Asia is one of the endemic regions for *M. orygis*. Further investigation including a larger sample size and different host interaction will help to understand the ecology and epidemiology of *M. orygis* in Nepal.**

Key Words: Mycobacterial interspersed repetitive units-variable number of tandem repeats, *Mycobacterium orygis*, Spoligotyping, Wild animals

Introduction

Mycobacterium orygis was initially described as oryx bacillus and had a distinct phylogenetic position in the MTC phylogeny[7,11,16)]. However, recently this species has been reclassified as a member of the *Mycobacterium tuberculosis* complex (MTC)[19)]. Although the exact host range is yet to be defined, *M. orygis*, or oryx bacillus, has been reported to affect a wide variety of hosts, namely, African wild buffalo[5)], antelope, oryx, water buck, deer, cows, rhesus monkeys and human[19)]. In addition, it was reported in New Zealand to be transmitted from a human patient suffering from tuberculosis (TB) to a dairy cow[4)].

Nepal is a TB endemic country with a high burden of TB in humans[10,14,22)].

*Corresponding author: Yasuhiko Suzuki, Division of Bioresources, Hokkaido University Research Center for Zoonosis Control, Kita 20 Nishi 10, Kita-ku, Sapporo 001–0020, Japan
Phone: +81–11–706–9503. Fax: +81–11–706–7310. E-mail: suzuki@czc.hokudai.ac.jp
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In addition, although many accounts of TB lesions in livestock tissues found during postmortem or at slaughter are unverified, cattle and buffalo are reportedly infected with *M. bovis*[8]. Moreover, TB infection in Nepal has been documented not only in human and livestock, but also in wild animals such as elephants[13] and monkeys[21].

In this study, we report the identification of *M. orygis* isolates in TB-infected lesions of a spotted deer (*Axis axis*) and a blue bull (*Boselaphus tragocamelus*), the largest Asian antelope, at a captive wild animal facility in Nepal. Comprehensive and comparative molecular analyses of the isolates were carried out.

Materials and methods

Deer and blue bull isolate: MTC isolates from a deer and a blue bull with TB lesions, which died on February 4, 2013 and February 18, 2014, respectively, were included in this study. In the deer, samples were collected from the lungs and extrapulmonary granulomatous lesions (Fig. 1). In the blue bull, samples were collected only from the lung lesion. In either case, no detailed postmortem was conducted due to the potential risk of spread of the organism into the environment. Thus, comprehensive descriptions of TB lesions from other organs were not available.

M. tuberculosis isolate: One *M. tuberculosis* isolate from a human patient was randomly selected from the culture stock of *M. tuberculosis* at the German Nepal Tuberculosis Project (GENETUP), Nepal, and its morphology was compared with the isolated *M. orygis*.

Microscopy and Culture: Tissue samples were processed as described by the European Society of Microbiology[6]. 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 $\times g$ 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 $\times g$ for 20 minutes, the supernatant was discarded, and the sediment used for further analysis. Afterwards, fluorescence microscopy analysis was carried out as follows. 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 under a fluorescence microscope. For sample culturing, an aliquot from the sediment was inoculated into Löwenstein-Jensen (LJ) growth media. The inoculant was grown for 8 weeks.

DNA extraction: Colonies of the deer isolate were collected with an inoculum loop, suspended in 300 μL of distilled water and heated for 20 min at 95°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°C. Afterwards, cells in the suspension were lysed by incubation for 15 min in an ultrasonic bath, followed by centrifugation at 13,000 $\times g$ for 5 min. The supernatant containing the bacterial DNA was used for further analysis.

Genetic analysis: A region of difference (RD) analysis was conducted to determine three genetic regions, namely, *cfp32*, RD9 and RD12, as previously described with some modification[12]. 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 carried out according to a previous publication[9] and interpreted using the international spoligotyping database (SpolDB4) to determine spoligo-international types (SIT)[3].

