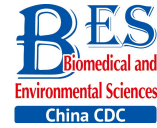# Original Article

First Report in China on the Identification and Drug Sensitivity of *Mycobacterium elephantis* Isolated from the Milk of a Cow with Mastitis[*]

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Abstract

Objective   In this study, milk from a cow with mastitis was analyzed to determine the presence of mycobacterial infection. Milk quality and security problems pertaining to the safe consumption of dairy products were also discussed in this study.

Methods   Milk was preprocessed with 4% NaOH. Then, mycobacteria were isolated from the milk sample on L-J medium. The isolate was identified using multiple loci Polymerase Chain Reaction (PCR) and multi-locus sequence analysis with *16S rRNA*, *sodA*, *hsp65*, and *ITS* genes. The drug sensitivity of the isolate to 27 antibiotics was tested through alamar blue assay.

Results   Smooth, moist, pale yellow colonies appeared on the L-J medium within a week after inoculation. Based on the results of multiple loci PCR analysis, the isolate was preliminarily identified as non-tuberculous mycobacteria. The *16S rRNA*, *SodA*, *hsp65*, and *ITS* gene sequences of the isolate exhibited 99%, 99%, 99%, and 100% similarities, respectively, with those of the published reference strains of *Mycobacterium elephantis (M. elephantis)*. The drug sensitivity results showed that the strain is resistant to isoniazid, p-aminosalicylic acid, and trimesulf but is sensitive to ofloxacin, rifampicin, amikacin, capreomycin, moxifloxacin, kanamycin, levofloxacin, cycloserine, ethambutol, streptomycin, tobramycin, rifabutin, ciprofloxacin, linezolid, cefoxitin, clarithromycin, and minocycline.

Conclusion   To the best of our knowledge, this study is initially to report the isolation of *M. elephantis* from the milk of a cow with mastitis in China.

Key words: Non-tuberculosis mycobacterium; *Mycobacterium elephantis*; Milk; Cow mastitis

INTRODUCTION

The group of nontuberculous mycobacteria (NTM) comprises 169 recognized species and 13 subspecies that are widely distributed in various environments (http://www. bacterio.net/mycobacterium.html). In recent years, the reported number of NTM species and the incidence of their associated diseases have increased globally. NTM have been isolated from water, soil, air, food, protozoa, plants, animals, and humans[1-2]. Under certain conditions, opportunistic NTM may become pathogenic; by contrast, saprophytic NTM never or very rarely cause diseases[3]. Nevertheless, approximately one-third of NTM species are associated with human diseases[4-5]. *Mycobacterium elephantis* (*M. elephantis*), a fast-growing mycobacterial species, was first isolated from a lung abscess of a Sri Lankan elephant that had died of chronic respiratory disease[6]. *M. elephantis* has been isolated from patients in developed countries, including Canada, Italy, and Belgium; nevertheless, its clinical relevance remains unknown[7-10]. Thus far, the isolation and identification of *M. elephantis* from milk specimens has not been reported in China. In this paper, we report the first Chinese case of the isolation of *M. elephantis* from the milk a cow with mastitis. In addition, we discuss the identification and drug sensitivity of this bacterium in this paper.

MATERIALS AND METHODS

Milk Sample

Fresh milk was collected from a cow with mastitis by a technician of a dairy cattle breeding company in Shaanxi Province, China. The sample was not sterilized.

Antibiotics and Chemicals

Cation-adjusted Mueller-Hinton broth (CAMHB) was purchased from Difco (Detroit, MI, USA). Alamar blue was purchased from AbD Serotec (Oxford, UK). Twenty-seven types of antibiotics were purchased from Sigma-Aldrich Company (St. Louis, MO, USA). Gatifloxacin and rifabutin were procured from Toronto Research Chemicals Inc. (Toronto, Canada). The details of the 27 antibiotics are shown in Table 1.

Reference Strains

The reference strain NTM95080 (originally purchased from National Institutes for Food and Drug Control) and H37Rv were provided by and stored at the Tuberculosis Laboratory, National Institute for Communicable Disease Control and Prevention, Chinese Center for Disease Control and Prevention.

