



# First Pathological, Molecular and Serological Investigation of Ovine Johne's Disease (Paratuberculosis) in Northeastern Algeria

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## RESEARCH ARTICLE

### Abstract

Paratuberculosis (PTCB) or Johne's disease (JD) caused by a slow growing acid fast bacillus *Mycobacterium avium* subspecies *paratuberculosis* (MAP), is an infectious disease of many species including humans. The disease is responsible for important economic losses to livestock industry worldwide. Although the disease is widespread, it had not been studied in Algerian sheep. In this study we inspected the presence of the infection in sheep (aged 2 years old or older) using histopathology and IS900-PCR techniques in randomly 378 tissues (ileum, ileocecale valve and lymph nodes) and fecal samples. Gross lesions were detected in 16 (4%) of samples. Histopathological examination revealed the presence of pathognomonic lesions of JD in 61 (14%) animals. Ziehl-Neelsen (ZN) staining of tissue samples was positive in 28 (7%) cases. MAP-DNA was detected in 34 (9%) fecal samples. 14 (4%) animals were tested positive for antibodies against MAP. This study showed that not only ovine paratuberculosis present in Algeria but infected animals are excreting the bacteria in feces. This could be a significant threat for other ruminants and humans. Other studies should be carried out in order to better understand the prevalence and the molecular epidemiology of MAP in the country.

**Keywords:** Paratuberculosis; sheep; histopathology; polymerase chain reaction (PCR); ELISA.

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## INTRODUCTION

Johne's disease (JD) or Paratuberculosis (PTBC) is a chronic wasting condition of ruminants and other domestic and wild animals, caused by *Mycobacterium avium* subspecies *paratuberculosis* (MAP) characterized by chronic enteritis of the small intestine diminishing an animal's ability to take up adequate nutrients resulting in the subsequent loss in body condition and eventual death (De Silva et al., 2013). PTBC is an economically important disease in sheep global industry due to lower milk production, reduced slaughter value due to profound weight loss of ewes, early culling of infected animals, and eventually death (Nielsen and Toft, 2009). Additionally, to direct losses, losses due to sheep infertility and value for animal sale caused by the disease was also reported (De Grossi et al., 2020; Gautam et al., 2018). Despite the presence of MAP in our food chain due to excretion of MAP in both milk and feces by either symptomatic and subclinically infected dairy cows (Garvey, 2018), several studies have linked the role of this bacterial species in human morbidity and therefore reported the detection of MAP DNA in a high percentage of patients with Crohn's disease (CD) (Mendoza et al., 2009). Ovine paratuberculosis has a worldwide prevalence and is a serious threat to sheep production due its silent progression and

the severity of the illness that are always accompanied to a too late clinical diagnosis, showing only indirect production effects (Juste and Perez, 2011). PTBC is more insidious in small ruminant and may occur from 2 years of age. Clinically, affected animals exhibit gradual weight loss and exercise intolerance termed as ‘an increase in the tail to the mob’, with soft feces in some animals (Windsor et al., 2014). Except in the final stages of disease where it is usually intermittent and unremarkable, diarrhea is not regarded to be pathognomonic of PTBC in small ruminants (Robbe-Austerman, 2011). This can make difficult to identify infected small ruminants compared to cattle, and may lead to the spread of the disease in herds/flocks unnoticed. The eradication of the disease is hindered by the lack of rapid, accurate and sensitive diagnostic tools (Garcia and Shaloo, 2015). The major problem for managing the disease is identifying subclinically infected animals in the herd to prevent further spread of the infection (Hemida and Kihal, 2015).

One of the greatest challenge for diagnosis of subclinical cases are the latency and slow growing of MAP and tests with high specificity and sensitivity are still missing (Nielsen and Toft, 2008). ELISA, bacterial culture of fecal samples, and PCR are widely employed for ante mortem diagnosis of PTBC (Clarke and Little, 1996), however the most ultimate diagnostic test for sheep and goats PTBC is post-mortem evaluation with histopathological confirmation (Stevenson, 2010).

