Survey of human and bovine tuberculosis infection on dairy farms in southwestern China

Shu Zhang (✉ dulianyouchao@qq.com)

Sichuan Center for Disease Control and Prevention <https://orcid.org/0000-0001-6123-5418>

Chuang Chen

Sichuan Center for Disease Control and Prevention, Chengdu, Sichuan, China.

Zhengyuan Rao

Sichuan Center for Disease Control and Prevention, Chengdu, Sichuan, China.

Aiguo Yang

Sichuan Animal Epidemic Prevention and Control Center, Chengdu, Sichuan, China.

Li Guo

Sichuan Animal Epidemic Prevention and Control Center, Chengdu, Sichuan, China.

Wei Hou

Sichuan Animal Epidemic Prevention and Control Center, Chengdu, Sichuan, China.

Liang Zhang

Hongya Center for Disease Control and Prevention, Hongya County, Meishan, Sichuan, China.

Xiulan Yang

Hongya Center for Disease Control and Prevention, Hongya County, Meishan, Sichuan, China.

Yan Liu

Hongya Center for Animal Epidemic Prevention and Control, Hongya County, Meishan, Sichuan, China.

Yumei Wu

Hongya Center for Animal Epidemic Prevention and Control, Hongya County, Meishan, Sichuan, China.

Research article

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Abstract

Background: Tuberculosis is a zoonotic chronic infectious disease caused by *Mycobacterium tuberculosis* complex (MTBC) that can be transmitted between humans and cattle. The aim of our study was to identify cross-infections of MTBC between humans and cattle on dairy farms in Sichuan Province, southwestern China. **Results:** We selected 6 large-scale and 5 small-scale dairy farms in Sichuan Province as research sites. A total of 378 dairy farm workers (except one pregnant woman) were screened for tuberculosis symptoms and examined by X-ray. One worker was diagnosed as tuberculosis, though the sputum culture was negative. In total, 99 of 10,224 (0.97%) cows showed positive results for the purified protein derivative (PPD) skin tests. Esophageal-pharyngeal (OP) secretions from PPD-positive cows were cultured and 21 isolates were obtained. Sequences of 16s rDNA, hsp65 and rpoB and 16s-23s rRNA spacer region were amplified and sequenced. BLAST analysis classified these isolates as nontuberculous mycobacteria (NTM) (18 *M. nonchromogenicum*, 1 *M. hiberniae*, 1 *M. arupense*, and 1 *M. chitae* isolates). **Conclusion:** This study indicates that the PPD-positive cows in these dairy farms were infected with NTM rather than MTBC. Tuberculosis cross-infection between humans and cows on dairy farms has been well controlled in this region.

Background

Tuberculosis is a chronic zoonotic disease caused by the *Mycobacterium tuberculosis* complex (MTBC), which includes *M. tuberculosis*, *M. bovis*, *M. africanum*, *M. pinnipedii*, *M. microti*, *M. caprae*, *M. canetti*, *M. mungi*, *M. orygis*. It mainly infects respiratory tract, digestive tract (food and milk with bacteria), and a low percentage of skin wounds. Humans are susceptible to *M. tuberculosis* and *M. bovis* [1]. Cattles are considered as a natural reservoir for *M. bovis* [1], and infection caused by *M. tuberculosis* [2,3] and *M. caprae* [4,5] in cattle were reported. The other members of MTBC could also cause tuberculosis in human and/or other animals, but are seldom found in bovine. Transmission of *M. tuberculosis* complex (mostly *M. tuberculosis* and *M. bovis*) between human and cattle are reported [6-8].

