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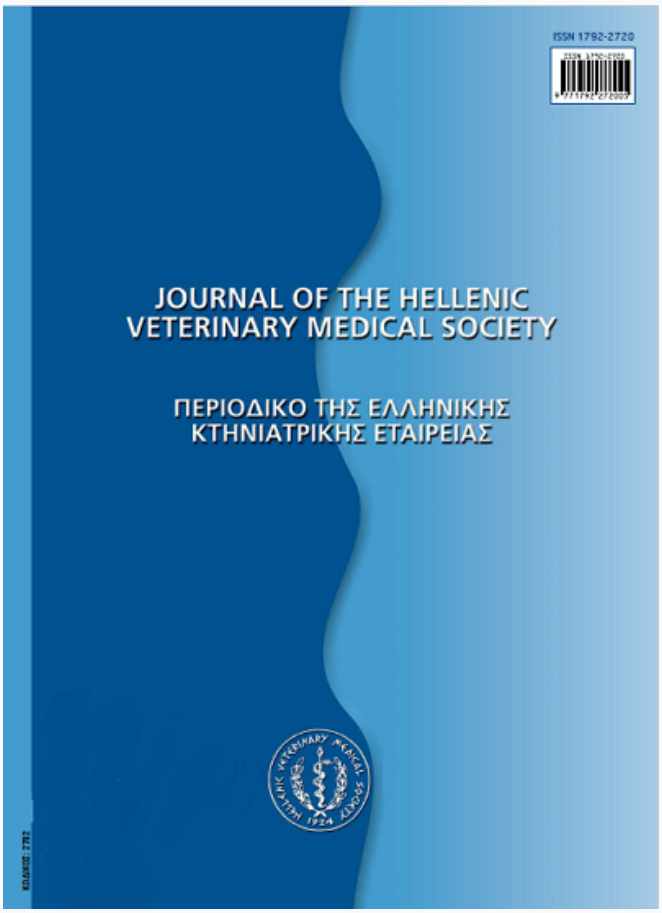

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Detection *of Mycobacterium avium subsp. paratuberculosis by se*veral diagnostics techniques in clinical suspected sheep

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ABSTRACT. A total of thirty sheep with clinical symptomatology of *paratuberculosis* (Johne's disease) were subjected to four diagnostic techniques: histopathological examination, bacteriological culture (in faeces and tissues), polymerase chain reaction (PCR) (in blood, tissue and faecal samples) and antibody responses (ELISA). Twenty-one (70.0%) animals showed histological lesions. Bacterial culture of both faeces and tissue revealed that 2 (6.7%) and 6 (20.0%) of the 30 sheep were infected, respectively. *Mycobacterium avium* subsp. 24 *paratuberculosis* (*Map*) was identified in 4 animals via PCR of faeces (13.3%), and in 19 (63.3%) by PCR in tissues. PCR in blood revealed 7 (23.3%) infected animals. Three (10.0%) animals showed antibodies against *Map.* A greater number of positive animals were detected by histopathological examination and PCR in tissues than by culture or ELISA. This study confirmed the clinical findings and results suggest that histopathology, PCR in tissues and in blood can improve the detection of *Map* in physically suspected animals and should be considered useful tools in the diagnosis of *Map* in suspected sheep.

Keywords: sheep, histopathology, culture, PCR, ELISA, paratuberculosis

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INTRODUCTION

Paratuberculosis (Johne's disease) is a chronic infectious disease caused by *Mycobacterium avium* subsp. *paratuberculosis* (*Map*) that affects ruminants. The disease is the most important infection of domestic ruminants affecting worldwide and responsible for substantial economic losses (Verdugo et al., 2014). The most important factor to achieve the eradication of this chronic disease is the difficult of ante-mortem diagnostic tests to detect animals during its latent period prior to their becoming infectious. Since, treatment of *paratuberculosis* is not considered a practical option, its control is mainly based on preventing measures to avoid the transmission of *paratuberculosis* as well the application of a test-and-slaughter program (Kawaji et al., 2011).

Regarding their diagnosis, several techniques have been described in the literature such as Ziehl-Neelsen staining (Coelho et al., 2008), bacterial culture (Whittington, 1999), histopathology analysis (Perez et al., 1996), ELISA (Whitlock et al., 2000) or PCR (Garrido et al., 2000). However, scarce information is available about their combination to improve the diagnostic of paratuberculosis.

