Multi-locus sequence typing of Mycoplasma bovis to assess its genetic diversity from 2009-2018 in Ningxia Hui Autonomous Region, China

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Research article

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**Abstract**

**Background:** *Mycoplasma bovis* (*M. bovis*) is a highly contagious cattle pathogen spreading worldwide and especially in Ningxia Hui Autonomous Region in China.

**Results:** Two types of ST, ST10 and ST134, were identified in Ningxia Hui Autonomous Region. Thirty-seven strains belonged to ST10 and 28 strains belonged to ST134. ST134 was a new ST and first found in 2009 and was only widely distributed in Ningxia Hui Autonomous Region at present. The *M. bovis* ST10 was widely spread in many provinces in China and was widespread in Ningxia Hui Autonomous Region since 2010. It is speculated that the prevalence of *M. bovis* ST10 in Ningxia Hui Autonomous Region began in 2010.

**Conclusion:** This study is the first report on the genetic diversity of *M. bovis* from 2009 to 2018 in Ningxia Hui Autonomous Region and provides the epidemiological information. These results may help further our understanding of the evolution of *M. bovis* and provide information that may be useful for the development of novel vaccines.

**Background**

*Mycoplasma bovis* (*M. bovis*) is an important pathogen causing severe pneumonia, mastitis, and arthritis in the world. Especially, pneumonia caused by *M. bovis* has high morbidity and mortality. It is becoming one of the most widely recognized pathogens in the world[1, 2]. The pathogen is highly contagious and can spread rapidly throughout the herd. *M. bovis* can not only cause pneumonia, mastitis, arthritis, and otitis but also induce postpartum infection of the uterus with a mortality rate of 80%[3]. Since there are no effective vaccines and drugs to prevent and cure the disease caused by the pathogen, the incidence of the disease is on the rise[4, 5].

With the completion of whole-genome sequencing of *M. bovis*, a variety of highly repeatable molecular typing methods have been developed for molecular epidemiology and population structure research, including arbitrarily primed PCR (AP-PCR), random amplified polymorphic DNA (RAPD) [6], amplified fragment length polymorphism (AFLP)[7], pulsed-field gel electrophoresis (PFGE)[8], insertion sequence (IS)[9], variable number of tandem repeats (VNTR)[10, 11], multiple-locus variable-number tandem repeat (MLVA)[10] and multi-locus sequence typing (MLST)[5]. Although AP-PCR, RAPD, AFLP, and PFGE methods can obtain a large amount of genetic information, they are subjective in the analysis of DNA fragments and require special equipment[5], so it is difficult to establish a standardized method.

MLST is a rapidly developing molecular biology analysis method with high resolution in recent years. It is suitable for both molecular epidemiological studies and molecular advancement studies. The MLST method compares the nucleic acid sequences of the core fragments of several housekeeping genes and then compares the diversity of the alleles of the strains. Different strains correspond to different sequence types (ST)[4]. Through the STs of *M. bovis* pathogens can be used to understand the genetic diversity, population structure, and evolutionary trend, which will be beneficial to the control of *M. bovis* and the development of vaccines, as well as providing a theoretical basis for the prevention and control of *M. bovis*[4]. MLST is a typing technique based on seven housekeeping genes of *M. bovis* to study the genetic diversity, population structure, and evolutionary trend of *M. bovis*, including alcohol dehydrogenase-1 (*adh-1*), glutamate tRNA ligase (*gltX*), glycerol-3-phosphate dehydrogenase (*gpsA*), DNA gyrase subunit B (*gyrB*), phosphate acetyltransferase-2 (*pta-2*), thymidine kinase (*tdk*) and transketolase (*tkt*)[12]. The MLST data were used to populate a newly created and publicly available database (www.pubmlst.org/mbovis) intended to serve as a tool for epidemiologic studies and further investigating the population structure of *M. bovis*[5]. MLST is a powerful, scalable, and highly standardized method that makes it easy to clearly distinguish housekeeping genes among different strains[13].

