



PCR INVESTIGATION OF THE VERTICAL TRANSMISSION OF *MYCOBACTERIUM BOVIS* IN MONTBÉLIARDE CATTLE IN GONBAD, NORTHEAST OF IRAN

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Summary

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Bovine tuberculosis is an important global zoonosis. The causative agent of the disease is *Mycobacterium bovis*, belonging to the *Mycobacterium tuberculosis* complex. This study aimed to identify vertical transmission of *Mycobacterium bovis* in tuberculin positive pregnant dairy cows referred to the slaughterhouse and commercial sperm used to inseminate them in Gonbad, Gorgan province, Iran, by PCR assay. From March to September 2016, one thousand and seven hundred artificially inseminated cows were subjected to the tuberculin test; then, tuberculin positive cows were referred to the Gonbad slaughterhouse. Samples containing milk, lymph node, placenta, foetal abomasal fluid, and amniotic fluid were prepared from all affected cows and stored at –80 °C until analysis. Bacterial DNA was extracted from all specimens by a commercial kit. Detection was performed by amplifying *pncATB* gene using special primers via the polymerase chain reaction method. Results of tuberculin test revealed 144 positive cases (8.47%). PCR results for 48 milk samples, 120 biopsy samples, 63 samples of fetuses showed 9, 93 and 43 positive cases, respectively which all belonged to tuberculin positive samples. No positive sperm samples were identified. It was concluded that the infection rate with *M. bovis* in Montbéliarde cattle was high and also, that vertical transmission was not seen.

Key words: cow, Gonbad, *Mycobacterium bovis*, PCR, *pncATB* gene

INTRODUCTION

Bovine tuberculosis is a chronic bacterial disease in animals caused by members of the *Mycobacterium tuberculosis* complex primarily by *M. bovis*, followed by *M. caprae* and *M. tuberculosis*. It is a major infectious disease among cattle, and it also affects other domesticated animals and

certain wildlife populations, causing a general state of illness, pneumonia, weight loss, and eventually death (Dibaba, 2019). Cattle are considered to be the major reservoir for *M. bovis* and the main infection source for humans. *M. bovis* attracts interest due to its potential spread from ani-

mals to humans, effects on animal health and trade. In 1945, *M. bovis* was isolated from dairy cows by Razi institute, and Khaleghian *et al.* (2015) reported its frequency as 0.6% in the Iranian population. Also, Arefpajoohi *et al.* (2015) reported that its prevalence among the bovine population in Iran was 0.18%.

The primary spread mode of bovine tuberculosis among herds is introducing infected animals into non-infected herds. Evidence exists for nose-to-nose transmission, which could play a key role in transmission between neighbouring facilities. The most significant factors determining the occurrence and spread of tuberculosis within a herd of cattle are the number of infected individuals, the number of young stock exposed to these infected animals, and the measures taken to prevent spread. The transmission mode in cattle is principally horizontal; however, not all infected animals transmit the disease. Nevertheless, most cattle suffering from pulmonary tuberculosis and tuberculous mastitis are infectious, and in some infected individuals, urine, vaginal secretions, semen, or faeces may also contain tubercle bacilli and act as a means of disease transmission between animals (Cousins, 2001).

Gonbad is located in the northeast of Iran and has two major breeds of cattle, including Holstein and Montbéliarde population. The latter is used for milk and dairy production. Although it is a dairy breed, it is useful for meat production as well; thus, Montbéliarde cattle are currently used for dual purposes. Montbéliarde population in France is the second dairy cow population after Holsteins. The quality and quantity of produced milk and meat by this breed are high.

Polymerase chain reaction (PCR), random fragments length polymorphism

(RFLP), sequencing and other genotyping methods are performed to understand disease transmission. The PCR analysis is perhaps the most widely used method to identify *M. bovis* due to the availability of DNA. RFPL, as a complementary PCR method, is directed against specific polymorphic regions within the mycobacterial genome and is capable of distinguishing between genotypic strains (O'Brien *et al.*, 2000). For the first time, Costello *et al.* (1999) used the RFLP method for epidemiological analysis of *M. bovis* in Ireland. Then, O'Brien *et al.* (2000) applied a new DNA probe for strain typing of *M. bovis* by RFLP in this country too. Zanini *et al.* (2001) identified *M. bovis* in bovine lymph nodes in Brazil. In Iran, Dehghanpour *et al.* (2016) identified and compared the genetic pattern of human and cattle *M. bovis* isolated in Zanjan province.

This study primarily aimed to confirm infection with *M. bovis* from *M. tuberculosis* complex in tuberculin positive cows at Gonbad, Iran. The research's second goal was to determine the precise *M. bovis* presence in clinical specimens, such as milk, lymph nodes, placenta, foetal abomasal fluid, amniotic fluid, and commercial Montbéliarde sperm. Finally, vertical transmission of *M. bovis* in Montbéliarde cattle breed was investigated in the study.