As stated in previous reports, the infection status of bovine tuberculosis is seriously, especially dairy cows are most susceptible bovines to tuberculosis [9]. Dairy cows can transmit tuberculosis to workers through respiratory secretions and feces. Milk secreted by cows with tuberculosis and dairy products that do not undergo pasteurization can also transmit tuberculosis to humans [10-11]. Approximately 3% of tuberculosis patients become infected with *M. bovis* by consuming unsterilized milk or dairy products [12-13], and slaughterhouse workers may also be infected by infected tissue [14]. In Asian and African, 4.7% to 30.8% of tuberculosis-positive cattle are infected with human *M. tuberculosis* strain [15]. Moreover, prevalence of human tuberculosis will rise when the proportion of cattle infected with human tuberculosis bacteria increases on a given farm [16]. According to reports from 2010, the prevalence of bovine tuberculosis in China was 0-15% [17]. Some researches indicate simultaneous infection of MTBC in humans and cattle [1,7,18]. Therefore, we conducted a survey of tuberculosis among cows and workers in large and small dairy farms in Sichuan Province, southwestern China, aiming to evaluate cross infections of MTBC between bovines and humans.

Results

Population survey

A total of 378 workers from 5 large-scale dairy farms and 6 small-scale dairy farms were screened for symptoms; 377 (a pregnant woman was not included) were examined by chest X-ray (DR). Based on the symptom screening results, all workers were asymptomatic; 1 person had an abnormal chest X-ray result with suspected tuberculosis lesions. Sputum samples collected from 5 workers (one pregnant woman; one abnormal chest X-ray person; three slight cough persons) were subjected to sputum smear and culture, and all samples showed negative results. One patient with abnormal chest X-ray result was clinically diagnosed and referred for tuberculosis management and treatment.

Investigation of dairy cows

A total of 10,224 cows were examined by PPD skin tests. Ninety-nine cows were PPD positive, but none of them displayed suspicious symptoms. Esophageal-pharyngeal (OP) secretions of the 99 PPD-positive cows were cultured on acid LJ medium, 21 bacteria isolations were obtained from these samples. OP secretions from 88 PPD-negative cows and 20 environmental samples that were adjacent to PPD-positive cows were also cultured, and all samples showed negative results.

Identification of species

The sequences of the 16S rDNA, *hsp65*, and *rpoB* genes and ITS region were sequenced to identify species by multilocus sequence analysis. The species of the 21 strains are presented in Table 1. All of the strains were nontuberculous mycobacteria (NTM).

Drug sensitivity testing results

Antimicrobial susceptibility testing results for the 20 isolates (*Mycobacterium arupense* did not grow) from the PPD-positive cows are shown in Table 2.

Discussion

The tuberculosis epidemic in China is high; however, it is not as severe as those in India and Indonesia. Sichuan Province is a populous province in southwestern China, and the epidemic of tuberculosis in this province is very concerning. In addition, many dairy cows raised in Sichuan Province. Tuberculosis is a zoonotic infectious disease caused by MTBC. Previous reports have shown evidence of tuberculosis cross infection between humans and cattle[1,2,6,8].

We screened dairy farm workers for tuberculosis symptoms and performed chest X-ray; all cows were screened by PPD skin tests. OP secretions were obtained from PPD-positive cattle, and the cows were fasted for 12 hours (with water accessible) to ensure that the OP secretions were not contaminated by ruminate. The results of the environmental sample cultures for the PPD-positive cattle were negative. Culture was also used to verify that the samples from OP secretions were not contaminated by environmental mycobacteria.

According to previous Chinese reports, the infection rate of MTBC in cows on dairy farms in China is 1% by the PPD methods. In our study, the PPD-positive rate was approximately 1%, too, which is consistent with previous reports. Through the culture of OP secretions from PPD-positive cows, 21 isolates were obtained; however, these isolates were classified as NTM after *16S rDNA*, *hsp65*, *rpoB* gene and *ITS* region sequencing and alignment. This finding indicates that there may be an error in the 1% MTBC infection rate reported in the previous paper mentioned above, as these cows were likely infected with NTM. According to our current data, tuberculosis in dairy cows has been well controlled, and NTM is the most likely infection source in PPD-positive cows. So, it is important to confirm the true MTBC infection rate of dairy cows in China by culture and PCR.

