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

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Research article

Keywords: Zoonotic disease; Nontuberculous Mycobacteria; Susceptibility; Bovine

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Abstract

Background Tuberculosis is a zoonotic chronic infectious disease that can be transmitted through respiratory secretions, causing simultaneous infection in both people and cattle. The aim of our study was to identify the co-infections of *Mycobacterium tuberculosis* between human and cattle in dairy farms in Sichuan Province, southwest China. **Results** We selected 6 large-scale dairy farms and 5 small-scale dairy farms in Sichuan Province, southwest China, as research sites. A total of 378 dairy farm workers (except one pregnant woman) were screened for tuberculosis symptoms and examined by X-ray radiographs. One worker was diagnosed with tuberculosis, but the sputum culture result was negative, and no strain was obtained. The purified protein derivative (PPD) skin tests of 10224 dairy cows showed that 99 cows were positive, with a positive rate of 9.7‰. The Oesophageal-Pharyngeal (OP) secretions of PPD-positive cows were cultured, and 21 strains were obtained. After amplifying and sequencing the 16SrDNA, hsp65 and rpoB genes and the ITS region, sequence alignment in BLAST showed that these strains were nontuberculous mycobacteria (NTM) (18 *Mycobacterium nonchromogenicum*, 1 *Mycobacterium hiberniae*, 1 *Mycobacterium arupense* and 1 *Mycobacterium chitae*). **Conclusion** This study indicated that PPD-positive cows on dairy farms were infected with NTM rather than *Mycobacterium bovis*. Cross-infection of tuberculosis between humans and cows on dairy farms has been controlled.

Background

Tuberculosis is a chronic zoonotic disease caused by the *Mycobacterium tuberculosis* complex (MTBC), which mainly includes *M. tuberculosis* (human-like), *Mycobacterium bovis*, *Mycobacterium africanum* and *Mycobacterium microti*. Humans and cattle share similar susceptibility to *M. tuberculosis* (human strain) and *M. bovis* [1-4].

As stated in previous reports, the infection status of bovine tuberculosis is grim. Livestock, cattle, especially dairy cows, are the most susceptible bovine to tuberculosis [5]. Dairy cows can transmit tuberculosis to workers through oral secretions and feces. Milk secreted by cows with tuberculosis and dairy products that do not undergo pasteurization can also transmit tuberculosis to humans [6-7]. Approximately 3% of tuberculosis patients are infected with *M. bovis* by drinking milk or dairy products that are not sterilized [8-9]. In Asian and African countries and regions, 4.7% to 30.8% of tuberculosis-positive cattle are infected with the human strain [10]. The more serious the prevalence of human tuberculosis on a farm is, the higher the proportion of cattle infected with human tuberculosis bacteria [11]. According to reports from 2010, the prevalence of bovine tuberculosis in China was 0-15% [12]. This indicates that there were simultaneous infections of tuberculosis in humans and cattle [13]. Therefore, we conducted a tuberculosis survey of dairy cows and workers in large and small dairy farms in Sichuan Province, southwestern China, to identify simultaneous infections of tuberculosis in cows and humans.

Full Text

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

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Background: Tuberculosis is a zoonotic chronic infectious disease that can be transmitted through respiratory secretions, causing simultaneous infection in both people and cattle. The aim of our study was to identify the co-infections of *Mycobacterium tuberculosis* between human and cattle in dairy farms in Sichuan Province, southwest China.

Results: We selected 6 large-scale dairy farms and 5 small-scale dairy farms in Sichuan Province, southwest China, as research sites. A total of 378 dairy farm workers (except one pregnant woman) were screened for tuberculosis symptoms and examined by X-ray radiographs. One worker was diagnosed with tuberculosis, but the sputum culture result was negative, and no strain was obtained. The purified protein derivative (PPD) skin tests of 10224 dairy cows showed that 99 cows were positive, with a positive rate of 9.7‰. The Oesophageal-Pharyngeal (OP) secretions of PPD-positive cows were cultured, and 21 strains were obtained. After amplifying and sequencing the 16SrDNA, *hsp65* and *rpoB* genes and the ITS region, sequence alignment in BLAST showed that these strains were nontuberculous mycobacteria (NTM) (18 *Mycobacterium nonchromogenicum*, 1 *Mycobacterium hiberniae*, 1 *Mycobacterium arupense* and 1 *Mycobacterium chitae*).