Ningxia Hui Autonomous Region is one of the most important raising regions for cow and beef cattle in China[14]. The feeding level of bovine is currently ranked second in China. In 2009, our team isolated *M. bovis* for the first time from the lung of cows in Ningxia Hui Autonomous Region. From 2009 to 2018, a total of 65 strains of *M. bovis* were obtained from samples of lung, synovial fluid, nasal swab, and milk in Ningxia Hui Autonomous Region. However, there is no related study on the molecular epidemiology and population structure of *M. bovis* in Ningxia Hui Autonomous Region. In this study, The MLST method was used to classify 65 isolates from different cities of Ningxia Hui Autonomous Region from 2009 to 2018, aiming to investigate the population structure of *M. bovis*[2, 5] and to explore the evolutionary relationship of Ningxia Hui Autonomous Region isolates with Chinese isolates and global isolates, which will lay a foundation for further prevention and control of *M. bovis* in the world.

**Results**

**Strains Identification**

Sixty-seven isolates were identified as *M. bovis* by PCR amplification using 16SrRNA and *uvrC* primers and sequencing of amplified products.

**MLST analysis of *M. bovis* isolates**

A total of 3 STs were identified among the 67 strains of *M. bovis*. Among the 65 isolates from Ningxia Hui Autonomous Region, 37 isolates belonged to ST10 and 28 isolates belonged to ST134. The HB0801 and PG45 isolates belonged to ST10 and ST17, respectively (Table 1).
According to Table 1, ST10 and ST134 strains could be isolated from samples of clinical mastitis (n=9), arthritis (n=22), and pneumonia (n=34). The main clinical symptoms of calves were arthritis and pneumonia. The clinical symptoms of dairy cattle were mainly mastitis and pneumonia, while beef cattle were mainly pneumonia and arthritis.

The NX001 strain isolated from Ningxia Hui Autonomous Region for the first time in 2009 was ST134, and the NX002 strain isolated in 2010 was ST10. Since then, the ST10 and ST134 have been isolated from different lesions of bovine in different cities in Ningxia Hui Autonomous Region.

**Phylogenetic analysis**

The phylogenetic tree constructed from the concatenated sequences of the seven target genes revealed two distinct lineages (Fig. 1). The ST173 and ST17 were in the same lineage. The other STs were in the other lineage including ST10, ST26, ST32, ST43, ST172, and the Ningxia Hui Autonomous Region isolates of ST134.

**Discussion**

Molecular epidemiological studies are of great significance to reveal the population structure, genetic diversity, and prevalence of *Mycoplasma spp* [4, 15], which facilitates the formulation of effective prevention and control measures, including the development of vaccines and diagnostic methods[4, 16]. MLST studies performed on 44 strains from nine Chinese provinces from 2008 to 2014 showed that ST10, ST32, and ST43 were found in Hubei province (n=25) and that ST10 was also found in Anhui (n=1), Fujian (n=2), Hunan (n=1), Henan (n=8), Inner Mongolia (n=1), Jiangxi (n=3), Guangzhou (n=2), and Shandong (n=1) province [4]. However, there are no reports about the MLST study of *M. bovis* in Ningxia Hui Autonomous Region. Therefore, this study is the first report on the molecular epidemiology of *M. bovis* from 2009 to 2018 in Ningxia Hui Autonomous Region.

At present, there are seven STs in *M. bovis* in China including ST10, ST26, ST32, ST43, ST134, ST172, and ST173[4]. ST10 is widely prevalent in all provinces reported in China, including Ningxia Hui Autonomous Region (n=37, 56.9%) where are no MLST reports. After the ST10 strain was first isolated in 2010 in Ningxia Hui Autonomous Region, the ST10 of *M. bovis* could be isolated and identified every year. And it has been widely diffused in different cities in Ningxia Hui Autonomous Region. Interestingly, the ST10 strains have been reported that were widely distributed in American, Australia, and Israel[4]. Previously, the international spread of contagious bovine pleuropneumonia was shown to be linked to the movement of cattle[4, 17, 18]. So, it is a similar transfer probably that through international movement of cattle and domestic movement of cattle caused the widespread distribution of the *M. bovis* ST10 in China and even in Ningxia Hui Autonomous Region. The reason for the widespread prevalence of ST10 strain in Ningxia Hui Autonomous Region may be that the Ningxia Hui Autonomous Region government strongly supports the construction of large-scale cattle breeding parks. For their expansion, intensive cattle farms had to purchase cattle from different provinces of China and different countries in the world, but *M. bovis* was ignored, which led to the widespread presence of exogenous *M. bovis* in Ningxia Hui Autonomous Region. It is speculated that the prevalence of *M. bovis* ST10 in Ningxia Hui Autonomous Region began in 2010.

