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Performance of immunohistochemistry versus real-time PCR method for detecting mycobacterial infections of cattle screened by comparative tuberculin test

Mojtaba Ashouri, Hossein Nourani*

Department of Pathobiology, Faculty of Veterinary Medicine, Ferdowsi University of Mashhad, Mashhad, Iran.

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Abstract

In addition to the fifty years since the test-and-slaughter program began in Iran and despite a significant reduction in the disease prevalence, positive tuberculosis cases are still being isolated from livestock farms across the country. Tests with 100% sensitivity and specificity are essential features for bovine tuberculosis diagnosis. The relationship between real-time PCR and immunohistochemistry (IHC) as two essential laboratory methods in the diagnosis of bacterial infections were aimed to evaluate single intradermal comparative tuberculin test (SICTT) results. One hundred thirty-eight cows in two groups were examined: Reactors (108 cows) and clean (as a control group; 30 cows). In the reactor group, 58(54.00%) cows were *Mycobacterium bovis* positive, 46(43.00%) were *Mycobacterium avium* subsp. *paratuberculosis* (MAP) positive, and 11(10.00%) were *Mycobacterium tuberculosis* positive. 32(55.00%) cows were co-infected with *M. bovis* and MAP and 5(4.55%) cows were co-infected with *Mycobacterium tuberculosis* and MAP in this group. Of 50 *M. bovis* negative cows of reactor group were 14(28.00%) MAP positive and 36(72.00%) negative, as well. Concurrent infection with all was observed in one reactor case. Comparing IHC and real-time PCR for the detection of bovine tuberculosis and Johne's disease showed very good agreement (Kappa values 0.81 - 1.00). The results also provided further confirmation on IHC and real-time PCR as a sensitive and reliable diagnostic screening approach for detection of bovine tuberculosis. The use of one laboratory method to detect bovine tuberculosis is not sufficient alone.

Introduction

Mycobacterium tuberculosis complex (MTC) causes tuberculosis in humans and animals, one of the top 10 causes of death in the world, and millions of people continue to fall sick each year. According to the World health organization (WHO) in 2017, Globally, 10.00 million people spread the disease and it caused 1.30 million deaths (16.00%).[1] The acquired immuno-deficiency syndrome (AIDS) co-infection, the failure of Bacillus Calmette–Guérin (BCG) vaccine and drug-resistant are factors contributing to the complication of the situation. The MTC includes *M. tuberculosis, M. bovis*, *M. microti*, *M. africanum* and *M. canetti*. Of these species, *M. tuberculosis* (MT), in the first place, and then *M. bovis* are highly pathogenic. Due to the abundance of *M. bovis* hosts more varied animal species are infected and become the source of infection.[2]

Another significant mycobacterial disease, namely paratuberculosis or Johne's disease (JD), is a chronic and progressive granulomatous enteritis of ruminants caused by *Mycobacterium avium* subsp. *Paratuberculosis* (MAP) and a disease affecting multiple species with socio-economic and/or public health importance. It may be associated with the etiology of Crohn's disease in humans.[1]

Although paratuberculosis available vaccines show a reduction in disease incidence, they are unable to prevent bacterial shedding or subsequent new cases in the herd. Unfortunately, these vaccines can interfere with the results of the tuberculin test.[3,4] A comparison between tuberculin test methods has shown that the use of comparative intradermal test method vs single intradermal test, decrease the percentage of cross-reaction from 6.55% to 0.15%.[5] Besides, in affected animals, the incubation period is 2 to 5 years (but up to 10 years), and the subclinical form of MAP infection usually appears several years before

***Correspondence:**

Hossein Nourani. DVM, DVSc
Department of Pathobiology, Faculty of Veterinary Medicine, Ferdowsi University of Mashhad, Mashhad, Iran
E-mail: nourani@um.ac.ir

the clinical form (diarrhea, reduced milk production, cachexia and severe emaciation) leading to death since no cure is available.[6]