Conclusion: This study indicated that PPD-positive cows on dairy farms were infected with NTM rather than *Mycobacterium bovis*. Cross-infection of tuberculosis between humans and cows on dairy farms has been controlled.

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Results

Population survey

A total of 378 workers in 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).The symptom screening results showed that all the workers were asymptomatic; 1 person had an abnormal chest X-ray result, with suspected tuberculosis lesions; all 5 worker samples that were subjected to sputum smear and culture were negative. One patient with active pulmonary tuberculosis was clinically diagnosed and was referred for tuberculosis management and treatment.

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A total of 10224 dairy cows were examined by PPD skin tests. Ninety-nine cows were PPD positive, but none of them had suspicious symptoms. The OP secretions of 99 PPD-positive cows were cultured on acid LJ medium, and 21 samples were cultured for bacterial growth. The OP secretions from 88 cows that were adjacent to PPD-positive cows and 20 environmental samples were cultured, with negative results.

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The sequences of the 16SrDNA, *hsp65*, and *rpoB* genes and the ITS region were used 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). The MTBC was not detected.

Drug sensitivity testing results

The 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 serious; however, it is better than those in India and Indonesia. Sichuan Province is a populous province in southwestern China, and the epidemic of tuberculosis is very serious in this province. There are also many dairy cows raised in Sichuan Province. Tuberculosis is a zoonotic infectious disease caused by *M. tuberculosis*. Previous reports have shown evidence of tuberculosis cross-infection between humans and cattle[4,9]. We screened dairy farm workers for tuberculosis symptoms and performed X-ray chest radiographs, and all the cows were screened by PPD skin tests. OP secretions were obtained from PPD-positive cattle; the cows were fasted for 12 hours (with water accessible) to ensure that the OP secretions were not contaminated by ruminant. The results of the environmental sample cultures of the PPD-positive cattle were negative. Culture was also used to verify that the samples from OP secretions were not contaminated by environmental tuberculosis.

According to previous reports in Chinese journals, the tuberculosis infection rate of cows on dairy farms in China is 10‰. In our study, the PPD-positive rate was approximately 10‰, which is consistent with previous reports. Through the culture of OP secretions from the PPD-positive cows, 21 isolates were obtained; however, they were recognized as NTM after sequencing and alignment the 16SrDNA, *hsp65*, *rpoB* gene and ITS region. This finding indicated that there may be an error in the 10‰ tuberculosis infection rate reported in the previous paper in dairy cows in China, as these cows are most likely infected with NTM. Currently, tuberculosis in dairy cows has been controlled, and NTM is the most likely infection source in PPD-positive cows.

In this study, 21 NTM strains(18 *Mycobacterium nonchromogenicum*, 1 *Mycobacterium hiberniae*, 1 *M. arupense*, and 1 *Mycobacterium chitae*) were obtained. Research in Northern Ireland also cultured *M. nonchromogenicum* (a member of the *Mycobacterium terrae* complex, a nonpigmented, slow-growing species) from cattle, indicating that *M. nonchromogenicum* is a dominant Mycobacterium of cattle strains[17]. In previous reports, *M. nonchromogenicum*, *M. hiberniae*, and *M. arupense* were also responsible for human infection and disease[18–22]. The minimum inhibitory concentrations (MICs) of 21 kinds of drugs against *M. nonchromogenicum*, *M. hiberniae* and *M. arupense* were relatively high in the drug sensitivity test; in contrast, the MIC of *M. chitae* was low. Therefore, it is difficult to select appropriate drugs for the treatment of these NTMs. Thus, these MICs of NTMs will provide some

suggestions for the further research. We believe that our study makes a significant contribution to the literature because it is the first time to identify that PPD-positive dairy cows were infected with NTM but not *Mycobacterium tuberculosis* in southwestern China.