The histopathological lesions of paratuberculosis in sheep have been reported in detail and various classification systems have been proposed (Thakur et al., 2017).

To the best of our knowledge, only one study (Hemida and Kihal, 2015) has reported the prevalence of cattle PTBC in Tiaret region in the Western Algeria. Although ovine PTBC could be found in many countries worldwide, no cases had been documented up to now among Algerian sheep herds. The purpose of the present study was to investigate the presence of sheep PTBC in Northeastern region of Algeria using three diagnostic methods.

## MATERIALS AND METHODS

During 2015-2017 periods, a total of 378 intestinal samples (ileum, mesenteric lymph nodes), serum and feces were randomly collected from 378 local adult sheep (2 to 6 years) from four provinces in the Northeastern Algeria for laboratory investigation (Table 1). Among these animals, 245 sheep were slaughtered in legal slaughterhouses, 95 were slaughtered at illegal places and 38 sheep were necropsied for different reasons. In all animals, clinical signs were carefully recorded either by ante mortem examination or through information supplied by the owners or the practitioners. Ileum and/or ileocecal valve and associated lymph nodes from each animal were observed macroscopically and lesions consistent with PTBC in terms of corrugation and thickening of the intestinal mucosa and hypertrophy of the mesenteric lymph (MLNs) nodes were recorded.

**Table 1.** Distribution of 378 studied animals and 61 positive cases (using Histopathology, PCR and ELISA), among four provinces located in North-eastern Algeria.

Province	No tested animals	No positive cases	Category	
			Clinical	subclinical
El Tarf	100	18	6	12
Annaba	92	9	3	6
Guelma	107	11	8	2
Souk Ahras	79	23	9	14

### Microscopic Examination of tissues samples

Tissue portions were taken from the distal ileum, ileocecal valve and associated MLNs. After that tissue samples were fixed in 10% neutral buffered formalin and dehydrated through graded alcohols and xylene before being embedded in paraffin wax blocks. Sections (5µm thick) were cut from each sample and stained with haematoxylin and eosin (HE) according to Bancroft et al. (2007), and by the Ziehl-Neelsen (ZN) method for acid fast bacteria (AFB) according to Huntley et al. (2005).

### DNA Extraction and PCR Reaction

DNA was extracted from fecal samples collected directly from the rectum of dead animals. The extraction of DNA was performed using ZR Fecal DNA MiniPrep™ (Zymo Research) and as described by Leite and Stabel (2012). Briefly, the method consisted of bead-beating 150 mg of fecal sample in lysing solution for 5 min at maximum speed using a vortex. The obtained lysed sample was centrifuged, and the supernatant was then added to a spin filter. After centrifugation, binding buffer was added to the filtrate, and the suspension was transferred to another spin column. Using centrifugation, DNA prewash and fecal DNA wash buffers were added to the spin column to clean the bound DNA. The final step of the protocol, after eluting the DNA in 100 µl, was filtration through a spin column into a new tube. Oligonucleotide primers P90 (5' - GAA GGG TGT TCG GGG CCG TCG CTT AGG-3') and p91 (5' -GGC GTT GAG GTC GAT CGC CCA CGT GAC-3') were used to amplify a 413bp fragment of IS900 insertion sequence specific for Map. Thermal amplification was performed using an initial denaturation step of 94°C for 1 min, followed by 30 amplification cycles at 94°C for 1 min, 55°C for 1 min, and 72°C for 10 min.

### Enzyme Linked Immunosorbent assay

Serum samples collected from live animals before slaughter and euthanasia (for necropsied cases) were tested by a commercially available PARACHEK® 2 ELISA (Prionics AG, Zurich, Switzerland). All samples were tested according to the manufacturer's instructions and as described by Mpenda and Buza (2014). Before testing, all serum samples were diluted and incubated in Green Diluent Buffer containing *Mycobacterium phlei* to remove cross-reacting antibodies. The absorbance was determined using Microplate Reader (BIO-RAD laboratories Inc., USA) with dual 450 nm and 650 nm filters.