Detection of bacteria from faeces and tissues samples by means of culture or molecular methods and the evaluation of histopathological lesions constitute the most effective methods of confirming a clinical diagnosis of *paratuberculosis* (Gonzalez et al., 2005, Perez et al., 1996) however, culture of bacteria from faeces and tissue samples is less sensitive and requires months of incubation before colony growth occurs. This means that a significant amount of time is needed before a diagnosis can be made (Huntley et al. 2005). In addition, the isolation of the bacteria is also difficult due to intermittent shedding and a low number of bacilli in faeces and tissues respectively (Pavlík et al., 2000, Reddacliff et al., 2003).

ELISA has been frequently used for *paratuberculosis* diagnosis however, its results should be interpreted with attention since its sensitivity and specificity is variable and affected by factors such as specie, type of sample or stage of disease (Nielsen et al., 2008). Thus, some reports indicated higher sensitivity and specificity values from serum or milk samples than from faeces (Liapi et al., 2015).

Diagnosis based on the detection of IS*900*-specific sequences of *Map* by polymerase chain reaction (PCR) from tissue, faeces and blood is considered to be very quick, and highly specific (Bhide et al., 2006, Coelho et al., 2008b, Garrido et al., 2000). The aim of this study was to describe and compare the performance of four diagnostic methods (Zielh-Nielsen stainign, faecal culture, ELISA and PCR) for the detection of *Map* in tissues, faeces and blood in clinically suspected animals.

MATERIAL AND METHODS

Animals and samples

A total of thirty adult sheep of both sexes with clinical signs of *paratuberculosis* were obtained from thirty different flocks of Trás-os-Montes e Alto Douro region (Northeast of Portugal). Sheep were euthanized by intravenous injection of barbiturate followed by exsanguination. Prior to euthanasia, blood samples to be used for serology and for DNA extraction were collected from each sheep. Serum was separated after clotting by centrifugation at 200 X *g* for 10 minutes and stored at -20ºC until use. Faecal samples weighing approximately 5 g were collected directly from the rectum using disposable gloves, transferred to individual sterile plastic containers, frozen, and then stored at -20ºC until processed.

Pathological examination

Animals were subjected to a full necropsy and gross lesions were recorded. The intestinal wall was carefully examined for thickness, corrugation and enlargement of the associated mesenteric lymph nodes. Segments of the jejunum, ileum, ileocaecal valve and mesenteric lymph nodes were removed aseptically using only sterile instruments and containers for bacteriological examination and DNA extraction and stored at -20ºC until used. Samples were fixed in a 10% buffered formol-saline-solution by immersion. Fixed tissue samples for histopathology were processed using routine techniques.

Tissue extracts were embedded in paraffin wax, sectioned at 4 μm, stained with haematoxylin and eosin (HE) and the Ziehl-Neelsen (ZN) technique. Histopathological lesions in the jejunum, ileum, ileocaecal valve and mesenteric lymph nodes with regard to the type of infiltrating cell and the presence of acid-fast organisms, were observed and recorded according to Clarke (1996).

Faecal and tissue culture

Processing of faeces and tissues for culture was carried out as described in the literature (Juste et al., 2001). Briefly, a 2 g sample of tissue or faeces was decontaminated with 0.75% hexadecylpyridinium chloride (HPC; Merck KGaA, Darmstadt, Germany) and homogenized in a Stomacher lab blender (Seward Stomacher 400) for 30 seconds. The decontamination procedure consists in the elimination of other microorganisms in competition with Map. Since these microorganisms growth faster than Map (Garrido et al., 2000b), the utilization of hexadecylpyridinium chloride made an unfavorable growth conditions for these microorganisms without negative effect against Map (Stabel, 1997). After being left to settle for approximately 18 hours at 22 ºC, 3ml of the suspension were transferred to a disposable pipette. The material was inoculated directly on to two slants with Löwenstein-Jensen® with mycobactin-J. For culture on Middlebrook® 7H11, 10 ml of the interface were transferred to 15 ml tubes after sedimentation and left for 18 hours. The suspension was centrifuged at 6000 X *g*, and the supernatant replaced with 3 ml of sterile water before being inoculated into the tubes. All tubes were incubated aerobically at 37ºC and examined every week for *Map* growth. Samples were considered negative in the absence of growth after 12 weeks.

