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Preliminary study of molecular identification of *Mycobacterium bovis* from cow's milk in Lorestan (Iran)

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Bovine tuberculosis is one of the most important common infectious diseases between humans and livestock. Cow's milk can be investigated as one of the transmission reservoirs of the disease. Our study was conducted to investigate the presence of *Mycobacterium bovis* (*M. bovis*) DNA in cow's milk from different regions of Lorestan province using Touch-down PCR (TD-PCR) method. So, 100 milk samples from industrial and traditional cattle farms were collected and evaluated according to the animal breed, the average age of the animal and the region where the animal was kept. Seven (26.9%) out of the 26 cow's milk samples contaminated with *Mycobacterium spp*., were diagnosed as *M. bovis* positive. The cattle with an average age of more than 5 years were most infected with *Mycobacterium*. Also, the crossbred cattle with an average age of 3 to 5 years, which were kept and raised in tropical areas, showed the highest rate of *M. bovis* infection. Based on our knowledge, this is the first study regarding the presence of M*ycobacterium* in cow's milk in Iran.

Keywords Milk, Cow, *Mycobacterium bovis*, Touch-down PCR, Iran

Abbreviations

M. bovis	Mycobacterium bovis
PCR	polymerase chain reaction
TD-PCR	Touch-down PCR
PICTT	Positive intradermal comparative tuberculin test
TBE	Tris/Borate/EDTA

Mycobacterium bovis (*M. bovis*) as the main causative agent of bovine tuberculosis, has the most host diversity among the members of the *Mycobacterium tuberculosis* complex[1]. It is estimated that more than 50 million cattle in the world are infected with this bacterium[2]. Before the general use of pasteurization, 20 to 40% of human cases of tuberculosis were attributed to *M. bovis*. Currently, the infection rate in the world is estimated to be around 1.4%. Although, this amount has been reported higher in different regions of the world such as Mexico, Africa, Brazil and Palestine[3,4]. In Turkey, Iran's western neighbor, about 4.3% of human tuberculosis cases have been determined to be caused by *M. bovis*[5]. In Iran, bovine tuberculosis was isolated for the first time in 1931 by the French veterinarian Carpantier from the carcasses of native cows in Tehran slaughterhouse[6,7]. Later similar reports were recorded by Iranian veterinarians in 1945 and 1946 and after that from other regions of the country[8]. Despite the implementation of the test and slaughter program, currently less than 8% of the country's cattle herd (the total cattle population of the country is estimated to be less than eight millions) are covered by this program. Besides, this program is mostly focused on controlling the disease in purebred and crossbred cows[9]. The presence of tuberculosis-infected cow carcasses in the annual reports of most slaughterhouses in Iran shows that the disease has become endemic in Iran and it does not seem possible to eradicate this disease in the country in a short time.

With more than 500 active industrial cattle farms and about 25,300 purebred cows in industrial and semi-industrial cattle farms, Lorestan province ranks sixth in the country's livestock population. In this province, the tuberculosis disease control program by performing the tuberculin test has been implemented for the past few decades. Dairy products in Lorestan province have a higher per capita consumption than other parts of Iran.

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Meanwhile, one of the popular dairy products used in this region is cheese obtained from the processing of raw cow's milk, which can be the cause of common diseases, such as tuberculosis, between humans and animals. It should be noted that the prevalence of human tuberculosis in this province has been estimated about 10 cases per 100,000 persons. Currently, there are no comprehensive studies that indicate the incidence of bovine tuberculosis or even human tuberculosis caused by *M. bovis* in this province. Tuberculosis caused by *M. bovis* is considered as one of the most important diseases occurring in the cattle population, and after being transferred to humans, it causes major risks in public health. Therefore, the present study was conducted to determine the proportion and extent of cow's milk contamination with *M. bovis* in different regions of Lorestan province by polymerase chain reaction (PCR).

