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SHORT REPORT
Occurrence of non-tuberculous mycobacteria species in livestock from northern China and first isolation of *Mycobacterium caprae*

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SUMMARY

We investigated the presence of *Mycobacterium* spp. in livestock in northern China. Of the 163 clinical samples selected for this study, 20 were from throat swabs of dairy cows, and 143 were tissue samples (including lung tissue from one reindeer, hilar lymph node tissue from 55 cows, and liver tissue from 87 sheep). A total of 41 mycobacterial isolates were identified including two isolates of *M. caprae* and 39 non-tuberculous mycobacteria (NTM) isolates. Multi-locus variable-number tandem repeat analysis (MLVA) profiles of the two *M. caprae* isolates proved to be unique. This is the first report of *M. caprae* isolates from livestock in China. This study also confirms previous reports that NTM is common in livestock in northern China.

Key words: Livestock, *Mycobacterium*, *M. caprae*, non-tuberculous mycobacteria.

Mycobacterium spp. are the causative agents of tuberculosis (TB) and other diseases. The main members of the genus are non-tuberculous mycobacteria (NTM) and the *M. tuberculosis* complex (MTBC), including *M. tuberculosis*, *M. bovis*, *M. caprae*, *M. africanum*, *M. microti*, *M. canettii* and *M. pinnipedii*. More than 125 species of NTM have been reported worldwide, of which 60 species are pathogenic to humans and/or animals [1, 2]. Moreover, the prevalence of *M. bovis* subsp. *caprae* has been extensively studied in France, Turkey and Spain. There have been several reports about its occurrence in animals and humans, where it is reported to account for about one-third of human *M. bovis*-associated cases of TB [3]. The prevalence of *M. caprae* was 1·6% in MTBC clinical isolates from 2007 to 2010 in Turkey [4]. In Spain, *M. caprae* represents 7·4% of all MTBC isolates from domestic and wild animals [5].

A total of 68 NTM isolates were recently isolated from 1067 throat swab samples of purified protein derivative (PPD)-positive cattle in northeast and northwest China [6], consistent with the reported prevalence of this large group of environmental mycobacteria in animals and their products [7]. With the emergence of drug-resistant strains and increasing numbers of self-employed, cattle-breeding households, the incidence of bovine tuberculosis (bTB) in China has increased annually. The average positive rate was 5·43% and 5·83% in 1985 and 1987, respectively [6]. More alarmingly, local epidemic surveys showed the positive

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Table 1. *Isolation and identification of* Mycobacterium *from clinical samples**

Samples	Locations	Animals	Organs	Sample numbers	Positive smears	Positive cultures	Species	Numbers
TB-like lesion samples	Hebei	Sheep	Liver	87	9	37	*M. caprae*	1
							M. engbaekii	1
							M. nonchromogenicum	4
							M. kumamotonense	4
							M. algericum	2
							M. arupense	2
							M. senuense	17
							M. peregrinum	1
							M. gordonae	5
Swollen lymph node samples	Beijing	Cow	Hilar lymph nodes	55	5	2	*M. intracellulare*	1
							M. kumamotonense	1
PPD-positive samples	Beijing	Cow	Throat swabs	20	0	1	*M. terrae*	1
PPD-positive samples	Heilongjiang	Reindeer	Lungs	1	1	1	*M. caprae*	1
Total				**163**	**15**	**41**		**41**

TB, Tuberculosis; PPD, purified protein derivative.

* Throat swab samples and tissue homogenates were liquefied, decontaminated with 4% NaOH [22] and neutralized with PBS. Finally the samples were centrifuged [11], grown in modified Lowestein–Jensen medium [23] and incubated at 37 °C for 6–8 weeks as appropriate. Once an acid-fast strain was confirmed, further purification was performed on 7H10 medium.

rate was 12·4% in Jilin province, northern China and 8·4% in Yunnan province, southern China in 2009 [6, 8]. *M. bovis* can be transmitted from cattle to humans through the consumption of contaminated milk and meat products [9]. Specific species act as a reservoir of the pathogen that can overflow to humans with zoonotic and economic consequences [10]. Although PPD-positive cases have been reported in northern China and a large number of suspected animals have been slaughtered without further identification, the PPD-positive reaction associated with TB or NTM infection remains unknown. Moreover, differentiating between TB and NTM infection in suspected animals will reduce the slaughter of uninfected herds. The present study aims to identify NTM and TB infection in PPD-positive animals and in organs with TB-like lesions.