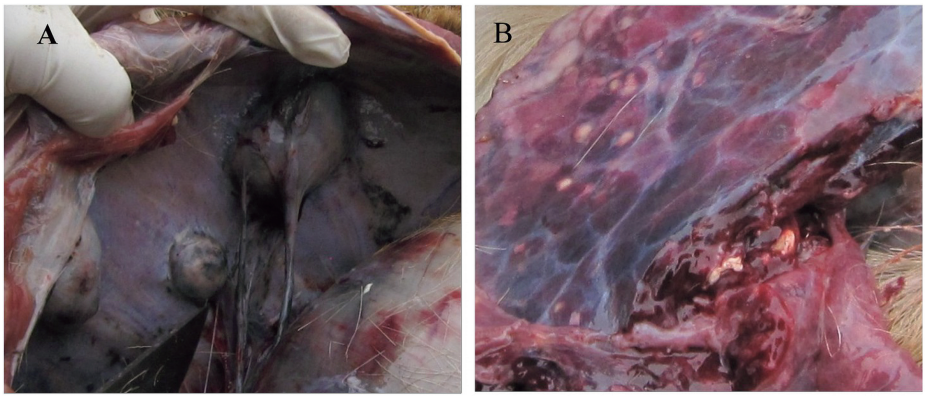

Fig. 1. Tuberculosis lesions from a dead spotted deer during postmortem. A) Extrapulmonary. B) Lung. Extrapulmonary tuberculosis lesions were of various sizes and capsulated with extensive liquefaction. Pulmonary tuberculosis lesions were of varying sizes from a single focal granuloma of 1 to 2 cm to extensive granulomatic lesions affecting a larger area of the lung tissue.

Typing by mycobacterial interspersed repetitive unit-variable number of tandem repeats (MIRU-VNTR) was carried out on 22 loci following a basic protocol as previously described[17]. The obtained MIRU-VNTR pattern was compared with previously reported MIRU-VNTR data[5,19] to construct an unweighed pair group method with an arithmetic mean (UPGMA) dendrogram using MIRU-VNTR*plus* (www.miru-vntrplus.org)[1,20]. A single nucleotide polymorphism (SNP) study was conducted on the genes *gyrB, mmpL6*, TbD1 and *PPE55*, and Rv2042c, as previously described[7,19].

Results

Microscopy and Culture

The result from the fluorescence microscopy analysis carried out on the tissue smear was positive for TB (data not shown). The culture from the tissues yielded smooth and moist colonies (Fig. 2).

Genetic analysis

Spoligotyping analysis of MTC isolates from a deer and a blue bull showed the SIT number 587 in the SpolDB4 database and was determined to be *M. orygis*, similar to the result from a previous study[19]. Spoligotyping analysis of both isolates showed spacer 3 with a very faint reactivity that was difficult to confirm by visual inspection. The presence of spacer 3 was confirmed by partial sequencing of the DR region as previously described[18]. Interestingly, spacer 3 had two-point mutations (Table 1).

The RD analysis showed that the isolates had *cfp32*, but lacked RD9 and RD12. The results of the SNP of *gyrB*, *PPEE5*, *mmpL6*, Rv2042c and the TbD1 genetic region corresponded to *M. orygis* (Table 2). Thus, the bacterial species of the isolates was determined to be *M. orygis*. The MIRU-VNTR types of both isolates were the same or similar to those of most reported isolates[5,19] and belonged to the same clade as that of most reported isolates in the UPGMA dendrogram (Fig. 3). Loci 2163b and 2165 were not amplified in any isolate, a result similar to that of most reported isolates[19].

Discussion

To the best of our knowledge, this report is the first to demonstrate the presence of *M. orygis* and TB in deer and blue bull in Nepal. Previously, personnel in the captivity facility had observed TB-like lesions during postmortem in some deer carcasses. Although it was likely that these TB-like lesions might have been due to *M. orygis* infection, information on TB in deer was