In 2009, our lab isolated *M. bovis* from the lung tissue of cows for the first time, which was ST134. From 2009 to 2018, ST134 (n = 28, 43.1%) was isolated and identified from different cities of Ningxia Hui Autonomous Region. However, it has not been identified in other provinces in China. Therefore, it confirmed that ST134 strains were closely related strains with the same origin in Ningxia Hui Autonomous Region, China, and has been widely distributed in Ningxia Hui Autonomous Region for many years.

To evaluate the evolutionary relationship of *M. bovis* between isolates from Ningxia Hui Autonomous Region and isolates from other provinces in China, a phylogenetic tree was constructed based on concatenated sequences. The evolutionary analysis showed that all ST10 strains were in the same lineage as ST26, ST32, ST43, ST172, including Ningxia Hui Autonomous Region ST134 strains. However, the ST173 strains were in the same lineage as the ST17 strain. The study results indicate that the *M. bovis* strains with STs different from ST173 and ST17 were closely related strains with the same origin.

**Conclusions**

This study revealed the genetic diversity of *M. bovis* from 2009 to 2018 in Ningxia Hui Autonomous Region and provides epidemiological information. ST10 strains were widely prevalent in Ningxia Hui Autonomous Region as well as all provinces of China that have been reported, and ST134 strains were also widely distributed in Ningxia Hui Autonomous Region.

These results may help further our understanding of the evolution of *M. bovis* and provide information that may be useful for the development of novel vaccines.

**Methods**

**Strains**
The detailed information of sixty-seven strains of *M. bovis* was listed in Table 1, including the host, isolation site, geographical location, and the number of strains. Sixty-five strains came from different farms and were isolated from nasal swab, or milk, or joint uid, or lung of *M. bovis*-infected cattle in Ningxia Hui Autonomous Region from 2009 to 2018. HB0801 strain was isolated from the lungs of beef cattle in Hubei Province in 2008 when *M. bovis* was first reported in China[19]. PG45 strain (ATCC 25523) was donated by Professor Aizhen Guo of Huazhong Agricultural University of China.

**Cultivation and identification of *M. bovis***

The *M. bovis* strains were cultured in PPLO broth BD DifcoTM, US California[4, 20]. *M. bovis* genomic DNA was extracted using a bacterial DNA extraction kit (Tiangen, Beijing, China)[21]. The 16S rRNA gene[22] and *uvrC* gene[23] were amplicated using two pairs of primers (Table 2). The PCR reaction mixture was 50 μL (5 μL 10×PCR buffer, 4 μL dNTP mixture, 0.25 μL rTaq, 2 μL primers, 1 μL DNA, 37.75μL ddH2O). The reaction mixture was incubated at 95 °C for 2 min, 35 cycles of 95 °C for 30 s, 50 °C for 20 s and 72 °C for 2 min, then a final incubation at 72 °C for 8 min[22, 23].

**Multi-locus sequence typing**

The genes of 67 isolates of *M. bovis* were amplified by PCR using MLST scheme (*adh-1, gltX, gpsA, gyrB, pta-2, tdk and tkt*)[4, 5] (Table 2). The PCR reaction mixture was 50 μL (5 μL 10×PCR buffer, 4 μL dNTP mixture, 0.25 μL rTaq, 2 μL primers, 1 μL DNA, 37.75μL ddH2O). The reaction mixture was incubated at 95 °C for 2 min, 35 cycles of 95 °C for 30 s, 55 °C for 30 s and 72 °C for 1 min, then a final incubation at 72 °C for 5 min[5]. The PCR amplification products were sent to Shanghai Bioengineering Co., Ltd for sequencing. The assembly sequences were aligned by the http://pubmlst.org/mbovis/ database to obtain the allele number and STs.