In recent years, the tuberculin skin test was the only tool for detecting mycobacterial infections especially in latent cases of infection. The tuberculin skin test is an intradermal injection of purified protein derivative (PPD) that is a complex of several antigens including antigens shared with non-tuberculous mycobacteria. The PPD is injected below the epidermal layer that causes the inflammatory reaction (a delayed-type hypersensitivity reaction) and results in the characteristic indurated area at the injection site. The tuberculin skin test has limitations including technical problems in administration, boosting of the immune response in the test reputation, false positive and false negative results, complicated, subjective interpretation and requirement in the second visit. Other methods, such as acid-fast staining and isolation of Mycobacterium spp. in culture, have many limitations. The acid-fast staining detection limit is more than 10^4 bacilli per slide. Mycobacterial culture requires several weeks, and in a few bacilli, its sensitivity is low.[7] In Iran, the test-and-slaughter program has been launched following the world plan since 1967.[8] Unfortunately, in addition to the fifty years since the program began, and despite a significant reduction in the disease prevalence,[8] still positive cases of tuberculosis are being isolated from livestock farms across the country. The success of the test-and-slaughter program for bovine tuberculosis control is directly dependent on the diagnostic ability of the laboratory tests.[9] Adopting tests with 100% sensitivity and specificity, high operating speed and low cost are essential features for a bovine tuberculosis diagnosis.[10] The use of real-time polymerase chain reaction (PCR) and immunohistochemistry (IHC) methods has been considered by researchers in the last decades. Direct real-time PCR has some advantages over the detection of mycobacterial species in animal tissue samples, which takes a few hours and highly analytical specificity. However, cross-reaction with unwanted microorganisms (contamination) is one of its main limitations.[11] The IHC is a powerful technique for detection and scoring of cellular macromolecules along with the microscopic observation of lesions in fresh or formalin-fixed tissue samples. Especially in the formalin-fixed form, it is a safe method to study many high-risk pathogens like mycobacteria in the laboratory. Human error, damage to some epitopes because of over-fixation, and difficulty in quantifying results are the most considerable limitations of IHC.[12] The relationship between real-time PCR and IHC as two essential laboratory methods in diagnosis of bacterial infections were aimed to evaluate single intradermal comparative tuberculin test (SICTT) results and to evaluate the SICTT sensitivity and specificity in Khorasan-Razavi province, Iran.

Materials and Methods

Samples. The minimum sample size (N) needed in this study was calculated using the following formula with a 95.00% confidence limit, 22.00% estimated prevalence (P), and 5.00% Tolerable error:[13]

$$N= 1.96^2 P (1-P) / 0.05^2$$

Thirty healthy and one hundred eight positive reactor cattle screened by SICTT for tuberculosis were included in this study. After inspection of carcasses, samples of ileum and bronchial, mediastinal and mesenteric lymph nodes were taken. After dividing the samples into two parts, the first part of the specimen was sent to the molecular laboratory of the Khorasan-Razavi Veterinary Office, ice packed. The second part was placed in 10.00% neutral buffered formalin (NBF; Sigma-Aldrich, St. Louis, USA) and sent to the above histopathological laboratory.

Preparation of tissues for histopathological examinations. After fixation in NBF, the specimens were placed in suitably labeled cassettes to segregate them from other specimens. For dehydration, series of increasing alcohol (Sigma-Aldrich) concentrations were used to avoid excessive distortion of the tissue, and then cleared with xylene (Merck, Darmstadt, Germany), and infiltrated with paraffin wax (Sigma-Aldrich). The infiltrated tissues were then embedded into wax blocks. Cutting of paraffin-embedded tissues was performed at 5.00 µm. Paraffin sections were properly mounted onto poly-L-lysine (Sigma-Aldrich) coated glass slides.

Immunohistochemistry. The tissue sections were deparaffinized and hydrated in water. One part trypsin concentrate (Abcam, Boston, USA) with one part buffer were mixed. Sections were incubated for 5 - 10 min at 37.00 ˚C to retrieve mycobacterium antigens. Then, they were rinsed thoroughly in Tris buffered saline (TBS; Sigma-Aldrich) and proceeded with the immunostaining procedure. The slides were washed two times for 5 min in TBS plus 0.025% Triton X-100 (Sigma-Aldrich) and then blocked in 10.00% normal serum with 1.00% BSA (Sigma-Aldrich) in TBS for 2 hr at room temperature. The slides were drained for a few seconds and wiped around the sections with tissue paper. Then, the rabbit primary antibody (anti-*M. bovis*, Bioss Inc., Woburn, USA /anti-MT, Abcam /anti-MAP, Daco, Glostrup, Denmark) was diluted to the manufacturer's recommendations (1/200), and the sections were incubated overnight at 4.00 ˚C. After two times for 5 min TBS plus 0.025% Triton X-100, the slides were incubated in 0.30% H_2O_2 (Sigma-Aldrich) in TBS for 15 min. After two times washing in TBS buffer, for enzymatic detection, enzyme-conjugated secondary antibody goat anti-rabbit (HRP; Abcam) was diluted in TBS with 1.00% BSA (1/200), applied to slides and incubated for 1 hr at room temperature. The samples were rinsed with in buffer TBS plus 0.025% Triton X-100, three times