## RESULTS AND DISCUSSIONS

A total of 378 samples (tissue samples, sera and feces) were analyzed using three tests for PTCB diagnosis. 26 (7%) sheep presented clinical signs suspecting the disease, all of them were with poor body condition score, 16/26 (61%) showed diarrhea and 5/26 (19%) presented soft pasty feces. In 3/26 (12%) sheep, intermandibular edema (bottle jaw) was observed. Moderate to complete loss of wool was seen in 4/26 (15%) cases.

### Gross findings

The intestinal wall was carefully examined for thickness, mucosal corrugation, congestion, as well as enlargement, adenopathy and calcification of the associated mesenteric lymph nodes lymph (MLNs). In our study, out of 378 animals only 16 (4%) were shown to have evident gross lesions as mild to moderate thickness and corrugation of the intestinal mucosa (ileum and ileocecal valve) as well as hypertrophy of associated mesenteric lymph nodes. These macroscopic changes were observed especially in animals with poor physical condition manifesting clinical signs as severe emaciation associated or no with diarrhea. Additionally, 8 (2%) apparently healthy sheep had lymphadenopathy of the mesenteric region with mild mucosal lesions.

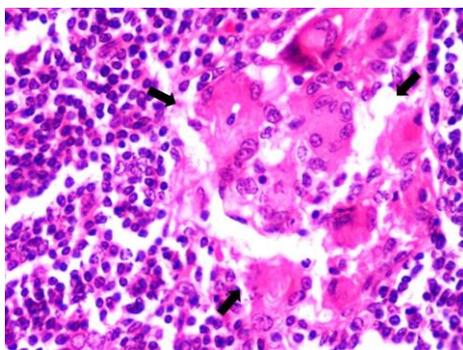
### Microscopic findings

The presence of focal, multifocal or diffuse granulomatous lesions in the intestinal mucosa, gut associated lymphoid tissue and/or lymph nodes were considered as typical for paratuberculosis as described previously by earlier workers (Gonzalez et al., 2005; Pérez et al., 1996). Out of 378 tissue samples (from ileum, ileocecal region and mesenteric lymph nodes), lesions associated with paratuberculosis were found in 61 animals (16%) (Table2).

**Table 2.** Summary of results of PCR, Histopathology and ELISA technique according to lesions types

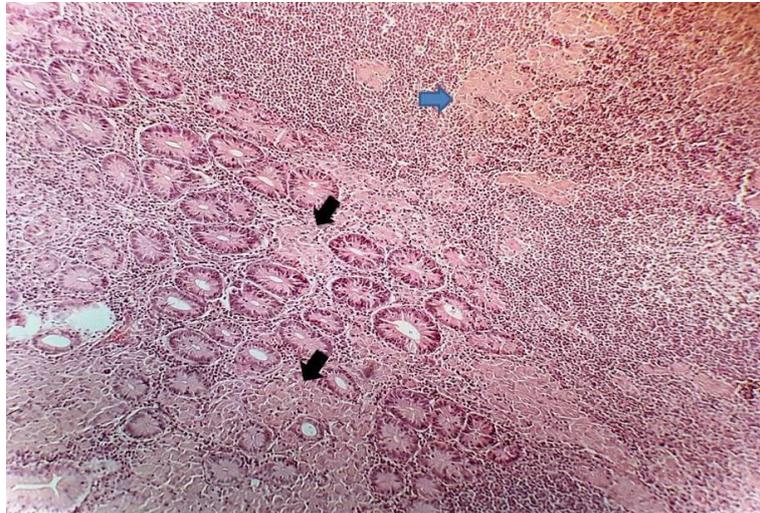
Lesions features	Histopathology		ZN		PCR		ELISA	
	No positive	%	No positive	%	No positive	%	No positive	%
<b>Focal</b>	14/61	23	0/14	0	2/14	21	0/14	0
<b>Multifocal</b>	21/61	34	2/21	14	5/21	24	1/21	5
<b>Severe diffuse</b>	26/61	42	25/26	96	26/26	100	13/26	5
<b>Total</b>	61/378	16	27/378	7	34/378	9	14/378	4