Materials and methods

Study design and research area

The present study investigated the presence of *M. bovis* in cow's milk obtained from industrial and traditional cattle farms in different regions of Lorestan province in 2020. Lorestan province, with an approximate area of 28,175 square kilometers, is located in the southwestern region of Iran between 46 degrees 50 min to 50 degrees 1 min east longitude and 32 degrees 40 min to 34 degrees 23 min north latitude from the Greenwich meridian. Due to the lack of previous studies regarding the amount of bovine tuberculosis in cow milk in Iran, the present study was conducted as a preliminary study in Lorestan province in order to provide the basis for more extensive research. Some of the reasons for choosing Lorestan province for this research are as follows: the presence of a favorable population of cows with a daily production of about 620 tons of milk, high-quality agricultural lands and various climates, including cold to temperate (Delfan, Selsele, Azna, Aliguderz and Borujerd) and tropical regions (Khorramabad, Dorud, Poldokhtar and Kohdasht) with a temperature difference of about 8 to 12 degrees Celsius (Fig. 1).

Milk samples

One hundred samples of raw milk were randomly collected from industrial and traditional cattle farms in Lorestan province from January to March 2020. After disinfecting each Cartier with 70% alcohol and discarding the first few cc, 50 ml of milk was collected from each cow, and then the age range and breed of the animal were recorded. Finally, the samples were transferred to the laboratory of the Faculty of Veterinary Medicine of Lorestan University on ice. It is evident that the study is preliminary, and the sampling method and the sample size were chosen for convenience.

DNA extraction

After vortexing each milk sample, 10 ml of that sample was transferred to a 10 ml plastic conical tube sterile (Falcon) and centrifuged at 6,000 rpm for 10 min. Then, the superficial fat layer was removed with a sterile swab and the supernatant was discarded. Finally, 200 µl of the sediment were transferred to a new micro tube and DNA extraction was performed using the Korean Gene All kit according to the manufacturer's instructions. The quality and concentration of the extracted DNA was measured using a nanodrop spectrophotometer (USA). The extracted DNA was stored at -20 °C until molecular work.

DNA amplification and detection of polymerase chain reaction (PCR) products

Using TD-PCR method, the specific *16 S rRNA* gene of the *Mycobacterium spp.* with a size of 543 bp[10] and the specific nucleotide sequence of the *M. bovis* species with a size of 500 bp were identified[11]. Forward (5'-ACG GTG GGT ACT AGG TGT GGG TTT C -3') and reverse (5'-TCT GCG ATT ACT AGC GAC TCC GAC TTC A – 3') primers were used for amplification of the *16 S rRNA* gene, and also JB21 forward (5'- TCGTCCGCTG ATGCAAGTGC – 3') and JB22 reverse (5'- CGTCCGCTGACCTCAAGAAG – 3') primers for amplification of the fragment sequenced of *M. bovis* species.

Touch-down (TD) PCR amplification was performed by using PCR master kit (Ampliqon Taq DNA Polymerase Master Mix RED 1.25 mL, Ampliqon Denmark) with 25 µL mixtures containing 12.5 μL of 2X master mix, 0.5 µL of each primer, and 4 µL of the extracted DNA. The BCG vaccine (supplied by the Iranian

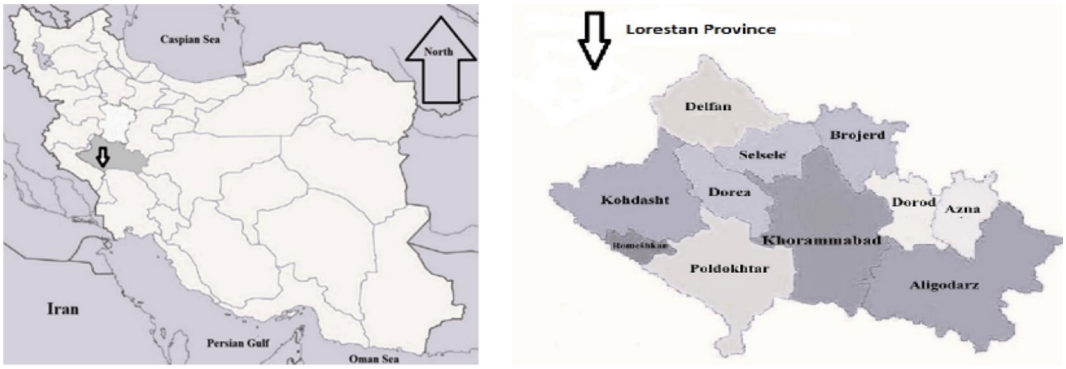

Fig. 1. Map of Iran showing the geographic location of the study areas mentioned in article.