Conclusions

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

Methods

Research sites

The study sites were five large-scale dairy farms and six small-scale dairy farms in Hongya County, Sichuan Province, southwest China.

Investigation methods

All the staff at the investigation sites were screened for clinical symptoms and examined by chest X-ray (DR). Then, 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 purified protein derivative (PPD) skin tests (purchased from Zhongmu CO., LTD., Chengdu, China). Then, Oesophageal-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 subjected to culture; 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 by the heating method using the culture strains. The culture strains were suspended in 100 µl of sterile distilled water and then heated at 99°C in a heating block for 20 min. The DNA was stored at –20°C.

Amplification and sequencing of the 16SrDNA, *hsp65*, and *rpoB* genes and the ITS region

According to previous reports[14–16], the 16SrDNA, *hsp65*, and *rpoB* genes and the internal transcribed spacer (ITS) region were used to design the primers for DNA amplification, which were used to accurately identify the *Mycobacterium* strains.

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 µl PCR mixture containing 25 µl of 2**Taq* Master Mix (purchased from Cwbiotech, CO., LTD., China), 1 µl of each forward and reverse primer (10 pmol/l) and 21 µl 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 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 µl PCR mixture contained 21 µl of double distilled water, 25 µl of 2**Taq* Master Mix (purchased from Cwbiotech, CO., LTD., China), 1 µl of each forward and reverse primer (10 pmol/l), and 2 µl 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 the amplification of the *rpoB* gene. The 50 µl PCR mixture contained 21 µl of double distilled water, 25 µl of 2**Taq* Master Mix (purchased from Cwbiotech, CO., LTD., China), 1 µl of each forward and reverse primer (10 pmol/l), and 2 µl of DNA template 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.

16SrDNA–23SrDNA internal transcribed spacer(ITS) PCR

ITS -F(5'- AAGTCGTAACAAGGTARCCG -3') and ITS -R(5'-TCGCCAAGGCATCCACC-3') were used for the amplification of the ITS gene fragment. A 50 µl PCR mixture contained 21 µl of double distilled water, 25 µl of 2**Taq* Master Mix (purchased from Cwbiotech, CO., LTD., China), 1 µl of each forward and reverse primer (10 pmol/l), and 2 µl 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 amplification products of the 16SrDNA, *hsp65*, and *rpoB* genes and the ITS region

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

Alignment of sequences and identification of species

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

Drug sensitivity tests

The in vitro drug susceptibility of these 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: Oesophageal-Pharyngeal; NTM: nontuberculous mycobacteria; *hsp65*: heat shock protein 65; *rpoB*: β subunit of bacterial RNA polymerase; ITS: 16SrDNA–23SrDNA internal transcribed spacer

Declarations

Ethics approval and consent to participate

This study was authorized by Sichuan Center for Disease Control and Prevention, and the Sichuan Medical Association. Its ethical approval was obtained from the Research Ethics Committee of Sichuan Center for Disease Control and Prevention. These animals in this study were taken for routine diagnostics and not used for experimental studies, and the material consisted of Oesophageal-Pharyngeal Secretions (OP Secretions) used in this study were collected by local vets after verbal approvals from the

owners for further researching. All the staff involved in this study gave the informed consent in oral. 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 the written informed consents for the participants, but the owners of the dairy farms believed that oral consent was faster and more convenient, and supported oral consent. 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), so we used oral informed consent.

Consent for publication

Not applicable.

Availability of data and materials

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

Competing interests

The authors declare that they have no competing interests.

Funding

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Authors' contributions

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

References

1. Michalak K, Austin C, Diesel S, et al. Mycobacterium tuberculosis infection as a zoonotic disease: transmission between humans and elephants. *Emerg Infect Dis.* 1998;4:283–287.
2. Ocepek M, Pate M, Žolnir-Dovč M, et al. Transmission of Mycobacterium tuberculosis from human to cattle. *J Clin Microbiol.* 2005;43:3555–3557.