MATERIALS AND METHODS

Comparative intradermal tuberculin test

A comparative intradermal tuberculin test (CITT) was conducted by the method of Boukary *et al.* (2011) with some modifications. Briefly, the test was performed on one side of the animal in the middle neck region. Two circular areas of about 2 cm² diameter, about 12–15 cm apart, on the skin of the cervical area, were clipped by a curved scissor and disinfected with 70%

ethanol. The initial skin thickness was measured with a caliper. Respectively, 0.1 mL (32500 IU/mL) of bovine tuberculin PPD (a purified protein derived from *M. bovis* AN5, Razi Institute, Iran) at one site and 0.1 mL (25000 IU/mL) of avian tuberculin PPD (produced from *M. avium* D4, Razi Institute, Iran) at the same side were injected into the dermis. Skin thickness was measured again at both injection sites after 72 h. The reaction at both injection sites was derived by measuring the difference of the skin thickness before and 72 h after the injection.

Sample preparation

The experiment took place between March and September 2016 in the dairy farms of Gonbad, Gorgan province, Iran. Commercial sperm specimens were collected randomly before artificial insemination. One thousand and seven hundred artificially inseminated Montbéliarde cows were screened by the tuberculin test using comparative intradermal tuberculin testing (CITT). Then, the tuberculin positive animals were referred to the slaughterhouse, and from each animal, specimens (retropharyngeal and mediastinal lymph nodes, placenta, amniotic fluid, foetal abomasal fluid), were collected. At first, the samples were stored at -80°C , then transferred to the bacteriology laboratory of the Faculty of Veterinary Medicine, Urmia University, in iceboxes.

Genomic DNA extraction

All samples were subjected to bacterial genomic DNA extraction using a commercial kit (Favorgen, Taiwan). The purity and quantity of extracted DNA were assayed by nanodrop apparatus (Thermo, USA) at 260 and 280 nm wavelength.

Polymerase chain reaction

A polymerase chain reaction was done using one set primers for amplifying *pncATB-1.2* (specific primers for *M. bovis* detection). The forward and reverse primers sequences for *pncATB-1.2* were 5'-ATGCGGGCGTTGATCATCGTC-3' and 5'-CGGTGTGCCGGAGAAGCCG-3', respectively. The PCR reaction mixture ingredients were 12.5 μL of 2 \times PCR master mix (Pishgam, Iran), 0.5 μL of each primer, 5 μL template DNA, and 6.5 μL DEPC water at a final volume of 25 μL . The reaction mixture was subjected to an initial denaturation at 94°C for 5 min, 35 cycles of denaturation at 94°C for 1 min, annealing at 68°C for 1 min, and extension at 72°C for 1 min followed by a final extension at 72°C for 10 min. The product was analysed on a 2% agarose gel (Sigma, USA) and stained with safe stain (Pishgam, Iran). The expected size of the amplicon for *M. bovis* was 186 bp (Spisitto *et al.*, 2014). Distilled water was used as negative control in the test.

RESULTS

Dairy cow tuberculin test revealed one hundred and forty four positive cases (8.47%) out of 1,700 tested animals.

Forty eight milk samples and one hundred and twenty biopsy samples of lymph nodes (retropharyngeal and mediastinal) were taken. Also, sixty three samples of amniotic fluid, foetal abomasal fluid, and placenta separately were prepared from pregnant animals. All samples were analysed by the PCR method. PCR was positive for *M. bovis* in 9 milk and 93 biopsy samples (retropharyngeal, mediastinal lymph nodes). Also, PCR was positive in 43 foetal specimens (placenta, amniotic fluid and foetal abomasal fluid) (Fig. 1).

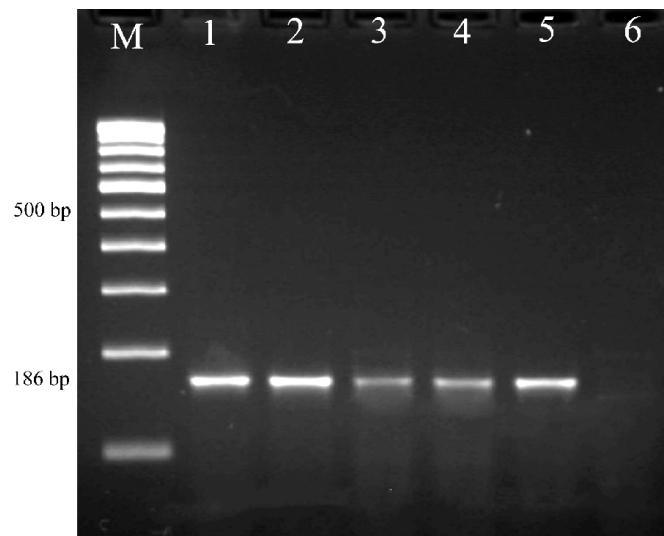


Fig. 1. Agarose gel electrophoresis of real-time PCR products. Lane M: 100 bp ladder; lanes 1–5: *M. bovis* (186 bp); lane 6: no template negative control.