Confirmation of growth included mycobactin dependence and the testing of a colony typical from each specimen by Ziehl-Neelsen staining and PCR assay for the DNA sequence at IS*900*. Slide smears prepared from faecal and tissues impressions and samples from primary colonies were stained by the Ziehl-Neelsen technique using standard methods.

Polymerase chain reaction (PCR)

Specific *Map* DNA was detected following the extraction of genomic DNA from frozen samples of faeces, mesenteric lymph node and segments of jejunum, ileum and ileocaecal valve. The PCR analysis procedure for colonies, faeces, tissues and blood was performed as described in the literature (Coelho et al., 2008b). Briefly, 1 g of faeces or tissue was homogenized in a Stomacher lab blender (Seward Stomacher 400) for 30 sec. in 20 ml of a buffer containing 5% sodium dodecyl sulfate (SDS). After sedimented for 15 min, the upper aqueous phase was transferred into 15 ml tubes and washed three times in PBS. The pellet was resuspended in 2 ml of PBS and transferred into a 2 ml screw-cap tube. The suspension was centrifuged at 9600 X *g* and the supernatant discarded. The pellet was transferred to an eppendorf tube and either processed immediately for DNA extraction or stored at -20ºC.

Blood samples were transferred to an eppendorf tube and the cellular fraction was incubated with 0.83% ammonium chloride (1:2, v/v) for 20 min to lyse the red cells. The tube was then centrifuged for 10 min at 200 X *g* and the cell pellet was washed twice in PBS by centrifugation under identical conditions (14). The cells were processed immediately for DNA extraction, or stored at -20ºC. The buffy coat was then transferred to 2 ml tubes. The DNA extraction was carried out by the freeze-boiling method[11]. Each sample was used in duplicate in a PCR assay. The primers RJ1 (GTT CGG GGC CGT CGCTTA GG) and PT91 (CCC ACG TGA CCT CGC CTC CA) flanking a region of 389 bp were used for amplification of the IS*900* sequence of *Map* as described by Moss (1992). A 5µl aliquot of DNA extract was added to a PCR mix containing 0.1M of each primer, 1.5mM of MgCl2 and 2U of Taq DNA polymerase (Qiagem, Germany) in a total volume of 25µl in 200µl tubes, covered by mineral oil. The cycling conditions with the thermal cycler (Perkin Elmer Cetus® 480) were one cycle at 96 ºC for 2 min, 40 cycles at 95 ºC for 30 s, 55ºC for 30 s and 72 ºC for 1 min and a final extension time of 72 ºC for 10 min. Aliquots of each amplification mixture were electrophoresed in a 2% agarose gel containing ethidium bromide. For each set of PCRs, an internal amplification control and a positive (*Map* DNA) and a negative (water) control were included. The results were analyzed blind, and a sample was scored as positive only if a 389-bp DNA fragment of the expected size was identified on gel, and no signal was obtained with the negative control. The identification of each positive culture was confirmed via PCR, as previously described, by selecting a colony from the agar surface with a sterile wooden toothpick and suspending it in 200 µl of distilled water.

Mycobacterium avium subsp. *paratuberculosis* isolates were differentiated by IS*1311* PCR-restriction endonuclease analysis. Briefly, in the reaction a segment of IS*1311* element was amplified and digested with *Hinf*I and *Mse*I endonucleases as reported in the literature (Marsh et al., 1999). DNA fragments were separated by electrophoresis in 4% (w/v) agarose gels

stained with ethidium bromide. Isolates were classified as sheep (S) strain types according to the criteria indicated in the literature (Sevilla et al., 2005, Whittington et al., 2001). This method was performed in the Instituto Vasco de Investigación y Desarrollo Agrario (NEIKER), Bizkaia, Spain.

ELISA

Sera were tested for *Map* antibodies by a commercial ELISA test (ELISA *Paratuberculosis* Antibody Screening, Institut Pourquier, Montpellier, France) used in accordance with manufacturer's instructions. This ELISA detects antibodies against *Map* in cattle, sheep and goats. Briefly, Blood (10 ml) was collected in the jugular vein and serum was separated after clotting by centrifugation at 200 g for 10 min and frozen at 20 C until use. All samples were subjected to a commercial ELISA test according to the manufacturer´s instruction. This ELISA detects serum antibodies against *M. avium* subspecies *paratuberculosis* using an absorption step to remove non-specific antibodies. On each 96-well plate, 94 serum samples were tested in single wells as well as a negative and a positive control provided by the manufacturer. Optical densities of the resulting products were measured in an ELISA reader at 450 nm. Test results were interpreted as indicated by the manufacturer and readings below 60% of the positive control serum OD were considered negative. Readings equal or greater than 70% as positive and readings between 60 and 70% were scored as doubtful. Positive and doubtful results were run into duplicate wells (one coated and one not-coated), with the same protocol. Following this step positive and doubtful results were considered as positive. According to the manufacturer, the kit has a sensitivity between 50 and 65% and a specificity above 99.5%.