3. Suzuki Y, Matsuba T, Nakajima C. Zoonotic aspects of tuberculosis caused by *Mycobacterium bovis*. *Kekkaku*. 2010;85:79–86.
4. AmeniGK, Tadesse E, Hailu Y, et al. Transmission of *Mycobacterium tuberculosis* between farmers and cattle in Ethiopia. *PLoS ONE*. 2013;8(Suppl 10):e76891.
5. Rebuma F, Rea T, Alehegne W, et al. High Prevalence of Bovine Tuberculosis in Dairy Cattle in Central Ethiopia: Implications for the Dairy Industry and Public Health. *PLoS One*. 2012; 7(12): e52851.
6. Grange JM. *Mycobacterium bovis* infection in human beings. *Tuberculosis (Edinb)*. 2001;81: 71–77.
7. de la Rua-Domenech R. Human *Mycobacterium bovis* infection in the United Kingdom: Incidence, risks, control measures and review of the zoonotic aspects of bovine tuberculosis. *Tuberculosis (Edinb)*. 2001; 86: 77–109.
8. Grange JM, Daborn C, Cosivi O. HIV-related tuberculosis due to *Mycobacterium bovis*. *Eur Respir J*. 1994;7: 1564–1566.
9. Cosivi O, Grange JM, Daborn CJ, et al. Zoonotic tuberculosis due to *Mycobacterium bovis* in developing countries. *Emerg Infect Dis*. 1999;4: 59–70.
10. Thoen CO, LoBue PA, I de Kantor. Why has zoonotic tuberculosis not received much attention. *Int J Tuberc Lung Dis*. 2010;14(9):1073–1074.
11. Klein EJ, Boster DR, Stapp JR, et al. Humans as source of *Mycobacterium tuberculosis* infection in cattle, Spain. *Emerg Infect Dis*. 2011;17(12):2393–2395.
12. Thoen CO, Steele JH, Kaneene JB. Zoonotic tuberculosis *Mycobacterium bovis* and other pathogenic *Mycobacteria*. *Mycobacterium bovis* infection and control in China, Wiley Blackwell, 2014.
13. Hezekiah KA, Victor O A, Mutolib AS. Knowledge and practices about zoonotic tuberculosis prevention and associated determinants amongst livestock workers in Nigeria; 2015. *PLoS One*. 2018; 13(6): e0198810.
14. Springer B, Erik C. Böttger, Philip Kirschner, et al. Phylogeny of the *Mycobacterium chelonae*-like organism based on partial sequencing of the 16S r RNA Gene and Proposal of *Mycobacterium mucogenicum* sp. nov. *Int J Syst Bacteriol*. 1995;45(2):262–7.
15. Kim H, Kim SH, Shim TS, et al. Differentiation of *Mycobacterium* species by analysis of the heat-shock protein 65 gene(hsp65). *Int J syst Evol Microbiol*. 2005;55(Pt 4):1649–56.
16. Slany M, Svobodova J, Ettlova A, et al. *Mycobacterium arupense* among the isolates of non-tuberculous mycobacteria from human, animal and environmental samples. *VeterinarniMedicina*. 2010;55(8):369–376.
17. Mc Corry TP, CM McCormick, MS Hughes, et al. *Mycobacterium nonchromogenicum* in nasal mucus from cattle in a herd infected with bovine tuberculosis. *Vet Microb*. 2004;99:281–285.
18. Sawai T, Inoue Y, Doi S, et al. A case of *Mycobacterium nonchromogenicum* pulmonary infection showing multiple nodular shadows in an immunocompetent patient. *Diagn Microbiol Infect Dis*. 2006;54(4):311–314.

19. Lai CC, Chen HW, Liu WL, et al. Fatal Meningitis Caused by *Mycobacterium nonchromogenicum* in a Patient with Nasopharyngeal Carcinoma. *Clinical Infectious Diseases*. 2008;46(2):325–326.
20. Kazda J, Cooney R, Monaghan M, et al. *Mycobacterium hiberniae* sp. nov. *IntJ Syst EvolMicr*. 1993; 43:352–357.
21. Cloud JL, Meyer JJ, Pounder JI, et al. *Mycobacterium arupense* sp. nov., a non-chromogenic bacterium isolated from clinical specimens. *Int J Syst Evol Microbiol*. 2006;56: 1413–1418.
22. Slany M, Pavlik I. Molecular detection of nontuberculous mycobacteria: advantages and limits of a broad-range sequencing approach. *J Mol Microbiol Biotechnol*. 2012;22(4):268–76.