Pasteur Institute) were used as the positive control. For the negative control, sterile water was added instead of nucleic acids. Further, the amplification was conducted by Bio-Rad thermo cycler (Model T- 100, USA) under the following conditions: A for *Mycobacterium spp.* and B for *M. bovis* species.

A. The initial step of 94°C for 5 min, followed by 5 cycles of 94°C for 1 min, annealing temperatures starting at 64°C for 45 s (decreasing 1°C/cycle), and finally, at 72°C for 45 s for the extension. This step was followed by 30 cycles of 94°C for 1 min, 60°C for 1 min, 72°C for 1 min, and finally, 72°C for 10 min.
B. The initial step of 95°C for 5 min, followed by 5 cycles of 95°C for 1 min, annealing temperatures starting at 72°C for 30 s (decreasing 1°C/cycle), and finally, at 72°C for 1 min for the extension. This step was followed by 30 cycles of 95°C for 30 s, 68°C for 30 s, 72°C for 1 min, and finally, 72°C for 10 min.

The TD-PCR products were separated in a 1.2% (w/v) agarose gel (Merck, Germany) containing 2.5 µg/mL gel stain (Smobio, Taiwan). Electrophoresis was performed in 0.5x Tris/Borate/EDTA (TBE) buffer for one hour at 100 V. The resulting PCR products were visualized under a UV translluminator (E-Box, Iran) and the 100 bp DNA ladder (Smobio, Taiwan) plus was used as the molecular size marker.

Statistical analysis

The obtained data were analyzed by Chi-square test using IBM SPSS 20 for Windows (SPSS Inc., Chicago, IL, USA). The P value < 0.05 was considered significant.

Results

Based on the results of the present study, out of 100 cow's milk samples taken from 8 cities of Lorestan province, 26 samples showed the presence of *Mycobacterium* species DNA, (Fig. 2) of which 7 were confirmed as *M. bovis* (Fig. 3). The highest presence of *Mycobacterium spp.* was seen in cows with an average age of more than 5 years at the rate of 32.6%, and in cows with an average age of 1–3 years and 3–5 years, this rate was 11.1% and 23.3%, respectively. This is while the proportion of *M. bovis* infection in cows with an average age of 3 to 5 years was higher than other cows (Table 1). The proportion of *Mycobacterium* species in the samples taken from tropical and cold to temperate regions was 25.4 and 26.6%, respectively. Examination of 13 native breed cows (Zebu) indicated that none of them had *M. bovis* contamination and only one cow's milk showed *Mycobacterium* contamination. In the study of 87 crossbred cows, it was found that 28.7% (25 cows) had milk contaminated with *Mycobacterium*, and *M. bovis* was detected in 7 of these cows (Table 2).