for 10 min, and then 3, 3-diamino-benzidine (DAB; Sigma-Aldrich) chromogen was used for 10 min at room temperature. The DAB produced a brown precipitate (where the secondary HRP antibody binds to the primary) that was insoluble in alcohol, xylene and other organic solvents most commonly used in the laboratories. The slides were rinsed in running tap water for 10 min and stained with nuclear counterstained Mayer's hematoxylin (Sigma-Aldrich) for better visualization of the tissue morphology. Dehydration, clearance, and mounting using a compatible mounting medium were carried out. Positive and negative controls were used with all samples. The analysis was performed in a blinded manner.

DNA extraction. Fifty mg of each sample were placed into 2.00 mL Eppendorf microcentrifuge 5420 tubes (Eppendorf, Hamburg, Germany) and digested by adding 200 µL of tissue lysis buffer (4.00 M Urea, 200 mM Tris, 20.00 mM NaCl, 200 mM EDTA, pH 7.40 at 25.00 ˚C) containing 40.00 µL proteinase K (200 µg mL^{-1}; Roche, Indianapolis, USA). Then each tube was incubated at 55.00 ˚C for 72 hr. Boiling (for 10 min) and immediately freezing (at – 20.00 ˚C for 30 min) were performed three times. According to the manufacturer's instructions, the extraction process was carried out using a DNA extraction kit (High pure PCR template preparation kit; Roche). Eluted DNA was stored at – 80.00 ˚C.

Real-time PCR. Real-time PCR was carried using a LightCycler® 480 (Roche) and LightCycler® 480 DNA SYBR Green I Master Mix (Roche). The oligonucleotides INS-1 and INS-2 were used to amplify a 245-bp fragment of the IS*6110* sequence present in members of the MTC, Jb21 and Jb22 were used to amplify a 500-bp fragment from the *RvD1Rv2031c* sequence present in members of *M. bovis*. P90 and P91 were used to amplify a 400-bp fragment from the IS*900* sequence present in members of MAP (Table 1).[10,14-16] Each reaction mixture of 20.00 µL was containing 10.00 µL of SYBR Green I Master Mix (Roche), 5.00 mM $MgCl_2$ (Roche), 2.00 µL of each primer, 5.00 µL of the template and molecular grade water up to 20.00 µL. The amplifications were carried out with the two following cycles: An initial step of 95.00 ˚C for 10 min and 45 cycles of amplifications 94.00 ˚C for 30 Sec, 55.00 ˚C for 35 Sec, and 72.00 ˚C for 45 Sec, for MTC and *M. bovis*; and an initial step of 95.00 ˚C for 10 min, 45 cycles of amplifications 94.00 ˚C for 30 Sec, 56.00 ˚C for 35 Sec, and 72.00 ˚C for 45 Sec for MAP. In this study, β-actin was used as an internal control (Table 1). If samples' results were negative for a target, the simultaneous DNA process control needed to be positive to prove that there was material in the reaction and that the real-time PCR was not inhibited. Positive and negative controls were used to assess accuracy in all PCR run. Real-time PCR data were analyzed to determine whether a significant difference was existed between samples and controls and to calculate the double delta cycle threshold (Ct) analysis by Livak and Schmittgen.[17]

Statistical analysis. The results of the real-time PCR and IHC assay were compared to those of SICTT using agreement and kappa tests. Analyses were done using SPSS Software (version 19.0; IBM, Armonk, USA).

Results

Macroscopic examination results. Out of the 108 reactor cows, 26(24.00%) were with macroscopically visible tuberculous granulomas and 82(76.00%) without macroscopically visible lesions. Based on the results of the post-mortem carcass inspection, the percentage of involvement in different tissues included: Liver (7.00%), diaphragm (14.00%), lung (43.00%), head (22.00%), pre-scapular (7.00%), and mesenteric (7.00%) lymph nodes.