From these 61 animals, 14 (23%) had focal lesions consisted of well demarcated small granuloma observed in the gut associated lymphoid tissue of the ileum (Peyer's patches), these micro granulomas are formed mainly by aggregation of epithelioid-macrophages, lightly stained by HE and sometimes with clear vacuoles (5 to 10 per granuloma) and few lymphocytes. Multinucleated giant cells were occasionally observed in the granuloma of some cases (Figure 1). No lesions were found in the associated mucosa and submucosa. Ziehl-Neelsen (ZN) examination of tissue samples was negative for all samples showing focal lesions distribution.



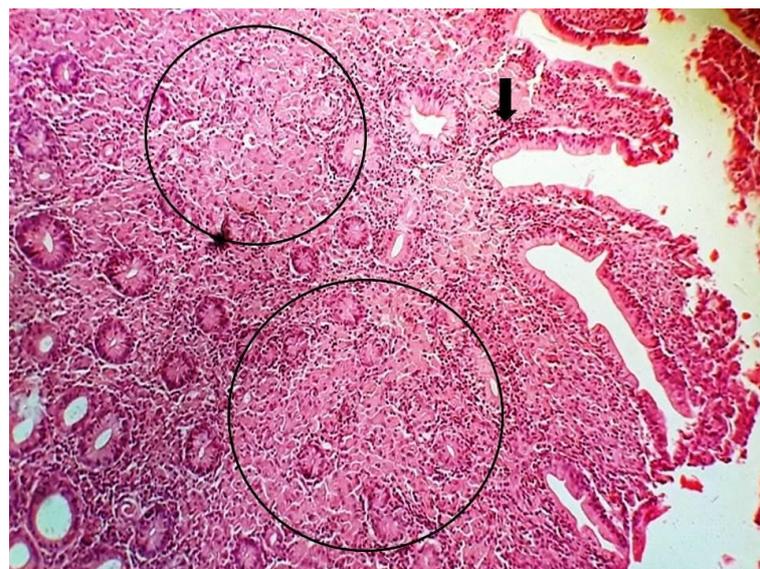
**Figure 1.** Ileocecal peyer's patches. Focal lesion composed of micro granuloma (area located between arrows). Hematoxylin and eosin (HE). (x400).

Tissues sections from 21 animals (34%) have shown another type of lesions with the presence of multifocal granulomas characterized by infiltration and aggregation of epithelioid cells, few multinucleated giant cells and surrounded by few lymphocytes. These lesions were seen either in the lamina propria and Peyer's patches (Figure 2). In some cases, granulomas were also seen in the apex of the villi causing thickening of the villi, as well as in the MLNs. Few AFB were demonstrated by ZN in three cases from this group of positive animals.



**Figure 2.** Ileum. Multifocal lesions. Granulomas invading the interfollicular zone of peyer's patches (blue arrow) and the lamina propria related to lymphoid tissue (black arrows). HE. (x 100).

26 animals (43%) recorded severe microscopic lesions characterized by moderate to severe granulomatous enteritis with thickening of the mucosa and variable infiltration of the intestinal wall by epithelioid macrophages, giant cells and lymphocytes (Figure 3). In addition to villous shortening and sloughing of the epithelial lining, intestinal villi and crypts were dilated due to a massive infiltration of mucosa and submucosa by numerous of epithelioid macrophages, lymphocytes, plasma cells and eosinophils (Figure 4). Congestion and edema were also seen in the mucosa in some cases. Lymph nodes from 6 cases showed focal zone of caseous necrosis, central calcification and a few giant cells surrounding the necrotic area. 25 samples from this group were positive for AFB staining by Ziehl-Neelsen. The cytoplasm of many epithelioid cells, especially in the mucosa and the upper zone of the submucosa, was packed with red to pink microorganisms (Figure 5).