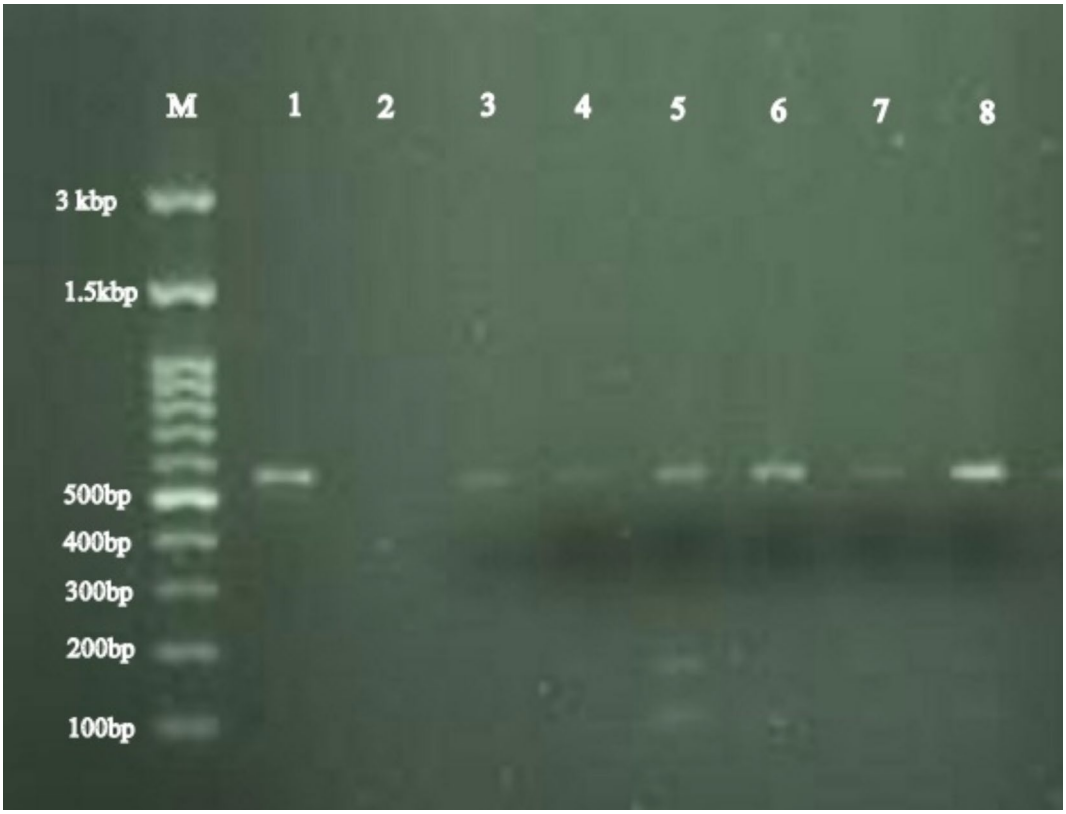

Fig. 2. Touch-down PCR assay for the detection of *Mycobacterium spp*. Lane M: Standard DNA marker; Lane 1: Positive control (543 bp); Lane 2: Negative control; Lanes: 3–8 Positive samples with *Mycobacterium spp.* DNA.

Fig. 3. Touch-down PCR assay for the detection of *Mycobacterium bovis*. Lane M: Standard DNA marker; Lane 1: Positive control (500 bp); Lane 2: Negative control; Lanes: 3–8 Positive samples with *Mycobacterium bovis* DNA.

Discussion

Bovine tuberculosis is a zoonotic disease of public health importance that affects livestock and humans worldwide. The most common cause of this disease is *M. bovis*, whose main host is cattle. The infected cattle play an important role in the transmission of the disease. In the present study, because of the limitations of the bacterial culture conditions and limited laboratory equipment, the presence of *M. bovis* DNA was investigated by tracing and identifying the specific gene of the species in cow's milk samples taken from different regions of Lorestan province. Considering the presence of impurities in milk, in the studies of Zumárraga and col[12,13] to prove the presence of *Mycobacterium* and also in some similar studies[14–19]. To identify the DNA of the target bacteria in milk, the TD-PCR method has been used successfully. Therefore, in the present study, this method was used to detection of cow's milk with *M. bovis*. Very limited studies have been done to identify cattle with tuberculosis in Iran. In 1959, using the intradermal comparative tuberculin test, the prevalence of bovine tuberculosis in Iran was estimated at 28%. This was while in 1984 this amount was estimated at about 3%[6]. In the study of Tadayon et al.[20], out of 470 samples obtained from the lymph nodes and lungs of cattle suspected of having tuberculosis (cattle with positive intradermal comparative tuberculin test (PICTT), 216 samples of *M. bovis* were isolated by culture method. Finally, 132 samples of these cases were investigated by genotyping methods and all of the strains were confirmed as *M. bovis*. In the study by Mosavari et al. in 2011[21], from the examination of slaughterhouse samples obtained from 213 PICTT cows from different regions of Iran using culture methods, acid fast staining, as well as PCR method with IS 6110 and also RFLP detection, a number of 67 heads (31.4%) showed infection with mycobacterium, and all isolates were *M. bovis*. In the study of Ghaderi et al. in 2020[22], from the examination of 50 pathological samples obtained from PICTT cows in the city of Shiraz (Iran) using culture and PCR method with 16 S rRNA gene detection, it was found that 13 heads (26%) were infected with mycobacterium species. In the examination of the positive mycobacterium samples by RD Typing method, it was found that all species were *M. bovis*. In the study of Qazvini et al. in 2023[23], from the examination of 123 slaughterhouse samples obtained from PICTT cows in northeastern Iran, 21 samples (17%) showed mycobacterium contamination, and in the genotyping study, all isolates were diagnosed as *M. bovis*.