Real-time PCR results. One hundred thirty-eight cows were examined in two groups: The reactor group (108 cows) and the clean group (30 cows; base of negative response to SICTT and the absence of clinical signs and no report of macroscopic lesions in the last two years as a control group). The results are shown in Table 2. In the reactor group, 58(54.00%) cows were *M. bovis* positive, 46(43.00%) cows MAP positive, and 11(10.00%) MTC positive (Fig. 1). 32(55.00%) cows were co-infected with *M. bovis* and MAP, and 5(4.55%) cows were co-infected with MTC and MAP in this group. Out of 50 *M. bovis* negative cows of reactor group, 14(28.00%) were MAP positive, and 36(72.00%) negative. Concurrent infection with all was observed in one reactor case. In the clean group, all samples were negative (Table 2). The sensitivity, specificity, positive predictive value (PPV) and negative predictive value (NPV) of SICTT using real-time PCR as the gold standard for detection of *M. bovis,* MTC and MAP in tissue sections are shown in Table 3.[7,18]

Table 1. Primers used in this study.

Target DNA	Primer sequence (5′→3′)	References
RvD1Rv2031c	Jb21 (TCGTCCGCTGATGCAAGTGC) Jb22 (CGTGAACGTAGTCGCCTGC)	10
IS6110	INS-1 (CGTGAGGGCATCGAGGTGGC) INS-2 (GCGTAGGCGTCGGTGACAAA)	14
IS900	P90(GAAGGGTGTTCGGGGCCGTCGTTAGG) P91(GGCGTTGAGGTCGATCGCCCACGTGC)	15
β-actin	β-actin-F (TCCCTGGAGAAGAGCTACGA) β-actin-R (AGGAAGGAAGGCTGGAAGAG)	16

Table 2. Results of the real-time PCR and immunohistochemistry (IHC) methods in two groups. Data are presented as number (%).

Groups	Method	Total	*M. bovis*[1]		MTC[2]		MAP[3]	
			Positive	Negative	Positive	Negative	Positive	Negative
Reactor group	Real-time PCR	108	58(54.00)	50(46.00)	11(10.00)	97(90.00)	46(43.00)	62(57.00)
	IHC	108	58(54.00)	50(46.00)	11(10.00)	97(90.00)	46(43.00)	62(57.00)
Clean group	Real-time PCR	30	0(0.00)	30(100)	0(0.00)	30(100)	0(0.00)	30(100)
	IHC	30	0(0.00)	30(100)	0(0.00)	30(100)	4(13.00)	26(87.00)
Total	Real-time PCR	138	58(42.00)	80(58.00)	11(8.00)	127(92.00)	46(33.00)	92(67.00)
	IHC	138	58(42.00)	80(58.00)	11(8.00)	127(92.00)	50(36.00)	88(64.00)

[1] *Mycobacterium bovis;* [2] *Mycobacterium tuberculosis* complex; and [3] *Mycobacterium avium* subsp. *paratuberculosis.*