**Phylogenetic analysis**

Seven gene sequences of 65 strains of *M. bovis* in Ningxia Hui Autonomous Region and the strains of different STs in China and PG45 reference strains were concatenated. A phylogenetic tree was constructed from concatenated sequences. The evolutionary history was inferred by using the Maximum Likelihood method based on the Kimura 2-parameter model[3] of MEGA 10.0. Initial tree(s) for the heuristic search was obtained automatically by applying Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using the Maximum Composite Likelihood (MCL) approach and then selecting the topology with superior log likelihood value[3].

**List Of Abbreviations**

*adh-1*: Alcohol dehydrogenase-1; AFLP: Amplified fragment length polymorphism; AP-PCR: Arbitrarily primed PCR; ddH2O: Doble distilled water; DNA: Deoxyribonucleic acid; *gltX*: Glutamate tRNA ligase; *gpsA*: Glycerol-3-phosphate dehydrogenase; *gyrB*: DNA gyrase subunit B; IS: Insertion sequence; *M. bovis*: *Mycoplasma bovis*; MLST: Multi-locus sequence typing; PCR: Polymerase chain reaction; PFGE: Pulsed-field gel electrophoresis; PPLO: Pleuropneumonia-like organisms; *pta-2*: Phosphate acetyltransferase-2; RAPD: Random amplified polymorphic DNA; RNA: Ribosomal ribonucleic acid; ST: Sequence types; *tdk*: thymidine kinase; *tkt*: Transketolase; VNTR: Variable number of tandem repeats.

**Declarations**

**Ethics approval and consent to participate**

This study was conducted in accordance with the Law on Animal Protection and Welfare of Ningxia Hui Autonomous Region of China. Samples were recovered after acquiring permission from study participants. All owners of farms provided their verbal consent to participate in this study based on the long and tight cooperative fellowship between the owners and our department. This study was submitted to and approved by the Laboratory Animal Ethical and Welfare Committee of Ningxia University (Approval No. NXU-ACAU-2018-124).

**Consent for publication**

Not applicable.

**Availability of data and materials**

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request. The information about strains during the current study has been uploaded to the MLST database (www.pubmlst.org/mbovis). The uploaded ids of NX001-NX065 strains are 1282-1346 respectively.

**Competing interests**

All authors declare that they have no conflict of interests.
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Authors’ contributions

YNG, SHH, and YL conceived and designed the experiments; YNG and HFL analyzed the data and drafted the manuscript; YNG, YYL, and SQG performed experiments and acquired data. YNG, SHH, and HFL revised the manuscript. All authors read and approved the final manuscript.