Specimen Processing and Mycobacterial Culture

A 30-mL milk sample was placed in a 50-mL centrifuge tube and centrifuged at 12,000 rpm for 15 min to obtain a precipitate. The precipitate was preprocessed with an equal volume of 4% NaOH (1:1) for 15 min to eliminate contaminants. Then, 45 mL of PBS (0.01 mol/L pH 7.2) was added to the sample to dilute NaOH. The sample was centrifuged again at 12,000 rpm for 15 min to obtain a precipitate. The precipitate was resuspended in 1.0 mL PBS. To culture mycobacteria, L-J medium was inoculated with 0.5 mL of the suspension.

Multiple Loci Polymerase Chain Reaction (PCR)

After three generations of culture on L-J culture, individual colonies were selected and placed in 1.5-mL screw-capped centrifuge tubes with 0.5 mL water. Bacteria were inactivated by incubating the tubes in a water bath at 80 °C for 30 min. Bacterial cells were collected by centrifuging the incubated tubes at 12,000 rpm for 5 min. The precipitate was resuspended in 300 μL water. To release genomic DNA from the bacterial suspension, the tubes were placed in a metal bath at 100 °C for 20 min, then centrifuged at 12,000 rpm for 5 min. The supernatant was then collected for PCR amplification. Multiple loci PCR with seven target genes, including *16S rRNA*, *Rv0577*, *IS1561*, *Rv1510*, *Rv1970*, *Rv3877*, and *Rv3120*, was used to identify the strain as *Mycobacterium tuberculosis* complex or NTM. Refer to reference[11] for the identification criteria and the PCR primers used to amplify the target gene loci from the chromosomal DNA of the isolate.

Multi-locus Sequence Analysis

Multi-locus sequence analysis (MLSA) was used to identify the *Mycobacterium* species of the isolate based on the sequences of the *16S rRNA, SodA, hsp65*, and *ITS* genes. *hsp65* was amplified using the *hsp65F* and *hsp65R* primers reported by Huard Richard C, et al.[11]. *SodA* was amplified using the *SodIgF* and *SodIgF* primers reported by Adékambi Toïdi, et al.[12]. *16s-23s ITS* was amplified using the *16sF* and *23sR* primers reported by Roth A, et al.[13]. A total of 2 μL of DNA, 1 μL of primers, and 8.5 μL distilled water were added to 12.5 μL 2 x Taq PCR

MasterMix to a final volume of 25 μL. PCR products were sent to Tsingke Company (Beijing, China) for two-way sequencing and sequence splicing to obtain the complete sequence of the PCR products. The sequencing results of *16SrRNA/SodA/hsp65/ITS* were submitted to the National Center for Biotechnology Information (NCBI) website for homology comparison. Those that displayed a similarity of over 98% were accepted as species[14]. MEGA 6.01 software was used for cluster analysis. Alignment and evolutionary tree construction were conducted using MUSCLE with Maximum Likelihood method.

Drug Sensitivity Testing

We performed the alamar blue assay to test the sensitivity of the isolate ShaanX15001 and the reference strain NTM95080 to 27 antibacterial drugs. Minimum inhibitory concentration (MIC) testing was performed in accordance with the guidelines established and approved by Clinical and Laboratory Standards Institute[15]. We combined the existing MIC thresholds of the tested drugs to estimate the susceptibility of the isolate to different antibacterial drugs.

Preparation of Microbial Dilution   Colonies that were growing well in L-J medium were scraped and placed in normal saline, suspended to 0.5 McIntosh concentration (approximately 0.5 mg/mL), and diluted with liquid medium at a ratio of 1:200 to a final concentration of approximately 10^5 CFU/mL of inoculant suspension.

Establishment of a Drug Concentration Gradient A total of 100 μL liquid CAMHB was added to each well of a 96-well plate except to border wells. Two-fold diluted drugs were added to the plate to establish the corresponding drug test range, as shown in Table 1. Finally, 100 μL of bacterial suspension was added to each well. Three negative controls were set. The drug-free control well (CAMHB + inoculum) was used as a reference for the addition of alamar blue. The well containing CAMHB without inoculum was used as reference to decide the interference of CAMHB with alamar blue. A series of control wells containing different concentration gradients of each drug and drug-CAMHB mixture were used as a reference for the interference of CAMHB mixture with alamar blue. The plates were sealed in individual Ziploc bags and then incubated at 37 °C.