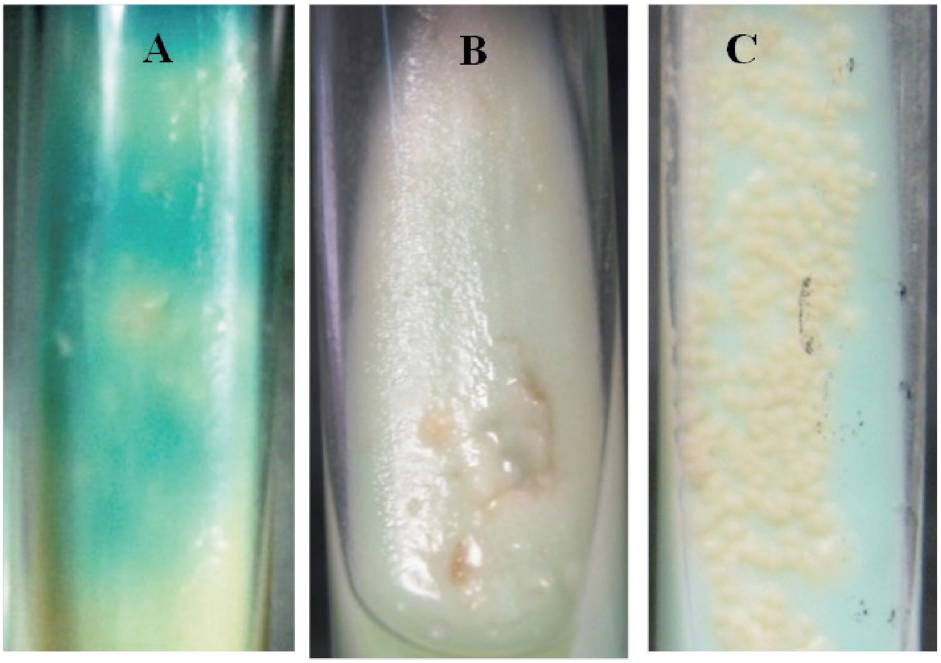

Fig. 2. Culture of the deer isolate (A), the blue bull isolate (B) and a comparative culture of an *M. tuberculosis* isolate from human (C). All cultures were in Löwenstein-Jensen medium in the same laboratory but at different times. Colonies of deer and blue bull isolates were smooth and moist, whereas the culture of the *M. tuberculosis* isolate from human was rough and relatively dry.

Table 1. Comparative analysis of spacer 3 sequencing

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 studies (Kamerbeek *et al.* 1997; van Embden *et al.* 2000).
Bold and underline letters denote SNPs.

limited and that on blue bulls not available. Indeed, individual clinical histories were not recorded because both deer and blue bulls were in a herd, and as the facility staff became aware of the TB infection among the deer population, regular postmortems were not conducted.

Deer and blue bulls confined in the facility originated from Chitwan and Bardia national parks, south of Nepal, but information concerning the exact date they were introduced into the facility is unknown. In Nepal, there is no ongoing TB control program in livestock, and such programs in wild animals are beyond disease control capabilities. As a result, there are no previous reports in Nepal of a) suspected TB lesions found in deer and blue bulls from national parks and forest areas, b) *M. orygis* isolated from human patients and livestock, or c) TB in human handlers in captivity facilities. Moreover, extensive tracing of the origin *of M. orygis* was also beyond the scope of this study.

Genetic analysis of the isolates by spoligotyping, region of difference and species specific mutations showed that they belonged to *M. orygis*. We failed to detect spacer 3 in spoligotyping despite it usually being identified in this species, perhaps due to the two-point mutations found in the spacer (Table 1). The MIRU-VNTR results showed that 22 loci of deer and blue bull isolates were exactly the same.

Table 2. Summary of comparative analysis of *M. orygis*-related SNPs with other members of MTC

Isolates	*gyrB*^{oryx}		*mmpL6*^{551}	TbD1^{171}	*PPE55*		Rv2042c^{38} (18)
	1113	1450			2162	2163	
	(7)						
M. tuberculosis	G	G	AAC	C	T	C	GTC
M. bovis	G	T	AAG	C	T	C	GTC
M. orygis	A[a]	T	AAG	G	G	T	GGC[a]
Deer and blue bull isolates	A[a]	T	AAG	G	G	T	GGC[a]

[a]*M. orygis* specific SNP.
Numbers in parenthesis indicate reference number.