In the study of Ashouri and Noorani in 2020[24], from the examination of 108 slaughterhouse samples in PICTT cows using histopathology and Real-Time PCR methods, they showed that the level of infection with *Mycobacterium* were as follow: 58(54.00%) cows were *M. bovis* positive, 46 (43.00%) were *Mycobacterium avium subsp.* paratuberculosis positive, and 11(10.00%) were *Mycobacterium tuberculosis* positive. Although other studies conducted in Iran[20–23] have detected only *M. bovis* in cattle samples, but in the Ashouri and Noorani study[24], they confirmed infection with other *Mycobacterium* species, which may be due to the difference in sampling areas and diagnostic methods. It has been confirmed that most strains of *Mycobacterium avium subsp.* paratuberculosis do not have the ability to grow in culture media, and a large number of its strains take

Row	Sample code Mycobacterium spp. positive	Mycobacterium bovis	Regions	Climate conditions of the region	Age	Breed type
1	3.1.1	−	Delfan	Cold to temperate	3	Crossbreed Cattle
2	3.1.18	+	Delfan	Cold to temperate	5	Crossbreed Cattle
3	3.1.3	−	Delfan	Cold to temperate	6	Zebu local breeds
4	3.2.14	−	Selsele	Cold to temperate	5	Crossbreed Cattle
5	5.1.1	−	Azna	Cold to temperate	7	Crossbreed Cattle
6	5.1.17	−	Aligodarz	Cold to temperate	4	Crossbreed Cattle
7	5.1.13	−	Aligodarz	Cold to temperate	6	Crossbreed Cattle
8	5.1.5	−	Aligodarz	Cold to temperate	7	Crossbreed Cattle
9	4.2.16	−	Brojerd	Cold to temperate	4	Crossbreed Cattle
10	4.1.18	−	Brojerd	Cold to temperate	2	Crossbreed Cattle
11	4.1.1	+	Brojerd	Cold to temperate	6	Crossbreed Cattle
12	4.1.11	−	Brojerd	Cold to temperate	6	Crossbreed Cattle
13	2.1.19	+	Kohdasht	Tropical	7	Crossbreed Cattle
14	2.2.5	−	Kohdasht	Tropical	4	Crossbreed Cattle
15	2.2.1	−	Kohdasht	Tropical	7	Crossbreed Cattle
16	2.1.6	+	Kohdasht	Tropical	5	Crossbreed Cattle
17	1.1.16	−	Khorramabad	Tropical	6	Crossbreed Cattle
18	1.1.18	−	Khorramabad	Tropical	7	Crossbreed Cattle
19	1.1.10	−	Khorramabad	Tropical	6	Crossbreed Cattle
20	1.2.15	−	Khorramabad	Tropical	5	Crossbreed Cattle
21	1.1.14	−	Khorramabad	Tropical	7	Crossbreed Cattle
22	1.2.1	+	Khorramabad	Tropical	6	Crossbreed Cattle
23	1.2.10	−	Khorramabad	Tropical	9	Crossbreed Cattle
24	4.2.18	+	Dorod	Tropical	7	Crossbreed Cattle
25	4.2.10	+	Dorod	Tropical	5	Crossbreed Cattle
26	4.2.19	−	Dorod	Tropical	8	Crossbreed Cattle

Table 1. *Mycobacterium* positive cases according to genus/species, sampling location, age and breed of cattle.

Parameters	1 to 3 years old	3 to 5 years old	5 years up	Tropical region	cold to temperate region	Crossbreed Cattle	Zebu local breeds
Number of samples	18	30	52	55	45	87	13
Mycobacterium SPP. infection	2	7	17	14	12	25	1
Mycobacterium bovis infection	0	3	4	5	2	7	0