In this study, 21 NTM strains (18 *M. nonchromogenicum*, 1 *M. hiberniae*, 1 *M. arupense*, and 1 *M. chitae*) were isolated. A study from Northern Ireland also reported culture of *M. nonchromogenicum* (a member of the *M. terrae* complex, a nonpigmented, slow-growing species) from cattle, indicating that *M. nonchromogenicum* is a dominant mycobacterium among cattle strains[22]. In previous studies, *M. nonchromogenicum*, *M. hiberniae*, and *M. arupense* were also found to be responsible for human infection and disease[23-27]. The minimum inhibitory concentrations (MICs) of 21 types of drugs against *M. nonchromogenicum*, *M. hiberniae* and *M. arupense* were relatively high in the drug sensitivity test; in contrast, the *M. chitae* MIC was low. Therefore, it is difficult to select appropriate drugs for the treatment of these NTM. Therefore, these NTM MICs will provide some guidance for future research. We believe that our study makes a significant contribution to the literature because it is the first to identify infection with NTM but not MTBC in PPD-positive dairy cows in southwestern China.

Conclusions

Our study indicates that the PPD-positive rate of cows on dairy farms in Sichuan Province, which is located in southwestern China, was approximately 10‰, but these cows were infected with NTM rather than *Mycobacterium bovis* or *Mycobacterium tuberculosis*. Crossinfection of MTBC between humans and cows on dairy farms has been controlled.

Methods

Research sites

The study sites were in Hongya County, Sichuan Province, southwestern China. All dairy farms in this county were in this survey, including five large-scale dairy farms and six small-scale dairy farms.

Investigation methods

All the staff at the investigation sites were screened for clinical symptoms (coughing lasts two or more weeks, coughing up blood, chest pain, unintentional weight loss, Fatigue, Fever, night sweats, chills, loss of appetite) and examined by chest X-ray (DR). Sputum smears (night sputum, morning sputum and spot sputum) and sputum cultures were performed for those with suspected tuberculosis symptoms and suspected tuberculosis on chest X-ray.

All dairy cows at the survey sites were screened by single intradermal cervical comparative test (SICCT) using purified protein derivative (PPD) of *Mycobacterium bovis* tuberculin(purchased from Zhongmu CO., LTD., Chengdu, China). The cows were inoculated with 0.1ml of bovine PPD in the position of anterior neck, the results were computed as the difference in skin fold thickness in millimeters(mm) before and 72($\pm$ 4) hours after injection of the bovine PPD. The skin fold thickness increased 4 mm or more, or severe local clinical signs(pain, edema, necrosis, exudation, emphysema, swelling, and/or local lymph node hypertrophy) were observed, it was considered the reactor to be positive.

Esophageal-pharyngeal (OP) secretions obtained from dairy cows (PPD-positive cows, cows with suspected tuberculosis symptoms, and adjacent cows of PPD-positive cows) that were fasted (water was provided) for 12 hours were cultured; environmental samples of PPD-positive cows were collected for culture as well.

Culture and DNA extraction

After treatment with 4% NaOH, acid Löwenstein-Jensen (LJ) medium (purchased from Celnovte, CO., LTD., Zhengzhou, China) was used to culture the samples. Each sample was cultured in two tubes at 37°C for 1-2 months.

DNA was extracted from the cultured strains by the heating method. The culture strains were suspended in 100 μ l of sterile distilled water and heated at 99°C in a heating block for 20 min. The DNA obtained was stored at -20°C.

Amplification and sequencing of the 16S rDNA, hsp65, and rpoB genes and ITS region

Primers were designed to amplify the 16S rDNA, hsp65, and rpoB genes and internal transcribed spacer (ITS) region to accurately identify Mycobacterium strains, according to previous reports[19-21].