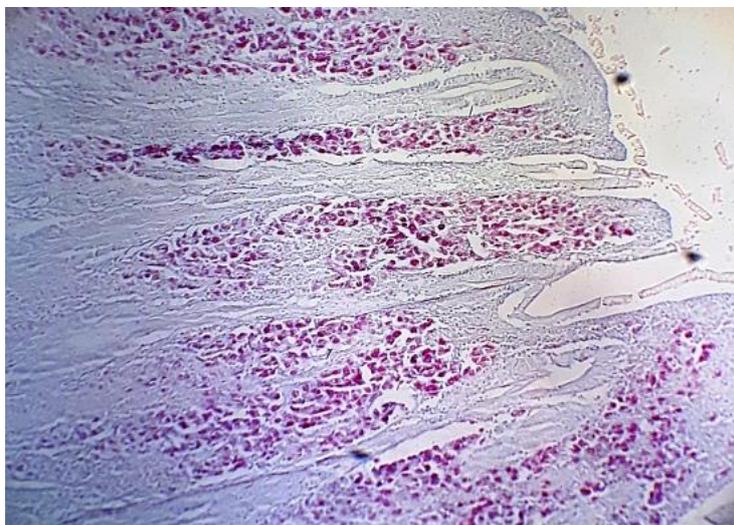
**Figure 3.** Ileum. Severe granulomatous enteritis. Diffuse infiltration of the mucosa and submucosa with epithelioid macrophages, few giant cells (circles) and lymphocytes (arrow). HE. (x 100)



**Figure 4.** Ileum. Atrophy and clubbing of the villi (arrows) due to heavy infiltration of the mucosa by epithelioid cells and lymphocytes (circles). HE. (x100).

All extracted DNA samples were tested by using PCR reaction that target the *IS900*, Analysis of the results reveals that 34/378 (9%) sheep samples yielded positive results in the *IS900* PCR assay with a band of approximately 413bp. The PCR results shows that MAP DNA was detected in 2/34 samples (6%) from the group with focal lesions, in 5/34 (15%) samples from the group with multifocal lesions, and in all samples 26/33 (79%) with severe diffuse lesions (Table 2).

Out of 378 sheep sera, 14 (4%) samples tested positive using ELISA technique (Table 2). These samples were taken from clinical and aged cases.



**Figure 5.** Ileum. Ziehl-Neelsen staining (ZN). Aggregates of intra and extra-cellular red to pink acid-fast bacilli in the mucosa and submucosa. (x40)

## DISCUSSION

Despite the non-practice of diagnosis and surveillance, Johne's disease is one of the disregarded diseases of ovine in Algeria. Paratuberculosis in Africa has been reported in many countries that have adequate laboratories to diagnose the disease as early as possible, in order to reduce economic losses and propagation of the risk to susceptible species including humans (Garcia and Shalloo, 2015; Okuni et al., 2020). Most of the researches focused on cattle while, the occurrence of paratuberculosis in sheep was not well investigated in diverse areas of the world (Khamassi et al., 2020). Reports of the disease in Algeria have been limited to the occurrence of bovine paratuberculosis in Western Algeria (Hemida and Kihal, 2015). This study was conducted to confirm the hypothesis that the disease will be found in Algerian sheep population. Indeed, out of 378 samples tested, 61 (16%) were positive for paratuberculosis by histopathology, 34 (10%) by PCR from fecal samples and 14 (4%) from 340 tested sera by ELISA.