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The primers *hsp65*-F(5'-ATCGCCAAGGAGATCGAGCT-3') and *hsp65*-R(5'-AAGGTGCCGCGGATCTTGTT-3') were used to amplify the *hsp65* gene. The 50 µl PCR mixture contained 21 µl of double distilled water, 25 µl of 2*Taq Master Mix (purchased from Cwbiotech, CO., LTD., China), 1 µl of each forward and reverse primer (10 pmol/l), and 2 µl 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 the amplification of the *rpoB* gene. The 50 µl PCR mixture contained 21 µl of double distilled water, 25 µl of 2*Taq Master Mix (purchased from Cwbiotech, CO., LTD., China), 1 µl of each forward and reverse primer (10 pmol/l), and 2 µl of DNA template 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.

16SrDNA-23SrDNA internal transcribed spacer(ITS) PCR

ITS -F(5'- AAGTCGTAACAAGGTARCCG -3') and ITS -R(5'-TCGCCAAGGCATCCACC-3') were used for the amplification of the ITS gene fragment. A 50 µl PCR mixture contained 21 µl of double distilled water, 25 µl of 2*Taq Master Mix (purchased from Cwbiotech, CO., LTD., China), 1 µl of each forward and reverse primer (10 pmol/l), and 2 µl 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 amplification products of the 16SrDNA, hsp65, and rpoB genes and the ITS region

Gene amplification products of the 16SrDNA, *hsp65*, and *rpoB* genes and the ITS region were sequenced at Tsingke(BeijingChina).

Alignment of sequences and identification of species

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

Drug sensitivity tests

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

Abbreviations

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

Declarations

Ethics approval and consent to participate

This study was authorized by Sichuan Center for Disease Control and Prevention, and the Sichuan Medical Association. It's ethical approval was obtained from the Research Ethics Committee of Sichuan Center for Disease Control and Prevention. These animals in this study were taken for routine diagnostics and not used for experimental studies, and the material consisted of Oesophageal-Pharyngeal Secretions(OP Secretions) used in this study were collected by local vets after verbal approvals from the owners for further researching. All the staff involved in this study gave the informed consent in oral. 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 the written informed consents for the participants, but the owners of the dairy farms believed that oral consent was faster and more convenient, and supported oral consent. 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), so we used oral informed consent.

Consent for publication

Not applicable.

Availability of data and materials

All data support 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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Authors' contributions

Chuang Chen supervised the project. Zhengyuan Rao, Aiguo Yang, Li Guo, Wei Hou, Liang Zhang, Xiulan Yang, Yan Liu, Yumei Wu and Shu Zhang were participated in the field investigation. Chuang Chen and Shu Zhang finished the lab work. Shu Zhang drafted and revised the manuscript. All authors read and approved the final manuscript.

References

1. Michalak K, Austin C, Diesel S, et al. Mycobacterium tuberculosis infection as a zoonotic disease: transmission between humans and elephants. *Emerg Infect Dis*. 1998;4:283–287.
2. Ocepek M, Pate M, Žolnir-Dovč M, et al. Transmission of Mycobacterium tuberculosis from human to cattle. *J Clin Microbiol* . 2005;43:3555–3557.
3. Suzuki Y, Matsuba T, Nakajima C. Zoonotic aspects of tuberculosis caused by Mycobacterium bovis. *Kekkaku*. 2010;85:79–86.
4. AmeniGK, Tadesse E, Hailu Y, et al. Transmission of Mycobacterium tuberculosis between farmers and cattle in Ethiopia. *PLoS ONE* . 2013;8(Suppl 10):e76891.
5. Rebuma F, Rea T, Alehegne W, et al. High Prevalence of Bovine Tuberculosis in Dairy Cattle in Central Ethiopia: Implications for the Dairy Industry and Public Health. *PLoS One*. 2012; 7(12): e52851.
6. Grange JM. Mycobacterium bovis infection in human beings. *Tuberculosis (Edinb)*. 2001;81: 71–77.