In this study, a total of 163 clinical samples collected from the PPD-positive livestock in northern China were used to isolate *Mycobacterium* spp. (Table 1). Of these samples, 20 throat swabs were collected from 3-year-old PPD-positive dairy cows from a Beijing suburb. Fifty-five dairy cows aged 3–5 years that were observed to have progressive weight loss, dyspnoea and reduced milk production tested positive in a skin test survey. At necropsy, swollen hilar lymph nodes were collected from dairy cows suspected of having bTB from observation at an illegal cattle slaughterhouse. Furthermore, 87 tissue specimens with tubercle-like nodules were collected during post-mortem examination at an illegal sheep slaughterhouse in Hebei province (Fig. 1). Clinically, the suspected sheep presented with progressive weight

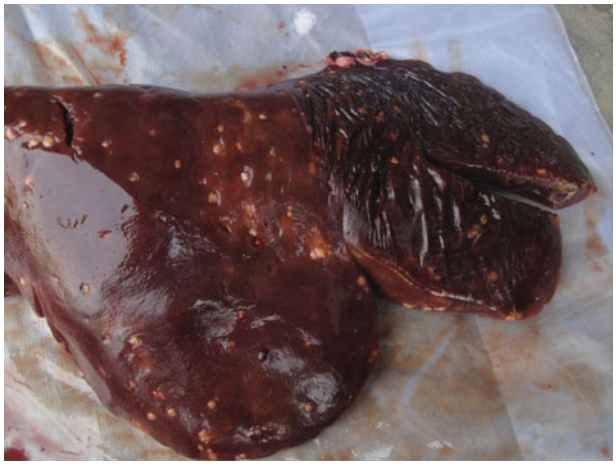

Fig. 1 [*colour online*]. Liver specimen showing pale and white nodules with a diameter of about 1 cm on the surface.

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Table 2. *Gene sequencing analysis of non-tuberculous mycobacteria isolates**

Strain no.	Maximum identity reference strain by *hsp65* sequencing	Identity (%)	Strain no.	Maximum identity reference strain by *hsp65* sequencing	Identity (%)
BJA10001	*M. senuense* (DSM 44999)	97	BJA10021	*M. senuense* (DSM 44999)	97
BJA10002	*M. senuense* (DSM 44999)	97	BJA10022	*M. gordonae* (AM398480)	99
BJA10003	*M. senuense* (AM398480)	97	BJA10023	*M. gordonae* (AM398480)	98
BJA10004	*M. senuense* (DSM 44999)	97	BJA10024	*M. kumamotonense* (DSM 45093)	97
BJA10005	*M. senuense* (DSM 44999)	97	BJA10025	*M. senuense* (DSM 44999)	97
BJA10006	*M. kumamotonense* (DSM 45093)	97	BJA10026	*M. senuense* (DSM 44999)	97
BJA10007	*M. senuense* (DSM 44999)	97	BJA10028	*M. arupense* (DSM 44942)	100
BJA10008	*M. kumamotonense* (DSM 45093)	98	BJA10029	*M. gordonae* (AM398480)	99
BJA10009	*M. senuense* (DSM 44999)	97	BJA10030	*M. nonchromogenicum* (ATCC 19530)	100
BJA10010	*M. senuense* (DSM 44999)	97	BJA10031	*M. senuense* (DSM 44999)	98
BJA10011	*M. senuense* (DSM 44999)	98	BJA10032	*M. terrae* (DSM 45454)	99
BJA10012	*M. nonchromogenicum* (ATCC 19530)	100	BJA10033	*M. senuense* (DSM 44999)	97
BJA10013	*M. nonchromogenicum* (ATCC 19530)	99	BJA10034	*M. arupense* (DSM 44942)	100
BJA10014	*M. nonchromogenicum* (ATCC 19530)	100	BJA10035	*M. senuense* (DSM 44999)	97
BJA10015	*M. senuense* (DSM 44999)	97	BJA10036	*M. bejaia* (DSM 45454)	98
BJA10016	*M. senuense* (DSM 44999)	97	BJA10037	*M. gordonae* (AM398480)	99
BJA10017	*M. peregrinum* (ATCC 14467)	99	BJA10039	*M. kumamotonense* (DSM 45093)	99
BJA10018	*M. engbaekii* (EU140946)	98	BJA10040	*M. intracellulare* (ATCC 13950)	100
BJA10019	*M. kumamotonense* (DSM 45093)	99	BJA10041	*M. terrae* (AY550212)	99
BJA10020	*M. senuense* (DSM 44999)	97			