Macroscopic lesions observed in clinical cases of the current study are in agreement with earlier works, where positive cases of JD were characterized by intestinal thickening associated with longitudinal and transverse mucosal corrugations (Alharbi et al., 2012)

Previously, examination of tissues samples by histopathology has been reported as the most important tool for the diagnosis of paratuberculosis in ruminants (Coelho et al., 2017). In our investigation, typical pathological lesions of the disease were observed among tissues samples from either apparently healthy or symptomatic animals. Three microscopic features compatible with paratuberculosis were revealed by histopathological examination, as recorded by several researchers (Hemalatha et al., 2013)

In our study, the focal lesions observed correspond to type 1 lesions in sheep and goats (Pérez et al., 1996; Thakur et al., 2017). These criteria have already been described in experimental infections of small ruminants, in which focal granulomas appeared more frequently in the intestinal lymphoid tissue (Peyer's patches) which seem to represent an important site of paratuberculosis lesions in sheep and goats (Delgado et al., 2013). In addition, this form of lesions has also been reported in subclinical and early stages of natural paratuberculosis in sheep and goats and could not be found in others intestinal locations until the infection had progressed to more advanced stages (Valheim et al., 2002).

Since all animals used in the present study were adult, it was suggested that in goats and sheep these lesions developed early in life and detected firstly in the intestinal lymphoid (Peyer's patches), represent latent and persistent forms that are limited by a strong cell-mediated immunity (CMI) reaction (González et al., 2005). The confinement of these lesions exclusively to the ileal and ileocaecal Peyer's patches; supports the suggestion that MAP were transported through M cells in the dome of gut-associated lymphoid tissue (García et al., 1992).

Multifocal lesions appeared in 21 animals have already been reported as characteristic for paratuberculosis lesions in sheep and cattle (Delgado et al., 2013), this type of lesions was classified as intermediate form in goats (Copra et al., 2000) and as type 2 and 3a in sheep infection (Pérez et al., 1996). Following the development of focal granulomas in the intestinal lymphoid tissue, these lesions identified mainly in subclinical disease, are considered as the following stages of lesion progression in the intestinal mucosa (Balseiro et al., 2008; Windsor et al., 2014). Siguardardottiret al. (1999) reported that due to other possible routes of infection, this type of lesions could also be considered to be primary lesions occurring outside the gut associated lymphoid tissue. In the present study multifocal lesions were observed in animals without any clinical signs indicating the infection and also, with no evident thickening of the intestinal wall or hypertrophy of the mesenteric lymph nodes, similar observations were reported by earlier works (Balseiro et al., 2008; Pérez et al., 1999) but contrasted with the findings of others (Jatav et al., 2018; Reddy et al., 2012)

Diffuse lesions described in the current study were consistent with Map infection and are in accordance with earlier works. (González et al., 2005; Sikandar et al., 2013). In current study this type of lesions was associated with mild to moderate thickening of the intestinal mucosa from only animals showing clinical signs consistent with paratuberculosis. Similar findings have also been postulated by earlier workers (Jatav et al., 2018). Contrary, it has been stated that unlike in cattle, where thickened intestinal mucosa, particularly the terminal ileum, is the main gross lesion during clinical paratuberculosis, these lesions are less conspicuous in MAP-infected sheep and goats. Even clinically affected sheep can also have macroscopically normal intestines but hypertrophied mesenteric lymph nodes (Robbe-Austerman, 2011).

Acid-fast staining technique of suspected tissues is rapid and requires little optimization and can be used as a screening method to detect bacilli either in feces or in tissues samples (Huntly et al., 2005; Coelho et al., 2008). In present study, ZN staining identified more positive samples among tissues samples from animals showing clinical signs and diffuse histopathologic lesions, similar findings were also observed by others (Hemalatha et al., 2013). However, failure to detect acid-fast bacteria in tissues samples with focal and multifocal lesions stained with ZN was observed in our study, this does not indicate that the animal is negative for paratuberculosis infection, since it was reported earlier that many factors may influence microscopic detection of the bacilli through tissues sections from subclinically infected animals, as the strong cellular immune response that limited multiplication of the bacilli, the rarity of organisms in cytoplasm, modification its morphology due to disruption of the cell wall; or the poor sensitivity of staining method (Pérez et al., 1999; Reddy et al., 2012). Delgado et al. (2013) reported that acid fast staining method detect only bacilli with an intact cell wall and therefore is unable to detect the bacterium with deficient cell wall or as spheroplasts form. Michel and Bastianello (2000) suggested that it is usually difficult to find acid fast organism through tissue samples with type 1 lesions and with low magnification 100 x for tissues samples showing type 2 lesions from subclinical infection.