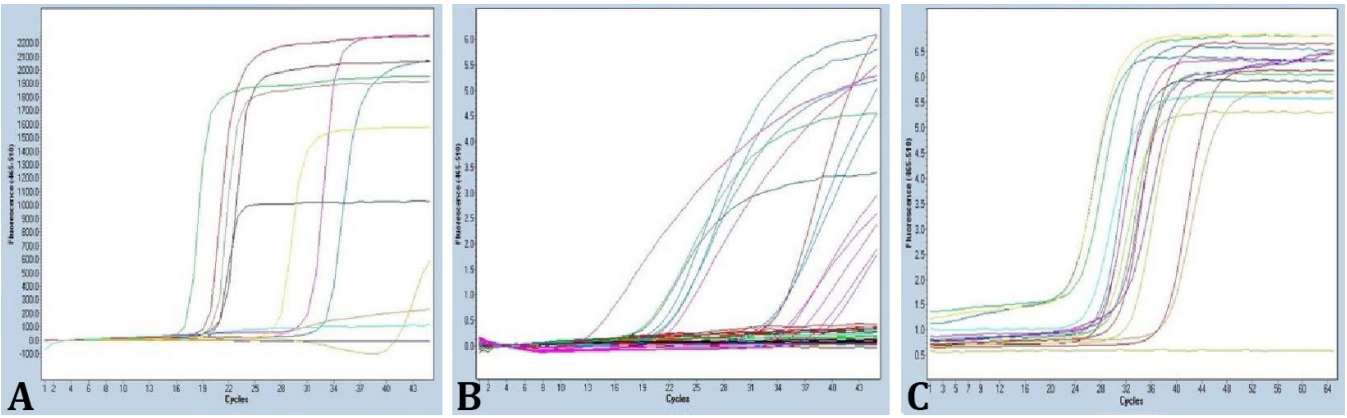

Fig. 1. Fluorescence curves of SYBR-Green real-time PCR. Amplification results after 45 cycles are shown for detection of **A)** *Mycobacterium tuberculosis* complex, **B)** *Mycobacterium bovis* and **C)** *Mycobacterium avium* subsp. *paratuberculosis*.

Table 3. Statistical evaluation of SICTT using real-time PCR and immunohistochemistry (IHC). Data are presented as percentage.

Type	Method	Sensitivity	Specificity	Accuracy	Prevalence	PPV[4]	NPV[5]	Kappa ($p < 0.0005$)
***M. bovis*[1]**	Real-time PCR	53.70	100	63.80	78.26	100	37.50	0.34
	IHC	53.70	100	63.80	78.26	100	100	0.34
MTC[2]	Real-time PCR	100	100	100	8.00	100	100	1.00
	IHC	100	100	100	8.00	100	100	1.00
MAP[3]	Real-time PCR	60.50	100	78.30	55.00	100	67.40	0.58
	IHC	60.50	93.60	75.40	55.00	92.00	65.90	0.52

[1] *Mycobacterium bovis;* [2] *Mycobacterium tuberculosis* complex; [3] *Mycobacterium avium* subsp. *paratuberculosis*; [4] Positive predictive values; and [5] Negative predictive values.

The agreement between the SICTT tests for *M. bovis*, MTC, MAP and real-time PCR was fair (κ = 0.34, 95.00% CI, 0.242 to 0.498, $p < 0.0005$), very good (κ = 1.00, 95.00% CI, 1.158 to 0.842, $p < 0.0005$) and moderate (κ = 0.58, 95.00% CI, 0.372 to 0.688, $p < 0.0005$), respectively.

Immunohistochemistry detection. Out of 108 reactor cows, 58 (54.00%) were detected as*M. bovis* positive, 11(10.00%) MTC positive, and 46 (43.00%) MAP positive by IHC. In the clean group, 4(13.00%) MAP positive cows were detected by IHC. Out of 58 *M. bovis* positive (Table 2), 32(55.00%) were MAP positive and 26(45.00%) negative. In the other words, co-infection with *M. bovis* and MAP was detected. Out of 50 *M. bovis* negative, 14(28.00%) were MAP positive, and 36(72.00%) negative. Out of 11 MTC positive cows, 5(45.50%) were MAP positive and 6(54.50%) were negative. Concurrent infection with all was observed in one reactor case. The sensitivity, specificity, positive predictive value (PPV) and negative predictive value (NPV) of SICTT using IHC as the gold standard are shown in Table 3. The agreement between the SICTT for *M. bovis*, MTC, MAP and IHC was fair (κ = 0.34, 95.00% CI, 0.242 to 0.498, $p < 0.0005$), very good (κ = 1.00, 95.00% CI, 1.158 to 0.842, $p < 0.0005$) and moderate (κ = 0.52, 95.00% CI, 0.872 to 1.088, $p < 0.0005$), respectively. No false-positive signals were founded (Fig. 2).

Comparison between immunohistochemistry and real-time PCR detection. Comparison between IHC and real-time PCR for detection of *M. bovis* showed very good (κ = 1.00, 95.00% CI, 1.135 to 0.865, $p < 0.0005$) agreement. Comparison between IHC and real-time PCR for detection of MT showed very good (κ = 1.00, 95.00% CI, 1.005 to 0.945, $p < 0.0005$) agreement. Comparison between IHC and real-time PCR for detection of MAP (Johne's disease) showed very good (κ = 0.94, 95.00% CI, 1.085 to 0.795, $p < 0.0005$) agreement.