The presence of *M. bovis* was screened in 50 commercial sperm by PCR; the results showed that none was contaminated.

DISCUSSION

Comparative intradermal tuberculin test is the standard method to identify infection with *M. bovis*, and it is also currently the most widely applied screening test for detecting bovine tuberculosis in living animals. The sensitivity and specificity of the tuberculin skin test are between 77–95% and 98–99.9%, respectively (Proano-Perez *et al.*, 2009). In this study, precautions are taken to minimise factors influencing the skin test result by carefully applying CITT and using high-quality well-maintained PPD products. It should be noted that calves younger than 2 months and cows 6 weeks before and after parturition were excluded; this is since cattle with tuberculosis go through a period of desensitisation immediately before and after calving and 30% show false-

negative reactions, returning to a positive status 4 to 6 weeks later. The loss of sensitivity is probably a result of the general immunologic hyperactivity associated with parturition. Calves drinking colostrum from infected dams give positive reactions for up to 3 weeks after birth, although they may not be infected (Constable *et al.*, 2016).

Although genital and congenital transmission is not common, the congenital route is still important in areas with high bovine tuberculosis prevalence. There is evidence for congenital tuberculosis in calves. The calves probably acquire the infection from their mother during the gestation or at birth. For example, Del Moral *et al.* (2018) reported congenital tuberculosis in a 25-day-old female calf. The researchers observed pathological alterations within the respiratory tract. Indeed, bovine tuberculosis lesions were observed only in the lungs; thus, the calf probably had aspirated contaminated am-

niotic fluid *in utero* close to parturition, producing the primary lung lesions. Consequently, the disease had developed during the first weeks after birth. Also, Vural & Tunca (2001) documented that tuberculosis can generally occur congenitally through umbilical veins or postnatally via respiratory, alimentary, genital, and cutaneous routes; they believe that congenital tuberculosis was rare. Ozigit *et al.* (2007) declared that congenital tuberculosis might occur as a result of maternal tuberculosis when the disease involves the genital tract or placenta. The infection spreads via the umbilical veins or the infected amniotic fluid ingested or aspirated *in utero* or at birth. Given that the dam had a positive result in the intradermal tuberculin test, the calf was probably infected *in utero*. In the present study, tuberculin positive slaughtered dairy cows and calves had tuberculosis lesions on some organs, such as placenta, retropharyngeal and mediastinal lymph nodes. In tuberculosis, primary lesions were seen in specific organs, including lung, lymph node, and liver; this is related to the route of the bacterial entrance. The udder is usually infected with *M. bovis* at the next stage of the disease; thus, the presence of bacterial in milk can be less, indicating that the animal is affected for a long time. The presence of *M. bovis* in milk is a risk factor for public health and newborn animals. In tuberculosis lymph nodes infected with *M. bovis*, retropharyngeal and mediastinal lymph nodes were reported to be contaminated with the bacterium in the present research. The mediastinal lymph nodes were more contaminated, proving that the main route of the bacterial entrance was the respiratory tract. *M. bovis* was shown to be present in placenta, amniotic and foetal abomasal fluid in tuberculin positive slaughtered cows. Placenta

showed higher contamination than amniotic and foetal abomasal fluid. Tuberculous lesions were observed on some placenta samples, indicating that animal was affected for a long time, thus suffered from chronic disease. Based on our observation, bacteria were present in the amniotic and foetal abomasal fluid of some cows with tuberculous lesions in the placenta. In general, the presence of *M. bovis* in the placenta, amniotic and foetal abomasal fluid could be a reason for congenital tuberculosis in the Montbéliarde breed. Probably, *M. bovis* was ingested from amniotic fluid by the foetus; thus, bacterial isolation from foetal abomasal fluid could document this fact.

PCR is a precise technique to detect pathogens; thus, it was used here to determine *M. bovis* in specimens. In the present study, *M. bovis* was identified in all positive collected samples, which showed that this strain may be local in Gonbad, Iran. PCR test did not amplify the *pncATB* gene in sperm samples. This showed that sperm samples were free from *M. bovis* and thus vertical transmission could not occur by artificial insemination through sperm to cows and subsequently to the foetus. More samples and studies are needed to prove or reject this theory.

In conclusion, to the best of authors' knowledge, this is the first study on congenital tuberculosis and vertical transmission of tuberculosis in dairy cows from the Montbéliarde breed in Iran. This study may, therefore, serve as a database for further evolution and characterisation of circulating strains. Along with epidemiological data, it may contribute to more effective transmission control measures, mainly in Iran.

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