Interpretation of Experimental Results   After incubation at 37 °C for 24 h, the first drug-free control wells were examined using an indicator (20 μL alamar blue and 50 μL sterile 5% Tween-80). The plates were then re-incubated for 24 h. If the control well turned pink, all of the other wells containing drugs received the indicator. After an additional 24 h of incubation, the colors of all wells were recorded. If the color of the first drug-free growth control well did not change to pink, the second drug-free control well received the indicator and the above steps were repeated. MIC was based on the range between the non-discolored hole that corresponded to the lowest drug concentration and discolored hole that corresponded to the largest drug concentration[16]. The MIC value reflected the bacteriostatic activity of the drug. A low value indicated high bacteriostatic activity. The final MIC of each drug was calculated as the mean of two or three tests. The MIC thresholds indicating sensitivity, moderate susceptibility, and resistance were interpreted in references[15,17-19].

Ethics Statement

Experimental animals were scientifically and rationally treated in accordance with the '3R' principle: 'Reduction, Replacement, and Refinement.' This study was performed in strict accordance with the recommendations provided in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. This study was approved by the Ethics Committee of the National Institute for Communicable Disease Control and Prevention, Chinese Center for Disease Control and Prevention. The company that provided the milk for this study also provided written informed consent.
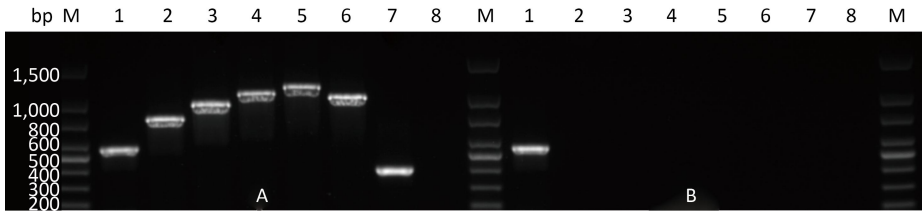
RESULTS

Culture Results

Several smooth and moist pale yellow colonies appeared on the L-J medium within a week after inoculation.

Multiple Loci PCR

The results of multiple loci PCR analysis were consistent with those for mycobacteria other than tuberculosis, e.g., NTM. In the results, only *16S rRNA* corresponded with the target band. The results are shown in Figure 1.

MLSA

The sequences of *16S rRNA*, *SodA*, *hsp65,* and *ITS* of ShaanX15001 revealed 99%, 99%, 99%, and 100% similarities, respectively, with those of the validated *M. elephantis* reference strains (NCBI). The