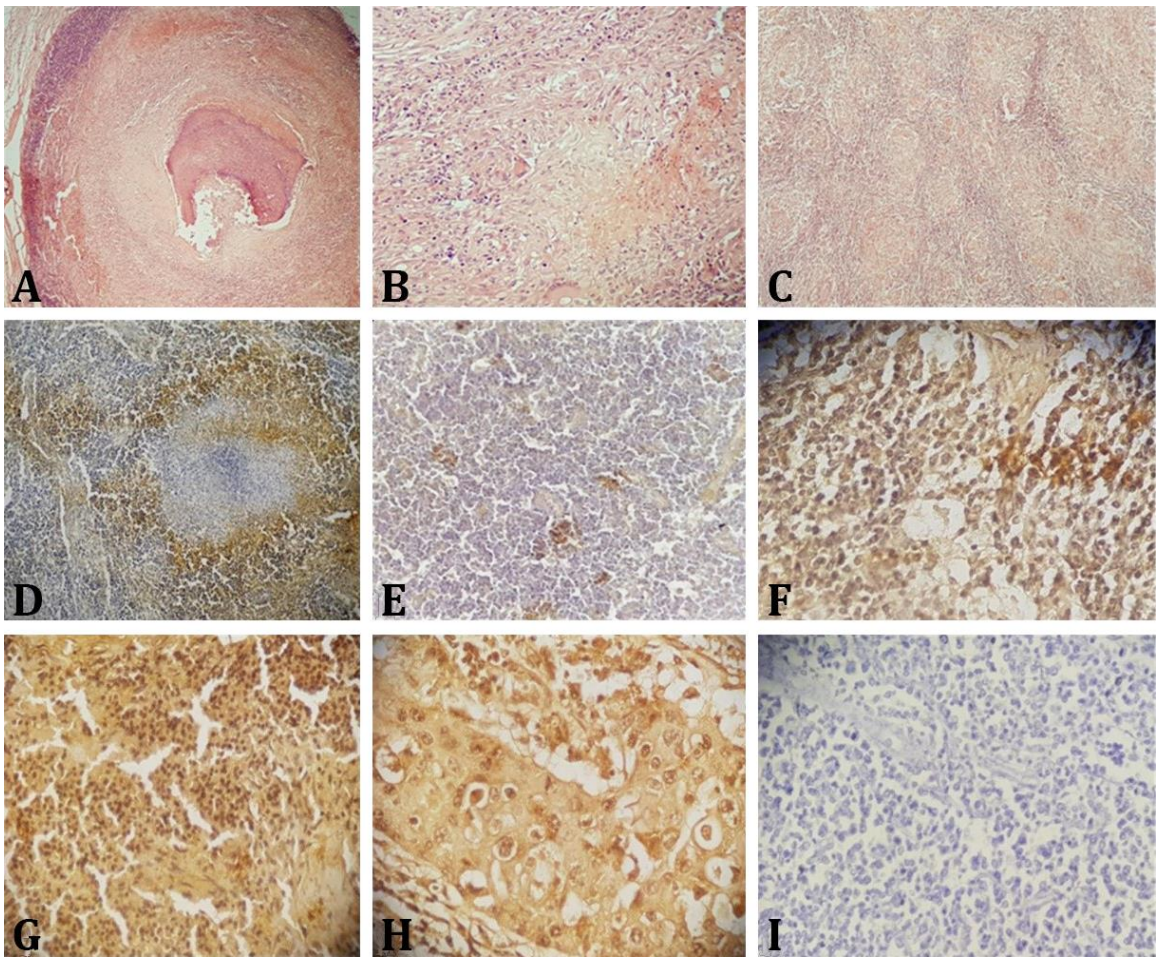

Fig. 2. A) Tissue sections from bronchial lymph node of positive SICTT reactor cow with macroscopic visible lesion, showing well-formed granuloma with central necrosis stained with Hematoxylin and Eosin (40×). **B** and **C)** Tissue sections from bronchial lymph node of positive SICTT reactor cow without macroscopic visible lesion, showing accumulations of epitheloid macrophages, small area of necrosis and low numbers of lymphocytes and neutrophils. Multinucleated giant cells were present, stained with Hematoxylin and Eosin, B (100×) and C (40×). **D** and **E)** Tissue sections from formalin-fixed paraffin-embedded bronchial lymph node showing the staining pattern of mycobacterial antigens with rabbit anti-MT antibody and secondary antibody goat anti rabbit (HRP) detected by DAB staining. Positive staining was seen as brown within cells and extracellular matrix, (IHC staining, 100×). **F** and **G)** Tissue sections from formalin-fixed paraffin-embedded bronchial lymph node showing the staining pattern of mycobacterial antigens with rabbit anti-*M. bovis* antibody and secondary antibody goat anti rabbit (HRP) detected by DAB staining. Positive staining seen as brown within cells and extracellular matrix are showing heavily *M. bovis*-infected lymphatic tissue, (IHC staining, 100×). **H)** Tissue sections from formalin-fixed paraffin-embedded mesenteric lymph node showing the staining pattern of mycobacterial antigens with rabbit anti-MAP antibody and secondary antibody goat anti rabbit (HRP) detected by DAB staining. Positive staining is seen as brown within cells and extracellular matrix, (IHC staining, 400×). **I)** Tissue sections from formalin-fixed paraffin-embedded control negative lymph node, (IHC staining 100×).

Discussion

Even though the test and slaughter programs based on SICTT and slaughter surveillance were adopted for eradication of *M. bovis* infection, the prevalence of infection has not substantially been diminished in several regions of Iran such as Khorasan-Razavi province. Slaughter surveillance for detection of mycobacterial infection requires infected cattle with visible macroscopic lesions, however, in many cases small lesions at early stages of infection are not visible at the abattoir among standard inspection in reactor animals.[19-21] In this study, 108 positive reactors were inspected and 26(24.00%) showed macroscopically visible lesions, 82(76.00%) no macroscopic lesion, and no lesion was seen in the clean group. These results were similar to other countries like UK and Ireland that 50.00 - 80.00% of reactor animals had no visible lesions.