* A 439-bp segment of *hsp65* was amplified using primers Tb11-forward (5′-ACCAACGATGGTGTGTCCAT-3′) and Tb12-reverse (5′-CTTGTCGAACCGCATACCCT-3′) [24]. The above data originated from the National Center for Biotechnology Information (NCBI) database and the Chinese Center for Disease Control and Prevention (CCDC) database.

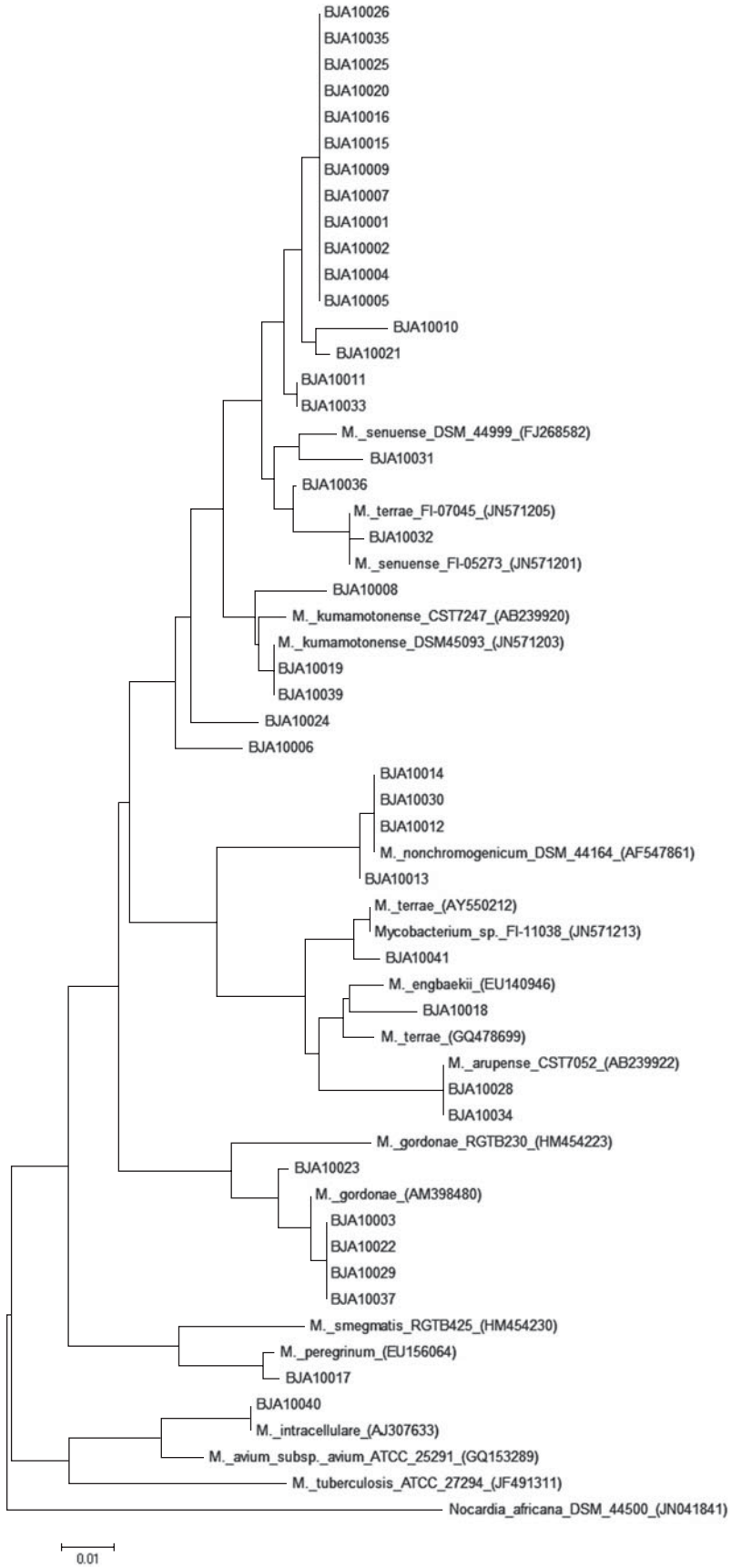

Fig. 2. Phylogenetic tree of the *hsp65* gene sequences of the isolates compared to the close species using the neighbour-joining method. The tree was rooted using *Mycobacterium tuberculosis* and *Nocardia africana* as outgroups. The accession number of each reference species appears within parentheses. The scale bar represents a 1 % difference in nucleotide sequences.

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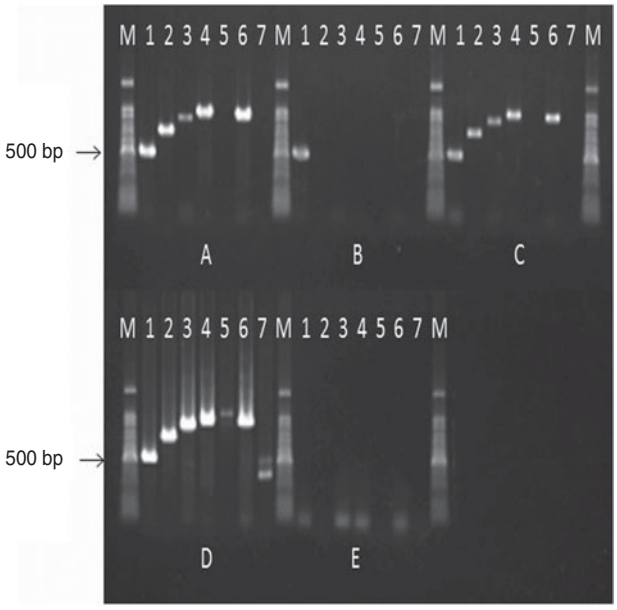

Fig. 3. Multi-loci polymerase chain reaction (PCR) typing of mycobacterial isolates. (A) BJA10038, (B) BJA10013, (C) BJA10027, (D) H37Rv as a positive control, (E) negative control. The PCR products were visualized by agarose gel electrophoresis and ethidium bromide staining. M represents a 100-bp DNA ladder. Lanes: 1, 16S rRNA; 2, RV0577; 3, IS1561; 4, Rv1510; 5, Rv1970; 6, Rv3877/8; 7, Rv3120. The results show that BJA10013 belongs to non-tuberculous mycobacteria, while BJA10038 and BJA10027 belong to *Mycobacterium caprae*.

loss and respiratory diseases. In addition, one lung with hyperplastic nodules was from a PPD-positive reindeer obtained in Heilongjiang province, but no clinical signs were observed in the reindeer.

Mycobacterium spp. were identified using standard procedures and further characterized using multi-locus variable-number tandem repeat analysis (MLVA), spoligotyping and *hsp65* sequence analyses. Fifteen of the 163 clinical samples were positive for the presence of *Mycobacterium* spp. in the primary acid-fast stain [11], as shown in Table 1. Post-cultivation, 41 acid-fast isolates were obtained: 37 isolates from 87 liver tissue samples with TB-like lesions, two strains from 55 hilar lymph nodes of slaughtered cows, one isolate from 20 throat swabs of the PPD-positive dairy cows, and one isolate from lung tissue of a PPD-positive reindeer (Table 1).