Figure 1. Multiple loci PCR results for H37Rv and ShaanX15001. A: H37Rv, B: ShannX15001, M: DNA marker. Molecular weights from the top to the bottom of the lane followed the order of 1,500 bp, 1,000 bp, 800 bp, 600 bp, 500 bp, 400 bp, 300 bp, and 200 bp. Lanes 1, 2, 3, 4, 5, 6, 7, and 8 correspond to *16S rRNA*, *Rv0577*, *IS1561*, *Rv1510*, *Rv1970*, *Rv3877*, *Rv3120*, and the negative control, respectively.
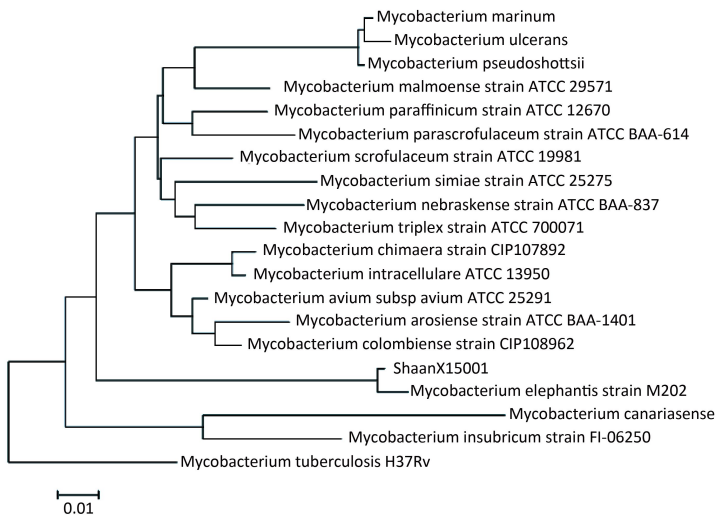
Figure 2. Sequence analysis of *hsp65* genes of partial nontuberculous mycobacterium strains and ShaanX15001 isolated from milk.
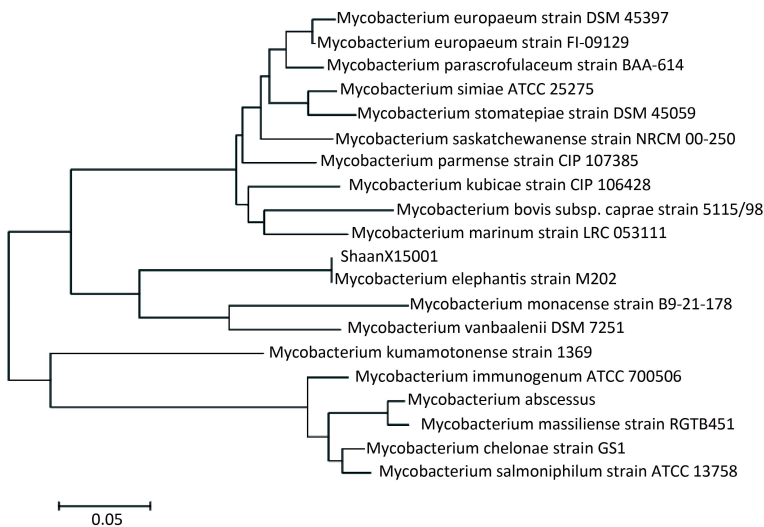
Figure 3. Sequence analysis of the *ITS* genes of partial nontuberculous mycobacterium strains and ShannX15001 isolated from milk.

clustering results for Hsp65 and ITS are shown in Figures 2 and 3, respectively. These results provide further evidence that ShaanX15001 is *M. elephantis.*

Drug Sensitivity Testing

The drug sensitivity results showed that the strain was resistant to isoniazid, p-aminosalicylic acid, and trimesulf. The strain was sensitive to ofloxacin, rifampicin, amikacin, capreomycin, moxifloxacin, kanamycin, levofloxacin, cycloserine, ethambutol, streptomycin, tobramycin, rifabutin, ciprofloxacin, linezolid, cefoxitin sodium, clarithromycin, and minocycline (Table 1).

DISCUSSION

Identifying NTM on the species level is difficult given the lack of a rapid and easy method for NTM identification. The *hsp65* gene has been used to identify mycobacteria since 1993[20] given its high conservation among mycobacterial species and interspecific differences. However, in this study, we found that the *ITS* gene is better at distinguishing between species than *hsp65* gene. Therefore, multiple genes should be used for species identification when the use of one gene could not provide definitive identification.

NTM can cause serious infections by direct transmission from the environment or after colonization. Bold *et al.* suggested that slowly growing mycobacteria are usually isolated from the lower respiratory tract of patients with NTM infections, whereas rapidly growing mycobacteria are more frequently isolated from other tissues[21]. Rapidly growing NTM species are more prevalent in