16S rDNA *PCR*

To amplify the *16S rDNA* of *Mycobacterium*, the following primers were used: 16S-F(5'-AGAGTTTGATCCTGGCTCAG-3') and 16S-R(5'-AAGGAGGTGATCCAGCCGCA-3'). Two microliters of culture-suspended DNA was used as the DNA template in a 50- μ l PCR mixture containing 25 μ l of 2**Taq* Master Mix (purchased from Cwbiotech, CO., LTD., China), 1 μ l of each forward and reverse primer (10 pmol/l) and 21 μ l of double-distilled water. The PCR cycling parameters were as follows: 95°C for 5 min for initial denaturation; followed by 35 cycles of denaturation for 1 min at 95°C, annealing for 1 min at 58°C, and elongation for 1 min at 72°C; and a final extension at 72°C for 10 min.

hsp65 *PCR*

The primers *hsp65*-F(5'-ATCGCCAAGGAGATCGAGCT-3') and *hsp65*-R(5'-AAGGTGCCGCGGATCTTGTT-3') were used to amplify the *hsp65* gene. The 50- μ l PCR mixture contained 21 μ l of double-distilled water, 25 μ l of 2**Taq* Master Mix (purchased from Cwbiotech, CO., LTD.,China), 1 μ l of each forward and reverse primer (10 pmol/l), and 2 μ l of DNA template. The PCR cycling parameters were as follows: initial denaturation at 95°C for 5 min; followed by 35 cycles of denaturation for 1 min at 95°C, annealing for 1 min at 63°C, and elongation for 1 min at 72°C; and a final extension at 72°C for 10 min.

rpoB *PCR*

rpob-F(5'-GGCAAGGTCACCCCGAAGGG-3') and *rpob*-R(5'-AGCGGCTGCTGGGTGATCATC-3') were used for amplification of the *rpob* gene. The 50- μ l PCR mixture contained 21 μ l of double-distilled water, 25 μ l of 2**Taq* Master Mix (purchased from Cwbiotech, CO., LTD., China), 1 μ l of each forward and reverse primer (10 pmol/l), and 2 μ l of DNA template from the culture suspensions. The PCR cycling parameters were as follows: initial denaturation at 95°C for 5 min; followed by 35 cycles of denaturation for 1 min at 95°C, annealing for 1 min at 60°C, and elongation for 1 min at 72°C; and a final extension at 72°C for 10 min.

16S rDNA-23S rDNA internal transcribed spacer(ITS) *PCR*

ITS-F(5'-AAGTCGTAACAAGGTARCCG-3') and ITS-R(5'-TCGCCAAGGCATCCACC-3') were used for amplification of the ITS gene fragment. The 50- μ l PCR mixture contained 21 μ l of double distilled water, 25 μ l of 2**Taq* Master Mix (purchased from Cwbiotech, CO., LTD., China), 1 μ l of each forward and reverse primer (10 pmol/l), and 2 μ l of DNA of the culture suspensions. The PCR cycling parameters were as follows: initial denaturation at 95°C for 5 min; followed by 35 cycles of denaturation for 1 min at 95°C, annealing for 1 min at 60°C, and elongation for 1 min at 72°C; and a final extension at 72°C for 10 min.

Sequencing of 16S rDNA, hsp65, and rpoB genes and the ITS region amplification products

Gene amplification products of the *16S rDNA*, *hsp65*, and *rpoB* genes and the *ITS* region were sequenced by Tsingke (Beijing, China).

Alignment of sequences and identification of species

The sequences of the amplified products (*16S rDNA*, *hsp65*, *rpoB* and *ITS*) were analyzed using the NCBI BLAST platform (<https://blast.ncbi.nlm.nih.gov/Blast.cgi>) with MegaBLAST for species identification.

Drug sensitivity tests

The in vitro drug susceptibility of the strains was evaluated by the broth dilution method according to the guidelines of the Clinical and Laboratory Standards Institute (CLSI).