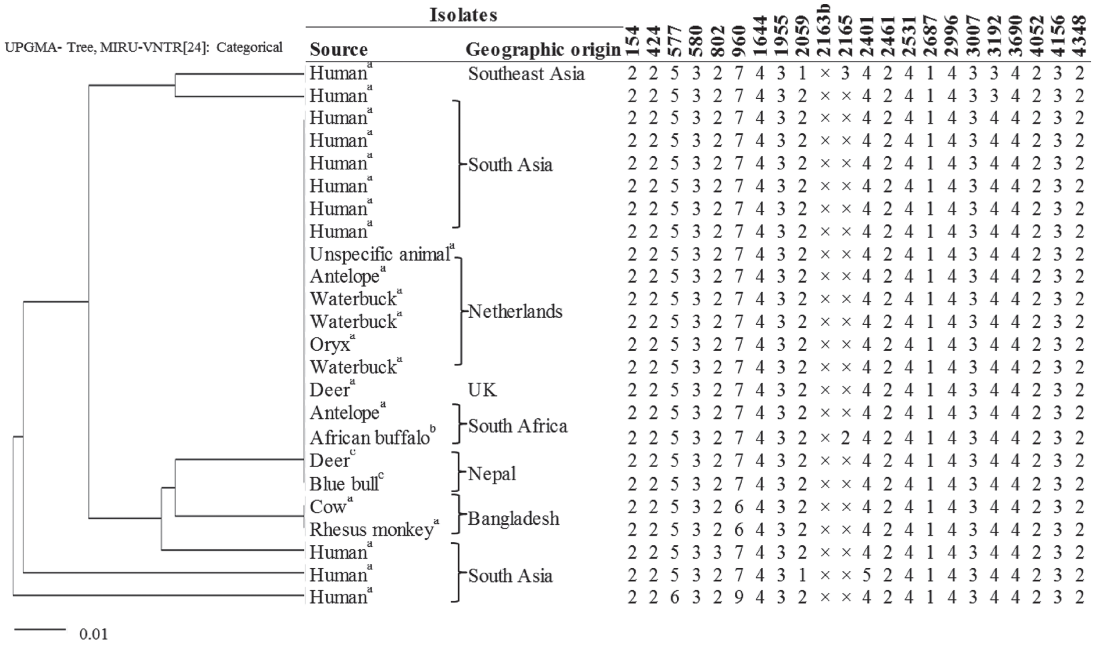

Fig. 3. UPGMA dendrogram showing phylogeny of *M. orygis* isolates based on the MIRU-VNTR results of 22 loci. The order of MIRU-VNTR is as follows, left to right: 154, 424, 577, 580, 802, 960, 1644, 1955, 2059, 2163b, 2165, 2401, 2461, 2531, 2687, 2996, 3007, 3192, 3690, 4052, 4156 and 4348.
[a]Isolates in van Ingen *et al.*[18)]
[b]Isolate in Gey van Pittius *et al.*[5)]
[c]Isolate from this study.

Therefore, it is likely that the same strain of *M. orygis* had infected these animals and may have been transmitted across animals in the captivity facility. Indeed, although the deer and the blue bull were housed in different enclosures, around 200 meters apart, these animals may have had contact opportunities if placed together for a short time during repair and maintenance of the enclosures. The results suggested that there may be an ongoing transmission of *M. orygis* in the captivity facility. Also, the possibility of aerosol transmission and contamination via food, utensils and other unknown sources is not ruled out.

The dendrogram showed that the deer and blue bull isolates shared the same phylogenic position with most of the compared isolates (Fig. 3). Most of the isolates from humans originated from South Asia, but no specific country has been stated[19)]. Nonetheless, in a different study in Bangladesh[15)], *M. africanum* was reported in cows, which was identified by spoligotype ST587 and Genotype MTBC assays

(Hain lifescience GmbH, Nehren, Germany). Along with spoligotype ST587, *M. orygis* isolates in the present study also shared the *gyrB*^{1450} (G to T) mutation with *M. africanum*[7], making it impossible to differentiate between species by the Genotype MTBC assay. Hence, it is likely that the *M. africanum* reported in that study was actually *M. orygis*. Separately, *M. orygis* has also been described in an Indian immigrant in New Zealand[4]. These results seem to suggest an endemic prevalence of *M. orygis* in animals and humans in South Asia, mainly Nepal, India and Bangladesh. It can therefore be inferred that *M. orygis* may have been historically present in the sub-continent, but misidentified with other members of the MTC, an error not identified until now due to advances in molecular markers and an increased awareness of TB in animals.

In this study, only two isolates from a deer and a blue bull were studied, but a more comprehensive study with a larger sample size would be desirable as it would help better the molecular characterization of *M. orygis* in Nepal. Ascertaining the prevalence of *M. orygis* in other animals of Nepal is also important. In addition, our results suggest a zoonotic transmission of *M. orygis* in Nepal, thus, this possibility should not be ruled out. To monitor and prevent a possible zoonosis of *M. orygis*, humans working in animal facilities should be tested for TB and the best biosecurity measures available implemented. Finally, based on the results of this study, a new member was added to the documented list of the MTC in Nepal. Further comprehensive studies will be needed to fully understand the ecology and epidemiology of *M. orygis* in Nepal.

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