Case definition - Individual sheep were classified as infected with *Map* if any one of the culture slants from their faeces or tissues were positive, histopathology was positive with a ZN positive or if a PCR assay detected *Map* DNA in any of their tissues, faeces or blood.

RESULTS

A total of 30 sheep were analyzed using eight tests for *paratuberculosis* diagnostic. The tests results are summarized in Table 1. Seven sheep presented soft pasty faeces and 15 of them also presented diarrhea during the clinical examination. 29 of 30 sheep of the study were females and all of them presented normal appetite, however, *paratuberculosis* was confirmed in 26 (86.7%) sheep. No positive results were observed in any of the tests used in the 4 sheep left. All of the suspected sheep presented a poor body condition. During necropsy, most of sheep presented macroscopic changes compatible with paratuberculosis. In those 26 (86.7%) previously confirmed, a thickening of the ileum mucosa was evident. In 9 cases (34.6%), the thickening was mild, in 5 (19.2%) it was moderate and in 12 (46.2%) severe. Six (18.8%) sheep showed mucosal congestion. Prominent transverse ridges and crevices that could not be reduced by stretching were observed in the ileum of 15 sheep (50.0%). 13 (43.3%) sheep presented serosal lymphatic dilation and small whitish lesions in the lymph vessels were observed in 6 (20.0%) sheep. Thickening of the intestinal wall and lymphangitis was less prominent in 9 cases. Changes in the caecum and colon were less severe in comparison with terminal ileum. Mesenteric lymph nodes

Table 1. Number of positive samples diagnosed by four diagnostic tests (culture, histopathology, ELISA and PCR) for detection of *Mycobacterium avium* subsp. *paratuberculosis* in blood, tissues and fecal samples

	Method							
	HP	ZN	CF	CT	PCR-f	PCR-t	PCR-b	ELISA
Positive	21/30	16/30	2/30	6/30	4/30	19/30	7/30	3/30
Total (%)	70.0	53.3	6.7	20.0	13.3	63.3	23.3	10.0

HP: Histopathology; ZN: Ziehl-Neelsen (smear); CF: culture of faeces; CT: culture of tissues; PCR-f: polymerase chain reaction in faeces; PCR-t: polymerase chain reaction in tissues; PCR-b: polymerase chain reaction in blood; ELISA: Enzyme-linked Immunosorbent Assay

were markedly enlarged and oedematous in 20 sheep (66.7%). Calcifications were only present in the lymph nodes of two animals. There were no visible macroscopic lesions in 3 sheep. The terminal ileum and ileocaecal valve had more severe lesions.

Paratuberculosis was confirmed by histopathological examination on the basis of specific granulomatous cell infiltrates and stainable acid-fast bacilli in 21 sheep (70.0%) that showed signs of granulomatous enteritis with marked cellular infiltrate (Figure 1). The histopathological characterisitics observed in sheep were: villous atrophy (22 – 73.3%), necrosis (16 – 53.3%), infiltrate composed of epithelioid cells (14 – 46.7%), lymphocyte infiltrate in the mucosa and submucosa (25 – 83.3%), hyperplasia of Peyer's patches (18 – 60%). Histopathological lesions in the ileocaecal valve and ileum (4-13%) were observed in the 13% of the paratuberculosis-infected sheep

In some cases, submucosa was thickened with epithelioid cellular infiltrate but other sheep presented an irregular accumulation of lymphocytes and epithelioid cells. Also, submucosal oedema and Multinucleate Langhans-type giant cells were observed in 3 and 2 sheep respectively. The presence of acid-fast bacilli bore a relation to the extension of the epithelioid cellular infiltrate. In 3 cases, numerous acid-fast bacilli packed in the cytoplasm of the many large epithelioid cells that infiltrated the mucosa were revealed by ZN staining.