List Of Abbreviations

MTBC: *Mycobacterium tuberculosis* complex; PPD: purified protein derivative; OP: esophageal-Pharyngeal; NTM: nontuberculous mycobacteria; *hsp65*: heat shock protein 65; *rpoB*: β subunit of bacterial RNA polymerase; *ITS*: *16S rDNA*-*23S rDNA* internal transcribed spacer

Declarations

Ethics approval and consent to participate

This study was authorized by the Sichuan Center for Disease Control and Prevention and the Sichuan Medical Association. Ethical approval was obtained from the Research Ethics Committee of Sichuan Center for Disease Control and Prevention. The animals in this study were taken for routine diagnostics and not used for experimental studies, and the material consisting of esophageal-pharyngeal secretions (OP secretions) used in this study was collected by local vets after verbal approval from the owners for further research. All the staff involved in this study gave oral informed consent. According to the review of biomedical research ethics (SC/DL-GZ023) of Sichuan Centers for Disease Control and Prevention, collecting animal sputum culture isolates does not require ethical review. We drafted written informed consent for the participants, but the owners of the dairy farms believed that oral consent was faster and more convenient and supported oral consent. As the ethical review committee agreed to this practice (Sichuan CDC ethical committee ethical review 4.7: Oral informed consent is allowed when signature would bring trouble to someone), we obtained oral informed consent.

Consent for publication

Not applicable.

Availability of data and materials

All data supporting the results of this paper are included in this published article.

Competing interests

The authors declare that they have no competing interests.

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Author contributions

Chuang Chen supervised the project. Zhengyuan Rao, Aiguo Yang, Li Guo, Wei Hou, Liang Zhang, XiulanYang, YanLiu, YumeiWu and Shu Zhang participated in the field investigation. Chuang Chen and Shu Zhang completed the lab work. Shu Zhang drafted and revised the manuscript. All authors read and approved the final manuscript.

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Tables

Table 1. The species and strains obtained from the PPD-positive cows.

NTM species identified	Number of strains
<i>Mycobacterium nonchromogenicum</i>	18
<i>Mycobacterium hiberniae</i>	1
<i>Mycobacterium arupense</i>	1
<i>Mycobacterium chitae</i>	1

Table 2. Distribution of antimicrobial susceptibility of the isolates.

Antimicrobial agent	Drug concentration range (µg/ml)	The 18 isolates of <i>M. nonchromogenicum</i>		MIC (µg/ml) of the isolate of <i>M. hiberniae</i>	MIC (µg/ml) of the isolate of <i>M. chitae</i>
		MIC ₅₀ (µg/ml)	MIC ₉₀ (µg/ml)		
moxifloxacin (MOX)	0.0625-64	2	4	2	<0.0625
gatifloxacin (GAT)	0.0625-64	4	4	4	<0.0625
tigecycline (TIG)	0.0625-64	>64	>64	32	<0.0625
clofazimine (CFM)	0.03125-32	>32	>32	>32	<0.0625
cefoxitin (CFX)	0.25-256	64	>256	>256	<0.0625
vancomycin (VCM)	0.25-256	>256	>256	>256	<0.0625
streptomycin (SM)	0.0625-64	8	>64	8	<0.0625
amikacin (AMK)	0.0625-64	16	64	16	<0.0625
azithromycin (AZM)	0.0625-64	>64	>64	>64	0.125
sulfamethoxazole (SMZ)	0.25-256	256	>256	>256	<0.25
rifabutin (RFB)	0.0625-64	8	32	32	<0.0625
linezolid (LZD)	0.0625-64	64	>64	>64	<0.0625
minocycline (MIN)	0.25-256	64	>256	64	<0.25
clarithromycin (CLA)	0.0625-64	16	>64	>64	<0.0625
meropenem (MEM)	0.25-256	32	64	64	<0.25

ethambutol (EMB)	0.0625-64	1	1	1	<0.0625
capreomycin (CAP)	0.0625-64	8	16	4	<0.0625
rifampicin (RIF)	0.0625-64	32	64	32	<0.0625
imipenem (IMP)	0.25-256	>256	>256	>256	<0.25
levofloxacin (LFX)	0.0625-64	8	64	16	<0.0625
Bedaquiline	0.015625-16	>16	>16	>16	<0.015625