Table 1. Drug Sensitivity Test Results of the Isolate from Milk Samples

No	Antibiotics	Concentration range (µg/mL)	95080 (µg/mL)	ShaanX15001 (µg/mL)	RGM Dividing Value (µg/mL)		
					S	I	R
1	Ofloxacin	64-0.031	0.062-0.125	0.125-0.25	-	-	2
2	Isoniazid	256-0.125	2-4	4-8			5
3	Rifampicin	256-0.125	0.125-0.25	< 0.125	-	-	1
4	Amikacin	16-0.008	0.125-0.25	0.125-0.25	16	32	64
5	Capreomycin	16-0.008	0.25-0.5	0.25-0.5	-	-	2.5
6	Moxifloxacin	16-0.008	0.008-0.015	0.015-0.031	1	2	4
7	Kanamycin	64-0.031	0.25-0.5	0.125-0.25	-	-	4
8	Gatifloxacin	16-0.008	0.015-0.031	0.031-0.062	-	-	-
9	Levofloxacin	16-0.008	0.031-0.062	0.062-0.125	2	4	8
10	Cycloserine	128-0.063	16-32	16-32	-	-	50
11	P-aminosalicylic Acid	16-0.008	> 16	> 16	-	-	2
12	Ethambutol	128-0.063	< 0.062	< 0.062	5	-	10
13	Streptomycin	128-0.063	0.125-0.25	0.062-0.125	5	-	10
14	Tobramycin	64-0.031	0.125-0.25	0.125-0.25	2	4	8
15	Rifapentine	32-0.016	4-8	4-8	-	-	-
16	Rifabutin	32-0.016	0.5-1	0.5-1	-	-	2
17	Ciprofloxacin	32-0.016	0.031-0.062	0.062-0.125	1	2	4
18	Linezolid	64-0.031	0.25-0.5	0.125-0.25	8	16	32
19	Sparfloxacin	16-0.008	0.008-0.015	0.031-0.062	-	-	-
20	Pasiniazid	64-0.031	0.5-1	0.25-0.5	-	-	-
21	Clofazimine	16-0.008	0.125-0.25	0.125-0.25	-	-	-
22	Cefoxitin	64-0.031	16-32	8-16	16	32-64	128
23	Meropenem	64-0.031	32-64	8-16	4	8-16	32
24	Clarithromycin	32-0.016	0.25-0.5	0.125-0.25	2	4	8
25	Minocycline	32-0.016	0.015-0.031	< 0.015	1	2-4	16
26	Trimesulf	256-0.125	128-256	> 256	38	-	76
27	Sulfamethoxazole	256-0.125	> 256	> 256	-	-	-

Note. '95080' is the reference *M. elephantis* strain, 'ShaanX15001' is the isolate, '-' represents a missing value. The reference values for the drug susceptibility test were reported in references[15,17-19]. RGM, rapid growing mycobacteria. S, sensitive. I, intermediated. R, resistant.

Eastern Asia[22]. Tortoli stated that the environment is the most probable reservoir of *M. elephantis* for either human or animal infections[8]. These reports are all consistent with our results, which showed that *M. elephantis* is a rapidly growing mycobacteria that can isolated from the milk of a cow with mastitis.

M. elephantis, a rapidly growing mycobacterial species, was first isolated in 2,000 from the lung abscess of an elephant. Since then, this mycobacterium has been occasionally isolated from the clinical samples of human patients and is most commonly isolated from sputum[6-8]. *M. elephantis* has also been isolated from a cervical lymph node sample, although its relationship with the clinical disease is unclear[7]. This study is the first to report a Chinese case of the isolation of *M. elephantis* from milk taken from a cow with mastitis.

Milk is pasteurized to ensure the safety of milk and dairy products for human consumption. However, to retain nutrients and active substances in milk, milk companies may apply temperatures lower than 60 °C during pasteurization. During pasteurization, milk is subjected to low temperatures for a long period or to high temperatures for a short period. The optimal growth temperature of *M. elephantis* is 42 °C; this species does not grow at 52 °C[7]. Nevertheless, we posit that *M. elephantis* may remain in milk due to incomplete pasteurization. Patients who drink milk as a nutritional supplement may acquire *M. elephantis* infections from milk, thus aggravating their illness. If the milk is used to produce milk powder for babies, babies who drink the milk powder could become seriously ill.

The control of cow tuberculosis must adhere to the policy of 'Prevention First' combined with the comprehensive preventative and control measures of monitoring, quarantine, culling, and disinfection. Cows should be regularly monitored, and tuberculosis-positive cattle should be immediately removed from the herd and subjected to clinical examination, and, if necessary, to bacteriological examination. Sick cows should receive treatment in accordance with the relevant provisions of the country. The pool, barn, stadium, and relevant vehicles and personnel should be disinfected.

Finally, more attention should be paid to the screening of milk products released into the market to ensure the safety of dairy products and avoid potential risks.

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