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**Tables**
| Sample ID | Year of isolation | Origin      | Host         | Sample type | Clinical status     | adh1 | gltX | gpsA | gyrB | pta2 | tdk | tkt | ST | Source       |
|-----------|------------------|-------------|--------------|-------------|---------------------|------|------|------|------|------|-----|-----|----|--------------|
| PG45      | 1961             | American    | Dairy cow    | Milk        | Clinical mastitis   | 3    | 2    | 4    | 2    | 1    | 3   | 2   | 17| This study   |
| HB0801    | 2008             | Hubei       | Beef cattle  | Lung        | Pneumonia           | 4    | 3    | 2    | 3    | 5    | 3   | 4   | 10| This study   |
| Hubei-1   | 2008             | Hubei       | Cattle       | Lung        | Pneumonia           | 4    | 3    | 2    | 3    | 5    | 4   | 4   | 26| MLST web     |
| EZ-8-NMH0962 | 2008   | Hubei       | Bovine       | Lung        | Pneumonia           | 4    | 5    | 2    | 3    | 5    | 3   | 4   | 32| MLST web     |
| CQ-W70    | 2009             | Chongqing   | Bovine       | Lung        | Pneumonia           | 4    | 5    | 2    | 3    | 5    | 3   | 4   | 32| MLST web     |
| EZ-2      | 2008             | Hubei       | Bovine       | Lung        | Pneumonia           | 4    | 3    | 2    | 12   | 5    | 3   | 4   | 43| MLST web     |
| NHD0986   | 2008             | Hunan       | Bovine       | Lung        | Pneumonia           | 4    | 3    | 2    | 12   | 5    | 3   | 4   | 43| MLST web     |
| NMH7      | 2018             | Inner Mongolia | Bovine   | Milk        | Mastitis            | 10   | 3    | 6    | 13   | 21   | 6   | 10  | 173| MLST web    |
| NMH03     | 2018             | Inner Mongolia | Bovine   | Milk        | Mastitis            | 10   | 3    | 6    | 13   | 21   | 6   | 10  | 173| MLST web    |
| HBHS01    | 2018             | Hubei       | Bovine       | Milk        | Mastitis            | 4    | 3    | 2    | 3    | 5    | 7   | 4   | 172| MLST web    |
| Shaanxi04 | 2018             | Shaanxi     | Bovine       | Milk        | Mastitis            | 4    | 3    | 2    | 3    | 5    | 7   | 4   | 172| MLST web    |
| NX001     | 2009             | Wuzhong     | Dairy cow    | Milk        | Clinical Mastitis   | 4    | 3    | 2    | 3    | 17   | 3   | 4   | 134| This study  |
| NX002     | 2010             | Wuzhong     | Dairy cow    | Lung        | Pneumonia           | 4    | 3    | 2    | 3    | 5    | 3   | 4   | 10| This study   |
| NX003     | 2010             | Wuzhong     | Dairy calf   | Joint fluid | Arthritis           | 4    | 3    | 2    | 3    | 5    | 3   | 4   | 10| This study   |
| NX004     | 2010             | Wuzhong     | Dairy calf   | Joint fluid | Arthritis           | 4    | 3    | 2    | 3    | 17   | 3   | 4   | 134| This study  |
| NX005     | 2010             | Wuzhong     | Dairy cow    | Nose Swab   | Pneumonia           | 4    | 3    | 2    | 3    | 5    | 3   | 4   | 10| This study   |
| NX006     | 2010             | Shizuishan  | Dairy cow    | Nose Swab   | Pneumonia           | 4    | 3    | 2    | 3    | 5    | 3   | 4   | 10| This study   |
| NX007     | 2010             | Wuzhong     | Dairy calf   | Joint fluid | Arthritis           | 4    | 3    | 2    | 3    | 5    | 3   | 4   | 10| This study   |
| NX008     | 2011             | Guyuan      | Beef cattle  | Lung        | Pneumonia           | 4    | 3    | 2    | 3    | 17   | 3   | 4   | 134| This study  |
| NX009     | 2011             | Yinchuan    | Dairy cow    | Nose Swab   | Pneumonia           | 4    | 3    | 2    | 3    | 5    | 3   | 4   | 10| This study   |
| NX010     | 2011             | Wuzhong     | Dairy cow    | Lung        | Pneumonia           | 4    | 3    | 2    | 3    | 5    | 3   | 4   | 10| This study   |
| NX011     | 2011             | Yinchuan    | Dairy cow    | Nose Swab   | Pneumonia           | 4    | 3    | 2    | 3    | 5    | 3   | 4   | 10| This study   |
| NX012     | 2012             | Wuzhong     | Dairy cow    | Nose Swab   | Pneumonia           | 4    | 3    | 2    | 3    | 5    | 3   | 4   | 10| This study   |
| NX013     | 2012             | Wuzhong     | Dairy cow    | Nose Swab   | Pneumonia           | 4    | 3    | 2    | 3    | 5    | 3   | 4   | 10| This study   |
| NX014     | 2012             | Guyuan      | Beef cattle  | Lung        | Pneumonia           | 4    | 3    | 2    | 3    | 5    | 3   | 4   | 10| This study   |
| NX015     | 2013             | Wuzhong     | Beef cattle  | Lung        | Pneumonia           | 4    | 3    | 2    | 3    | 5    | 3   | 4   | 10| This study   |