[22-26] Pal *et al.* showed that the probability of missing bovine tuberculosis lesions during routine slaughterhouse inspection was 95.00%.[27] Earlier studies indicated these probabilities as 84.00%,[28] 70.00%,[24] and 85.00%.[29] These results also revealed a much low

sensitivity of meat inspection to detect the tuberculous lesion, and it was depended on the anatomical sites and method of examination.[27,30-32] Factors including early infection, examination technique, location of the lesion, latent infection, and infection with other mycobacteria are the most important reasons.[33] In the present study, most lesions were in the lung (bronchial and mediastinal) lymph nodes, head and diaphragm, respectively. These results were similar to previous findings, which reported that many macroscopic visible lesions occurred in the respiratory system,[20,34-37] however, differed from that reported mesenteric lymph nodes by Ameni *et al*.[38] Generally, locations of tubercles depend on the route of infection, and transmission by ingestion is usually observed in calves and in the respiratory tract in cattle.[29]

Out of 108 extracted DNA of reactor cows, 58(54.00%) were positive using the species-specific primers Jb21 and Jb22. Rodríguez *et al*. obtained 100% concordance between culture and PCR with primers JB21/JB22.[39] Meikle *et al*. reported that PCR was a more specific and sensitive method than culture.[40] Vitale *et al*., Tortoli *et al*. and Mustafa *et al*. proposed that PCR was the gold standard for identifying mycobacterium species.[7,41,42]

In this study base on real-time PCR, 11(10.00%) reactor cows were MTC positive using primers INS1/INS2. Only 4 cases of 11 MTC positive cattle had a macroscopically visible lesion. It might be due to the transmission of MTC between farmers and cattle. In similar studies, Ameni *et al*. reported five out of the 16 isolates from farmers and cattle were members of the MTC.[38] Romero *et al*. reported MT infections in three cattle farms in Spain with humans as the source.[43] Ameni *et al*. at a preliminary study reported approximately 27.00% in grazing cattle in Ethiopia.[38] Thakur *et al*. in a study reported four cases of MT infections in India.[44] The results of this study and similar findings indicated the necessity of considering veterinary health measures in dairy farms to control bovine tuberculosis in humans.