Lesions in the lymph nodes were composed of a macrophage infiltrate within the subcapsular sinuses (12 - 40.0%) with extension to the paracortex and medulla (10 – 33.3%), granulomas (2 - 6.7%), and epithelioid cells in 7 lymph nodes (7 - 23.3%, 2 of them presented acid-fast bacilli). Seven sheep did not present histological changes in the intestinal tract and mesenteric lymph nodes. Lesions of sheep positive *paratuberculosis* (n=21) were classified accoding to Gonzalez (2005) as focal-grade 1 (2 – 9.5%), focal-grade 2 (2-9.5%), multifocal subtype 3b (diffuse multibacillary (9 – 42.9%), multifocal subtype 3c (diffuse paubacillary) (8-38.1%),

Faecal smears from 16 sheep were positive to ZN, however, only 2 of them were confirmed by culture. In *post mortem* samples, mycobacteria were observed by ZN in 13 (43.3%) cases from ileum mucosal scrapings. The ZN smear was also positive in 9 and 12 samples of jejunum and ileocecal valve respectively. In addition, lymph nodes were positive to ZN smear in 11 cases. Faecal culture identified *Map* infection in 6.7% of the sheep. Six (20.0%) sheep were positive to bacterial culture from tissue. Bacterial culture of the jejunum and ileal mucosa was positive in 4 and 3 sheep respectively.

The microorganism was also isolated from 3 (10.0%) lymph nodes. Identification of 3 culture isolates by IS*1311* PCR-restriction endonuclease analysis indicated that Map belonged to the S strain (Sheep type or Type I). Also, 4 (13.3%) sheep presented positive results in faeces PCR.

The PCR analysis was positive in blood (7), intestinal tissue (19) and mesenteric lymph nodes (7). By tissue localization, samples of ileocaecal valves (12), jejunum (9) and ileum (13) were PCR positive. However, PCR analysis was negative in 8 sheep although 4 of them presented positive results in other diagnostics test. Regarding ELISA diagnostic, 3 sheep were positive. However, 23 of the 27 seronegative were positive by other diagnostics techniques. .

Combination of all diagnostic methodologies

16 were found infected by Map both by histopathological examination and PCR analysis. Only 2 sheep were both positive by faecal culture and histopathology.

6 sheep that were positive for tissue culture also presented histopathological lesions compatible with paratuberculosis.

Regarding PCR, 3 sheep presented positive results in tissues samples without histopathological lesions. In contrast, 5 sheep that were positive to histopathological analysis were negative to PCR diagnosis in tissues. PCR using the IS*900* sequence detected 4 and 19 sheep, previously positive to faecal and histopathological analysis respectively. In contrast, positive results of PCR from faeces, blood or tissues samples were observed in only 5 sheep without positive results after histopathological and culture analysis. Combination of ELISA and histopathology techniques detected only 3 cases of paratuberculosis. Moreover, only 2 sheep were considered positive by histopathological analysis exclusively.

Combination of histopathological analysis and PCR in tissues detected 80.8% and 73.1% of suspected clinical sheep respectively, being the most sensitive techniques. However, faecal culture presented the lowest sensibility since it only detected 8.3% of the positive cases. Since it was assumed a specificity of 100% for all tests, PCR analysis applied in tissues or

blood detected 84.6% of infected sheep. Combination of histopathology analysis and PCR of tissues and blood detected 86.6% of the total of sheep studied.

Although 70% of sheep presented histopathological lesions, combination of ELISA and culture of faeces only detected 16.6%, indicated the low sensitivity of these techiniques.

DISCUSSION

Information about ovine *paratuberculosis* in Portugal is scarce (Coelho et al., 2007, Mendes et al., 2004). In the current study, *paratuberculosis* was confirmed in 26 of the 30 sheep using a combination of pathological, microbiological and serological methods. Since a high percentage of sheep showed lesions compatibles, the histopathological analysis revealed as one of the most important diagnostic techniques for those animals with clinical signs compatible with paratuberculosis. Necropsy revealed gross lesions in all infected animals with differing grades of mucosal thickness. The gross lesions observed were according as described in the literature (Clarke and Little, 1996, Perez et al., 1996). Multinucleate Langhans' giant cells were identified in few cases. These observations are according to other studies which suggest that such cells are uncommon in small ruminants (Clarke and Little, 1996). Our results are in accordance with previous studies that suggest that smears stained with Ziehl-Neelsen can be considered as good indicators of infection and could be used as a screening method using faeces and tissue as samples since this technique is inexpensive and low time consuming (Coelho et al., 2008). However, the low sensitivity of this technique, about 36%, (Zimmer et al., 1999) indicates that this diagnostic tool should be complemented with other diagnostic techniques.