Table 2. The number of milk samples collected and number of positive cases for *Mycobacterium spp.* and *Mycobacterium bovis.*

a long time (16–25 weeks) to show detectable growth in culture media[25–28]. On the other hand, *Mycobacterium tuberculosis* needs at least 3 weeks to grow, while some strains of *M. bovis* are able to grow within 5–6 days to show recognizable growth in the culture medium[29]. Since in the present study the rate of infection with *Mycobacterium spp.* and *M. bovis* species was determined to be 26% and 7%, respectively, our study shows that more than half of the target cows may be infected with other *Mycobacterium* species, including *Mycobacterium tuberculosis* and *Mycobacterium paratuberculosis*. Therefore, considering the limitations of culture, using PCR method to detect the source of infection with direct detection of DNA of mycobacteria, especially in sources such as milk, can be very useful for detecting infected farms. However, in terms of the importance of tuberculosis disease to public health, it is better to study more detailed plans regarding the identification, control and eradication of the disease in more comprehensive studies. In the studies conducted to identify *M. bovis* in cow's milk in some countries, the rates were determined to be as follows: 13.1% in India[30], 10.2% and 12.1% in Iraq[31,32], 4.9% in Tunisia[33], 2% in Brazil[34] and 2% in Egypt[35]. Compared to the studies conducted in some other countries[30–35], the current research indicates a high prevalence of *M. bovis* in Iran and some other developing countries, and this issue is not only important in the economy of animal husbandry, but also a threat to the health of the people in the society. It should be mentioned that some studies[36–38] have shown the contamination of people with tuberculosis in Iran by *M. bovis* species. In Yaghoubi et al. study, on 27 positive *Mmycobacterium* culture samples received from the Center for Pulmonary Diseases and Tuberculosis in Zanjan Province (Iran), 25 isolates of *Mycobacterium tuberculosis* and 2 isolates (7.4%) of *M. bovis* were detected[36]. In the study conducted by Soleimanpour et al. in Iran, based on the examination of 42 samples obtained from people with tuberculosis, one case (2.3%) of *M. bovis* was diagnosed[37]. In the study of Mozafari et al., 6 samples (0.48%) of *M. bovis* were identified from the examination of 1242 samples taken from people with tuberculosis infection in different provinces of Iran[38].

In the present study, the highest rate of *Mycobacterium* contamination is seen in the cows with an average age of more than 5 years (32.6%). This association could be due to the older age and the greater exposure to the bacteria. Therefore, it can be concluded that in conducting tests related to bovine tuberculosis, older cows are more likely to be carriers and can be considered as risk factors in the herd, so they should be considered more and the number of annual tests should be increased. Also, in the present study, the highest rate of contamination (10%) was observed in cows with an average age of 3 to 5 years and it was caused by *M. bovis* species. *M. bovis* infection rate was found to be 0.0% in cows with an average age of 1 to 3 years and 7.6% in cows over 5 years old. The recent finding indicates the higher pathogenic power of *M. bovis* for cattle compared to other *Mycobacterium* species. Thus, it can be assumed that cows with bovine tuberculosis (caused by *M. bovis*) usually show symptoms of the disease such as cachexia earlier in the first months or years of infection, and the probability of removing them from the herd is higher. As mentioned earlier, cases of infection caused by *M. bovis* in cattle with an average age of 1 to 3 years were detected at 0.0%. This may be due to the less exposure of younger cows to this bacterium or the lack of milk production in some cows belonging to this age group and subsequently the lack of sampling from them in the present study. In evaluating the effect of regional weather conditions, the rate of infection with *M. bovis* species was higher in tropical regions (9%) than in cold regions (4.4%). Also, the results of the present study showed that the level of contamination of cow's milk with *bovis* species is higher in cows with an average age of 3 to 5 years that have been raised in tropical regions. Since in the present study, none of the native breed cows showed *M. bovis* infection, the present study in consistency with similar studies[6,7,39,40] indicates that crossbred cattle compare to native ones (Zebu breed) are more likely to be infected with bovine tuberculosis. Local breeds of cattle seem to show physiologically higher resistance than crossbred breeds to infection with *M. bovis*, this could be due to the fact that local breeds of cattle have a higher level of immunity due to long-term exposure to the pathogen. On the other hand, crossbred cows have a lower level of immunity due to a little or no previous exposure to the pathogen[8,9]. The detection of *M. bovis* in 7% of the milk samples in this study indicates that the testing and slaughtering programs of TB-affected cows were not very successful, which may be due to the following factors:

- Inability to definitively diagnose infected cows with tuberculin test: Not all cattle infected with bovine tuberculosis can be detected by the tuberculin test, so some of them may be remain as carriers for a long time, spreading the disease in the herd or even in the region, So that in the study of Zumárraga and col[12] in Argentina, the survey results of 177 milk samples taken from the tank (Bulk Tank Milk Samples) in herds with an official TB-free certificate showed that 38% of the samples have contamination with *M. bovis*. On the other hand, the tuberculin test is not performed in all cows in Iran and only about 10% of the total cow population of Iran is covered by regular tuberculin testing[6]. In addition, this program is mostly focused on disease control in purebred and crossbred cattle.
- Ignoring reservoirs of the disease that are not investigated and controlled: in Iran, there are still no complete studies of the level of contamination of sheep and goats with *M. bovis*. While in studies conducted in Ethiopia 5.29% of small ruminants[41], in Palestine 2.7% of goat milk samples[42], in Nigeria (tissue samples of lung and liver in goat) 4.47%[43], showed contamination with *M. bovis*. In 2016, for the first time, *M. bovis* was isolated from a case of a dead European fallow deer (Dama dama dama) in Howeizeh Zoo (Khuzestan - Iran)[44]. Also, in another research and after isolating acid fast bacteria from house mice with a biological colony in a cattle farm in Isfahan city (Iran), it was determined in a molecular investigation that one of these mice was infected with *M bovis*[9]. In the study by Akhtar et al.[45] in Pakistan, after laboratory examination of the carcasses of a number of animals that had been died in the zoo, it was found that 3.7% and 1.1% of the carcasses were contaminated with *M. bovis* and *Mycobacterium tuberculosis*, respectively. These studies show that one of the important reasons that prevent the eradication of bovine tuberculosis is the role of vector animals in the spread of this disease, perhaps because in Lorestan province and most parts of Iran, sheep and goats are kept next to cows. Therefore, this case should be investigated and the necessary plans should be made regarding the identification of carrier animals.
- Another influential factor in the permanence of bovine tuberculosis in Iran is the insufficient support of the government to the rancher in order to kill positive reactor cattle and pay compensation. This causes other ranchers to refrain from conducting tests for their herds. It can also be mentioned that there are no sufficient reference veterinary laboratories in the diagnosis of infected animals[6–9,20,21].

The present study is in sync with some similar studies (including:[9,24,41,42]) and indicates that unpasteurized milk consumed by humans can be an important source of infection with *Mycobacterium* species, especially *M. bovis*. Because in Lorestan province and even many other regions of Iran, people tend to prepare and consume dairy products, especially cheese, in a traditional way, and considering that traditional cheese is made from raw milk, and unfortunately, the consumers do not have the necessary health conditions. Like cheese made from raw milk, they do not keep it in salt water for two months, so raw milk is considered as a risk factor in the transmission of bovine tuberculosis to humans. In Lorestan province and even many other regions of Iran, people tend to prepare and consume dairy products, especially cheese, in a traditional way. It should be mentioned that in the traditional method, cheese is prepared from raw milk. Unfortunately, most of the consumers do not follow the recommended health measures, such as keeping cheese processed from raw milk in salt water for at least two months. It is obvious that the consumption of raw milk and unsanitary products, including the mentioned traditional cheeses, will be risk factors for the transmission of bovine tuberculosis to humans.

Conclusions

It should be noted that the prevalence of human tuberculosis caused by *M. bovis* in Lorestan province and other regions of Iran is unknown, and only the prevalence of *M. tuberculosis* in humans has been investigated.

It should be noted that *Mycobacterium tuberculosis* has been isolated from the milk of ruminants[30,42] and this indicates that the consumption of milk and other unsanitary dairy products by humans may be effective in contracting tuberculosis caused by *M. tuberculosis* species. Considering the point that not all positive samples of bovine tuberculosis can be identified by the tuberculin intradermal test, and regarding the fact that it is not possible to perform molecular methods for all cows in the same herd due to the high cost of implementation, it is suggested in the initial investigation, a general sample of the milk of each cattle farm (milk tank specific to the same cattle farm) should be examined with the TD-PCR method, and if the result is positive, the whole herd should be tested and evaluated individually. Also, due to the transmission of tuberculosis from milk to humans and its health importance, it is suggested to investigate the presence of tuberculosis in the milk of sheep and goats and even other possible carriers such as dogs and cats, as well as the rate of people suffering from tuberculosis caused by *M. bovis*.

Data availability

All obtained data from this study was included in this manuscript and are available on request from the corresponding author.

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Declarations

Competing interests

The authors declare no competing interests.

Additional information

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