| UID     | Year | Location  | Species | Sample Type | Disease          | Age | Sex | Season | Year  | Disease | Age | Sex | Season | Year  | Result |
|---------|------|-----------|---------|-------------|------------------|-----|-----|--------|-------|----------|-----|-----|--------|-------|--------|
| NX016   | 2013 | Yinchuan  | Dairy cow | Milk | Clinical mastitis | 4   | 3   | 2      | 3     | 17       | 3   | 4   | 134    |       | This study |
| NX017   | 2013 | Shizuishan | Dairy cow | Milk | Clinical mastitis | 4   | 3   | 2      | 3     | 17       | 3   | 4   | 134    |       | This study |
| NX018   | 2013 | Yinchuan  | Dairy cow | Milk | Clinical mastitis | 4   | 3   | 2      | 3     | 17       | 3   | 4   | 134    |       | This study |
| NX019   | 2013 | Yinchuan  | Dairy cow | Milk | Clinical mastitis | 4   | 3   | 2      | 3     | 17       | 3   | 4   | 134    |       | This study |
| NX020   | 2013 | Yinchuan  | Dairy cow | Nose Swab | Pneumonia | 4   | 3   | 2      | 3     | 5        | 3   | 4   | 10     |       | This study |
| NX021   | 2013 | Guyuan    | Beef cattle | Lung | Pneumonia | 4   | 3   | 2      | 3     | 5        | 3   | 4   | 10     |       | This study |
| NX022   | 2013 | Yinchuan  | Dairy calf | Joint fluid | Arthritis | 4   | 3   | 2      | 3     | 17       | 3   | 4   | 134    |       | This study |
| NX023   | 2014 | Yinchuan  | Dairy cow | Nose Swab | Pneumonia | 4   | 3   | 2      | 3     | 17       | 3   | 4   | 134    |       | This study |
| NX024   | 2014 | Yinchuan  | Dairy cow | Milk | Clinical mastitis | 4   | 3   | 2      | 3     | 5        | 3   | 4   | 10     |       | This study |
| NX025   | 2014 | Yinchuan  | Dairy cow | Lung | Pneumonia | 4   | 3   | 2      | 3     | 17       | 3   | 4   | 134    |       | This study |
| NX026   | 2014 | Yinchuan  | Dairy cow | Nose Swab | Pneumonia | 4   | 3   | 2      | 3     | 17       | 3   | 4   | 134    |       | This study |
| NX027   | 2014 | Yinchuan  | Dairy cow | Lung | Pneumonia | 4   | 3   | 2      | 3     | 17       | 3   | 4   | 134    |       | This study |
| NX028   | 2014 | Yinchuan  | Dairy cow | Lung | Pneumonia | 4   | 3   | 2      | 3     | 17       | 3   | 4   | 134    |       | This study |
| NX029   | 2014 | Shizuishan | Beef cattle | Lung | Pneumonia | 4   | 3   | 2      | 3     | 5        | 3   | 4   | 10     |       | This study |
| NX030   | 2014 | Yinchuan  | Dairy cow | Nose Swab | Pneumonia | 4   | 3   | 2      | 3     | 5        | 3   | 4   | 10     |       | This study |
| NX031   | 2014 | Yinchuan  | Dairy cow | Milk | Clinical mastitis | 4   | 3   | 2      | 3     | 17       | 3   | 4   | 134    |       | This study |
| NX032   | 2014 | Yinchuan  | Dairy cow | Milk | Clinical mastitis | 4   | 3   | 2      | 3     | 17       | 3   | 4   | 134    |       | This study |
| NX033   | 2015 | Wuzhong   | Dairy calf | Joint fluid | Arthritis | 4   | 3   | 2      | 3     | 17       | 3   | 4   | 134    |       | This study |
| NX034   | 2015 | Wuzhong   | Dairy calf | Joint fluid | Arthritis | 4   | 3   | 2      | 3     | 17       | 3   | 4   | 134    |       | This study |
| NX035   | 2015 | Wuzhong   | Dairy calf | Joint fluid | Arthritis | 4   | 3   | 2      | 3     | 17       | 3   | 4   | 134    |       | This study |
| NX036   | 2015 | Wuzhong   | Dairy calf | Joint fluid | Arthritis | 4   | 3   | 2      | 3     | 17       | 3   | 4   | 134    |       | This study |
| NX037   | 2015 | Wuzhong   | Dairy calf | Joint fluid | Arthritis | 4   | 3   | 2      | 3     | 17       | 3   | 4   | 134    |       | This study |
| NX038   | 2015 | Shizuishan | Dairy cow | Milk | Clinical Mastitis | 4   | 3   | 2      | 3     | 17       | 3   | 4   | 134    |       | This study |
| NX039   | 2016 | Guyuan    | Beef cattle | Lung | Pneumonia | 4   | 3   | 2      | 3     | 5        | 3   | 4   | 10     |       | This study |
| NX040   | 2016 | Guyuan    | Beef cattle | Lung | Pneumonia | 4   | 3   | 2      | 3     | 5        | 3   | 4   | 10     |       | This study |
| NX041   | 2016 | Guyuan    | Beef cattle | Lung | Pneumonia | 4   | 3   | 2      | 3     | 5        | 3   | 4   | 10     |       | This study |
| NX042   | 2016 | Guyuan    | Beef cattle | Lung | Pneumonia | 4   | 3   | 2      | 3     | 5        | 3   | 4   | 10     |       | This study |
| NX043   | 2017 | Yinchuan  | Dairy calf | Joint fluid | Arthritis | 4   | 3   | 2      | 3     | 5        | 3   | 4   | 10     |       | This study |
Wuzhong, Shizuishan, Yinchuan and Guyuan are different cities in Ningxia Hui Autonomous Region of China. Hubei, Chongqing, Hunan, Inner Mongolia, and Shaanxi are different provinces of China.