The results of real-time PCR with primers P90/P91 showed that out of the total cows studied, 46(33.00%) were infected with MAP, which showed high contamination of Khorasan-Razavi province farms. Also, there were no macroscopic visible lesions in 28(60.00%) of these MAP positive cows that might be due to subclinical infection. Fathi *et al*. compared three methods in diagnosis of John's disease and concluded that molecular technique could be more valuable than culture.[45] In another study, 90 dairy cattle were tested by Johnin test and PCR. The results showed that the PCR technique was able to identify suspected cases. Out of 108 reactor cows, 32 cases of *M. bovis* and *MAP* co-infection, four cases of *MT* and *MAP* co-infection and one case of co-infection with all three mycobacteria were observed. Our results were similar to that of Kennedy *et al*. They reported that co-infection with *M. bovis* and *MAP* on a single animal induced cross-reactivity with PPD and then false responses to many types of diagnostic tests were observed.[46] Brahma *et al*. showed that *M. bovis* infection increased the animal susceptibility to MAP infection or vice versa, and co-infection was occurred with both agents.[2] Byrne *et al*. highlighted the co-infection of *M. bovis* and MAP infection as a potential factor in identifying and removing infected animals from endemic cattle farms.[26] Alvarez *et al*. described an increase in false-negative results in SICTT.[47] Amadori *et al*. showed when MAP was administered prior to *M. bovis*, animals did not react or poorly reacted to the skin test compared to *M. bovis* only.[48] The results of these researches showed the need for comprehensive and thorough research to identify the interactive effects of mycobacterial co-infection on routine diagnostic methods of tuberculosis.

In this study, the results of IHC confirmed real-time PCR findings in the reactor group and showed very good agreement ($\kappa = 1.00, 95.00\%$ CI, 1.135 to 0.865, $p < 0.0005$). In the clean group, 4(13.00%) MAP positive cows detected by IHC were negative in real-time PCR and SICTT. It might be due to the low concentration (under 8.00 pg μL^{-1}) of DNA in samples that remained undetected by PCR or due to early infection with MAP.[2] In this group, real-time PCR and IHC showed very good agreement ($\kappa = 0.94$, 95.00% CI, 1.085 to 0.795, $p < 0.0005$). It showed the role of IHC in the diagnosis of MAP infection compared to the tuberculin test and real-time PCR. In several studies, the important role of IHC in the diagnosis of mycobacterial infections in formalin-fixed paraffin-embedded tissues was reported.[49] In another study, there was 100% agreement between the real-time PCR and IHC for detection of MTC and 94.00% for detection of MAP.[2] Our results were different from study of Mustafa *et al*. that reported an 87.00% agreement between IHC and PCR as the gold standard.[7]

In Iran, in 1967, the test-and-slaughter program was instituted nationally based on the SICTT using both tuberculins provided by Razi Vaccine and Serum Research Institute (RVSRI), for intensive farms as well as their surrounding traditional farms. The operation of this program since 1981 has decreased the frequency of bovine tuberculosis in the cattle population of Iran from > 5.00% to < 0.14%.[8] In this study, the sensitivity and specificity of SICTT for *M. bovis* were calculated 53.70% and 100%, MTC 100% and 100%, and MAP 60.50% and 100%, respectively. These results were similar to Alvarez *et al*. study in Spain that the sensitivity and specificity of SICTT were highly variable (sensitivity v = 40.10 - 92.20% and specificity > 99.00%).[47] Similarly, Schiller *et al*. in a review of many international studies, reported 55.10 - 93.50% sensitivity and 88.80 - 100% specificity for SICCT.[50] According to a study by O'Hagan *et al*., in the British Isles, the sensitivity of the SICCT test was 88.61%, and the median estimated for the specificity was 99.80% that was different from our results in the specificity.[25]

In some circumstances, anergy and immunosuppression in the advanced stages of tuberculosis, could explain the decreased insensitivity. This low sensitivity is an essential issue for the success of the test-and-slaughter program that should be considered in the control program.

The success of the test-and-slaughter program for bovine tuberculosis control directly depends on the diagnostic ability of the laboratory tests. The SICTT is limited in its sensitivity and specificity especially in association with another *Mycobacterium* spp. In this study, one of the most important points was co-infection with two or more *mycobacterium*. This means that using one laboratory method to detect bovine tuberculosis alone is not sufficient, and it must use two or more laboratory methods. The results also provided further confirmation of IHC and real-time PCR as a sensitive and reliable diagnostic screening approach for the detection of bovine tuberculosis.

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Conflict of interest

The authors declare no conflict of interest.

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