Examination of fecal samples by using PCR was shown to be an accurate and reliable indicator for determining diseased animals, and can be performed faster (Kawaji et al., 2011). Recently this technique has also been suggested as an alternative method for the diagnosis of paratuberculosis (Stevenson, 2010), and thus it has been proposed for herd screening in many countries (Collins et al., 2006; Prendergast et al., 2018). Due to its multiplicity within the MAP genome, the IS900 element (present in the MAP genome in 14 to 18 copies), is an insertion sequence considered to be a MAP specific gene and is commonly targeted for rapid detection of MAP by PCR (Prendergast et al., 2018).

In our study, PCR method detected MAP DNA from fecal samples of animals with focal, multifocal and diffuse lesions; higher positive results were observed among fecal samples from animals with clinical signs and severe histopathological lesions suggesting presence of paratuberculosis. This finding is in accordance with a previous report that suggested a good sensitivity of PCR for the detection of symptomatic shedders of MAP in feces (Correa et al., 2017). Low shedder animals have been classified as the most challenging to any test sensitivity (Munjal et al., 2005).

Due to the presence of PCR inhibitors, irrelevant DNA and the thick waxy cell wall of MAP difficulty in detection of MAP DNA in fecal samples from apparently healthy animals by performing PCR was reported (Ozpinar et al., 2015). However, because of its high specificity and sensitivity, PCR has been reported to be best able for the diagnosis of subclinical MAP infection (Gümüşsoy et al., 2015; Kojima et al., 2002).

Although, sensitivity of PCR on fecal samples during early stages of the disease was shown to be low, PCR technique has been widely improved in recent years, leading to an increased sensitivity for the detection of low animal shedders. An interesting finding in the present study was that positive results, suggestive of MAP infection, were observed among samples from low shedder showing mild histopathological lesions.

This finding can be explained by the use of a commercial DNA extraction kit using bead-beating for physical lysis that has been improved a good sensitivity of PCR when used on fecal samples from either moderate and low animal's shedder (Leite and Stabel, 2012; Prendergast et al., 2018).

Detection of serum antibodies using ELISA test is the most frequently used technique for serological diagnosis, estimation of prevalence and screening of domestic animal's populations for paratuberculosis control programs (Coelho et al., 2017). Previously, it has been reported that the sensitivity and effectiveness of ELISA was low due to its inability to detect early positivity (Nielsen and Toft, 2008). In addition, it was stated that increase in humoral response against the disease occurs mainly in the late stage of the infection and were not related with early histopathological changes, thus ELISA detects immune response in advanced stage of the disease (Chen et al., 2020; Hemida and Kihal 2015). From 378 examined sera, serological investigation showed that only 14 (4%) animals were positive, these results were consistent with the low sensitivity of the technique reported by previous researchers (Stevenson, 2010).

## CONCLUSIONS

The present study is the first record of sheep paratuberculosis in Algeria. In view of the absence of known national strategy to deal with the disease in the country, the true prevalence among Algerian sheep population may be very high and the disease more widespread to other regions. Thus, it is for big concern to begin further investigations to provide best information on current herd and sheep level prevalence. The combined use of Histopathology and PCR tests was shown to give better results for the diagnosis of suspected clinical and subclinical animals, and thus can be useful for an effective disease management and surveillance.

The information in the current study can be regarded as a starting point for further epidemiological studies and implementation of successful control program to reduce economic losses as well as disease transmission to humans in Algeria.

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## Conflicts of Interest

The authors declare that they do not have any conflict of interest.

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