**Table 2.** Primers used for identification and amplification of MLST loci of *M. bovis.*
| Name       | Sequence                                      | Amplicon size (bp) |
|------------|-----------------------------------------------|--------------------|
| 16S rRNA forward | 5'-GAA TTC CGA GAG TTT GAT CCT GGC T-3'        | 1517               |
| 16S rRNA reverse  | 5'-AAG CTT GAG GTA ATC CAT CCC CAC GTT C-3'    |                    |
| uvrC forward  | 5'-GAA TTC AAT GTG TCT ACT AGT CCT GG -3'      | 1620               |
| uvrC reverse  | 5'-AAG CTT AGC GTC ATA GAT TTT TGC ATA-3'     |                    |
| adh-1 forward | 5'- GGA GTA ACT AGT TAC AAA GCA CTT A -3'     | 546                |
| adh-1 reverse | 5'- TGC TAG TTG TTC AAA CAC GT -3'            |                    |
| gltX forward  | 5'- TGG TGA GTA TTC AAT AAG GT-3'             | 530                |
| gltX reverse  | 5'- GTT TTG AGA ATC ATT GCA -3'              |                    |
| gpsA forward  | 5'- AAA ATG TGA GGA ATT GAT CA -3'           | 521                |
| gpsA reverse  | 5'- CCA ATT CCA ATT GCT AAA AC -3'           |                    |
| gyrB forward  | 5'- AGC TTG CTA ATT GCA CCA -3'              | 678                |
| gyrB reverse  | 5'- TAT TTT GAA CAA ATT TTG CAT -3'          |                    |
| pta-2 forward | 5'- AAT TCG TAA TGG CAA AGA AG -3'           | 490                |
| pta-2 reverse | 5'- CTT AGC TTT TCT TAC ATT TAG GT -3'       |                    |
| tdk forward  | 5' –ATG TAT TTA AAA AGT GGA TTA GG -3'       | 572                |
| tdk reverse  | 5'- TAT CTC ATA GCT TTT TTA GC -3'           |                    |
| tkt forward  | 5'- CCA ACT TAT ATT GTG CA -3'               | 533                |
| tkt reverse  | 5'- CCA CCA TAT AAA TTA ATG CC -3'           |                    |
Figure 1

Phylogenetic tree constructed using maximum likelihood and Kimura 2-parameter model based on concatenated MLST sequence data.