The *Map*-S strains, recovered in the present study, are more fastidious and presented a slower growth rate (Bannantine et al., 2012). Failure to isolate *Map* from faeces and tissues, in which numerous acid-fast bacilli were observed, could be associated to the infection by viable but not cultivable or hard to culture, sheep strains (Ayele et al., 2004). The PCR of lymph nodes also showed a lower sensitivity compared with their application in other tissues. Similar results were found by others authors, and were attributed to the large quantity of non-target DNA or to the differences in effectiveness of the extraction method in different tissues (Gwózdz et al., 2012).

The detection of *Map* by PCR in blood supports the hypothesis that *Map* spread by the circulatory system as described by other authors (Bhide et al., 2006, Coelho et al., 2008b, Gwózdz et al., 2012). Our results showed that blood samples of some cases had sufficient bacterial DNA to identify Map by PCR, even when faecal analysis, culture from tissue or PCR from these samples were negative. However, positive results in blood should be carefully interpreted since not all small ruminants exposed to pathogenic mycobacteria succumb to disease (Silva et al., 2010) and blood is not the target location of the bacteria.

Contrary, it was observed that PCR from tissues was positive while the histopathological analysis as well as other techniques used were negative. These differences could be explained by the fact that same tissue sample cannot be used for histopathology, culture and PCR because the portion sampled for microbiological purposes might not contain any lesions. In addition, PCR does not require the presence of viable organisms, whereas it is necessary for bacterial culture (Miller et al., 2002). Also, the sample freezing may cause loss of viable organisms (McKenna et al., 2005).

Histopathological examination presented a better performance of *paratuberculosis* diagnostics than bacteriological culture. Moreover, slightly higher positive results of tissues analysis by histopathological examination or PCR were observed than bacteriological culture. In a recent study, ELISA was presented as a good tool for diagnosis of *paratuberculosis* in cattle (Lavers et al., 2015) altough variations in the sensitivity and specificity in small ruminants have been reported (Nielsen et al., 2008). ELISA has been used as a screening test in domestic animals (Kumar et al., 2006) since culture is not useful in the routine screening of animals (Singh et al., 2014). A previous study showed that Pourquier ELISA kit appeared to be quite satisfactory for small ruminants (Gumber et al., 2006) but Pourquier ELISA was produced for cattle and for small ruminants. Since in the kit the *Map* antigen used was quite different to the one present in Portugal, this could explain the lower sensitivity observed. Thus, the ELISA test used in our study was not standardized using the correct type of the antigen from S bio-type strain. Previously, it was observed that antibodies against *paratuberculosis* were not related with histopathological lesions

(Hope et al., 2000). In addition, ELISA is a better test to detect infected sheep with focal lesions which had small numbers of acid-fast bacilli (Hope et al., 2000). Although the sensitivity of the ELISA technique used in the current study was quite similar as reported by Liapi et al., (2011), the lower sensitivity observed, when used as unique diagnosis technique, could be associated to differences in the stage of the disease that conditioned the immunological response of the animal. Previous studies indicated that both faecal microscopy and ELISA could be adopted to improve the diagnostic of *paratuberculosis* at field (Singh et al., 2009, 2013, 2014). However, the high specificity of this commercial ELISA, according to the manufacturer´s information, could be a valuable diagnostic tool in order to confirm positive cases obtained by others methodologies, mainly by PCR.

Due to the variable sensitivities of the diagnosis techniques studied in the current work, PCR analysis of tissues and blood displayed the best performance in the diagnosis of *paratuberculosis* in sheep compared to other techniques such as ELISA or bacteriological culture.

CONCLUSION

The current study presented the performance of different diagnosis tests for *Map* infection in sheep. Since the time to obtain a results and its cost are important factors in a diagnostic laboratory, the results observed by the application of PCR analysis in blood and tissues together with histopathology were the valuable diagnostics tools due to their effective, low-cost and low-time consuming. The present results suggest that combination of two diagnostic tools improve the detection of *Map* in clinically suspected animals and should be considered in the diagnosis of *Map* in suspected sheep.

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