The 41 mycobacterial isolates were identified as NTM (39) and MTBC (2) using the *p*-nitrobenzoic acid and thiophene-2-carboxylic acid hydrazide tests. Subsequently, speciation of these isolates was verified by multi-locus polymerase chain reaction (PCR) analysis based on the analysis of the following target genes: *16S rRNA*, *Rv0577*, *Rv3349c*, *RD4*, *RD7*, *RD1* and *RD12* [12]. NTM species were further determined by partial analysis of *hsp65*, based on 97% homology of the cut-off value for differentiating species [13]. These NTM strains were identified as *M. engbaekii* (1), *M. intracellulare* (1), *M. peregrinum* (1), *M. terrae* (1), *M. algericum* (2), *M. arupense* (2), *M. nonchromogenicum* (4), *M. gordonae* (5), *M. kumamotonense* (5), and *M. senuense* (17) (Table 2). The phylogenetic tree based on *hsp65* genes is shown in Figure 2. It can be seen that *M. senuense* accounted for 43·5%, indicating the most prevalent, while *M. gordonae*, *M. kumamotonense* and *M. nonchromogenicum* were 12·8%, 12·8% and 10·6%, respectively, in 39 NTM isolates. On the contrary, other subspecies were less prevalent in the clinical samples, such as *M. engbaekii*, *M. intracellulare*, *M. peregrinum*, *M. terrae* , *M. algericum* and *M. arupense*. Free-ranging sheep have a higher risk of exposure to environmental mycobacteria [14]. Moreover, illegal slaughtering of animals in backyards and unhygienic slaughterhouses also contributes to NTM infection.

Interestingly, the two MTBC strains were confirmed as *M. caprae* (Fig. 3): one was isolated from a sheep liver and the other was from a lung sample of a PPD-positive reindeer. To our knowledge, this is the first report of the isolation of *M. caprae* in China.

MLVA containing 15 mycobacterial interspersed repetitive units (MIRU)-variable-number tandem repeat (VNTR) genetic loci was applied to investigate the molecular epidemiology of the two *M. caprae* strains as described previously [15–17]. Spoligotyping was also performed [18]. The results were compared using the MIRU-VNTRplus web application (http://www.miru-vntrplus.org/). This analysis showed that the two *M. caprae* strains had an identical MIRU-VNTR profile with the same number of tandem repeats in all 15 examined loci, namely, ETR-A (13), ETR-B (4), ETR-C (5), ETR-D (4), ETR-E (4), MIRU10 (3), MIRU16 (3), MIRU23 (3), MIRU26 (4), MIRU27 (2), MIRU39 (2), MIRU40 (2), Mtub21 (2), Mtub30 (4) and Mtub39 (2). Both *M. caprae* strains were identified as SpolDB4 type 647, with a spacer property of 200003777377600.

The fact that *M. caprae* strains were isolated from different animal species in two geographically distant provinces, suggests that *M. caprae* might be present in livestock in northern China. Importantly, the presence of this pathogen might pose the threat of potential transmission to humans. Indeed, there have been several reports of human TB associated with

M. caprae infection in the last 10 years [19–21]. Although infectious cases of *M. caprae* have not been reported in humans in China, the potential for transmission from animals to humans warrants further investigation and surveillance.

There are few reports regarding NTM infection in animals in China. In this investigation, 39 NTM strains were successfully isolated from animals and identified. These NTM were isolated from tubercle-like samples and throat swabs of PPD-positive cows, suggesting the potential for misdiagnosis of bTB and NTM infections. Since NTM misdiagnosis causes unnecessary slaughter of livestock and significant economic loss for local farmers, the occurrence of NTM infection should be closely monitored by inspecting the quarantined animals when an outbreak of bTB occurs in the future.

In summary, both NTM and MTBC were isolated from livestock, although NTM was the main type of *Mycobacterium* isolated from tubercle-like lesions observed in clinical samples. Our study reports for the first time the isolation of *M. caprae* from both deer and sheep in northern China.

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DECLARATION OF INTEREST

None.

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