7. de la Rua-Domenech R. Human *Mycobacterium bovis* infection in the United Kingdom: Incidence, risks, control measures and review of the zoonotic aspects of bovine tuberculosis. *Tuberculosis (Edinb)*. 2001; 86: 77–109.
8. Grange JM, Daborn C, Cosivi O. HIV-related tuberculosis due to *Mycobacterium bovis*. *Eur Respir J*. 1994;7: 1564–1566.
9. Cosivi O, Grange JM, Daborn CJ, et al. Zoonotic tuberculosis due to *Mycobacterium bovis* in developing countries. *Emerg Infect Dis*. 1999;4: 59–70.
10. Thoen CO, LoBue PA, I de Kantor. Why has zoonotic tuberculosis not received much attention. *Int J Tuberc Lung Dis*. 2010;14(9):1073-1074.
11. Klein EJ, Boster DR, Stapp JR, et al. Humans as source of *Mycobacterium tuberculosis* infection in cattle, Spain. *Emerg Infect Dis*. 2011;17(12):2393-2395.
12. Thoen CO, Steele JH, Kaneene JB. Zoonotic tuberculosis *Mycobacterium bovis* and other pathogenic *Mycobacteria*. *Mycobacterium bovis* infection and control in China, Wiley Blackwell, 2014.
13. Hezekiah KA, Victor O A, Mutolib AS. Knowledge and practices about zoonotic tuberculosis prevention and associated determinants amongst livestock workers in Nigeria; 2015. *PLoS One* . 2018; 13(6): e0198810.
14. Springer B, Erik C. Böttger, Philip Kirschner, et al. Phylogeny of the *Mycobacterium chelonae*-like organism based on partial sequencing of the 16S r RNA Gene and Proposal of *Mycobacterium mucogenicum* sp. nov. *Int J Syst Bacteriol*. 1995;45(2):262-7.
15. Kim H, Kim SH, Shim TS, et al. Differentiation of *Mycobacterium* species by analysis of the heat-shock protein 65 gene(hsp65). *Int J syst Evol Microbiol*. 2005;55(Pt 4):1649-56.
16. Slany M, Svobodova J, Ettlova A, et al. *Mycobacterium arupense* among the isolates of non-tuberculous mycobacteria from human, animal and environmental samples. *VeterinarniMedicina*. 2010;55(8):369-376.
17. Mc Corry TP, CM McCormick, MS Hughes, et al. *Mycobacterium nonchromogenicum* in nasal mucus from cattle in a herd infected with bovine tuberculosis. *Vet Microb*. 2004;99:281-285.
18. Sawai T, Inoue Y, Doi S, et al. A case of *Mycobacterium nonchromogenicum* pulmonary infection showing multiple nodular shadows in an immunocompetent patient. *Diagn Microbiol Infect Dis*. 2006;54(4):311-314.
19. Lai CC, Chen HW, Liu WL, et al. Fatal Meningitis Caused by *Mycobacterium nonchromogenicum* in a Patient with Nasopharyngeal Carcinoma. *Clinical Infectious Diseases*. 2008;46(2):325-326.
20. Kazda J , Cooney R, Monaghan M, et al. *Mycobacterium hiberniae* sp. nov. *IntJ Syst EvolMicr*. 1993; 43:352-357.
21. Cloud JL, Meyer JJ, Pounder JI, et al. *Mycobacterium arupense* sp. nov., a non-chromogenic bacterium isolated from clinical specimens. *Int J Syst Evol Microbiol*. 2006;56: 1413-1418.
22. Slany M, Pavlik I. Molecular detection of nontuberculous mycobacteria: advantages and limits of a broad-range sequencing approach. *J Mol Microbiol Biotechnol* . 2012;22(4):268-76.

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