Identification of *Mycobacterium avium* subspecies paratuberculosis in sheep farms in Bayannaoer, Inner Mongolia, China

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Research Article

Keywords: sheep, Paratuberculosis, ELISA, PCR, Histopathological

Posted Date: November 15th, 2021

DOI: <https://doi.org/10.21203/rs.3.rs-944333/v1>

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Abstract

Background

Paratuberculosis, is a widespread chronic infection of *Mycobacterium avium* subsp. *paratuberculosis* (Map), causes significant economic losses to the sheep industry. The current study investigated this disease, which causes diarrhea in sheep, particularly, in Bayannaoer, Inner Mongolia. Diagnosis was based on clinical symptoms, pathological autopsy, histopathological inspection, and serological and molecular methods.

Results

Paratuberculosis was confirmed via a polymerase chain reaction using DNA extracted from tissue and fecal samples. Serum samples from 472 individual sheep were obtained to detect antibodies against MAP by ELISA test and MAP antibodies were separately detected in 17.86% (35/196) and 18.48% (51/276) of sheep herds at 6 months and 1 years of age respectively. The results of tissue lesion and pathological section were consistent with *paratuberculosis* infection.

Conclusions

This is the first report of *Mycobacterium avium* subspecies *paratuberculosis* seroprevalence in Bayannaoer sheep in Inner Mongolia. Our findings show that MAP was prevalent and potentially threaten in this region and more further investigations on long-term epidemiological

Background

Johne's disease (JD) or paratuberculosis (Ptb) is a chronic, progressive granulomatous enteritis disease that caused by *Mycobacterium avium* subspecies *paratuberculosis* (MAP)(1). The disease primarily affects ruminants, including cattle, sheep, goats, causes lower productivity and incurs significant economic losses of ruminant industries in world-wide(2).In addition, most evidences concluded that MAP is a zoonotic bacterium causing Johne's Disease and public health threats(3). The common clinical manifestations were chronic diarrhea or scours, decreased productivity, weight loss and eventually results in death of the animal(4). For sheep and goats, many countries have reported infection with the disease as well as the infection is more discrete and insidious. In Europe, approximately 4% infectious rate in sheep and goat herds has been reported(5, 6); In some Australian flocks, mortality approached 20% per annum(7).The economic study showed that MAP infection reduced the profit efficiency from 84 to 64% in Italian dairy sheep and goat farms(8). Other countries and regions also have varying degrees of economic losses because of the infection of *paratuberculosis*(9).

The infection of *paratuberculosis* of ruminants in China happen timely in recent years. Some studies indicated that Map has become a common pathogen in dairy farms, the herd level prevalence was 57.9 % in 19 dairy herds in Shandong province of China(10). The MAP infection in dairy cattle differs with farming modes at the animal level and herd level, and farming density could be an important risk factor associated with the presence of MAP infected cattle(11). The study has shown that the overall MAP seroprevalence in the tested Tibetan sheep was 11.29%(12). Recent studies showed that *paratuberculosis* was widely prevalent in dairy farms in Tai'an, Shandong Province(13). Therefore, more attention should be paid to the epidemic prevention and control of *paratuberculosis*. Mutton sheep industry plays an important role in Bayannaoer, which is located in the west of Inner Mongolia in China. About 10% of sheep have intermittent diarrhea every year, the pharmaceuticals treatment was ineffective, and the sick sheep eventually die of exhaustion in this area. This disease causes serious negative effect to economic development. Hence, we diagnosed the herds of infection with *paratuberculosis* through clinical inspection, molecular biological method, histopathological examination and serological detection, to understand the epidemic situation of MAP in Bayannaoer of Inner Mongolia in China.

Results

Clinical symptoms and necropsy findings

the sick sheep showed clinical signs of John's disease, including severe weight losses, chronic diarrhea, and runny noses (Fig.1 A, B). Necropsy findings in representative dead sheep showed enlarged and edematous abomasum (Fig.1C), besides mesenteric lymph node edema and liquefaction (Fig.1D). Mesenteric congestion and intestinal mucosa exfoliation had also found for intestinal lesions (Fig.1 E, F).

MAP detection via polymerase chain reaction (PCR)

The DNA of faeces and ileum samples of diarrhea sheep were used as a template. Finally, the results showed that the fragment size amplified by nested PCR (L/AV) for target IS900 were consistent with the expected size (298 bp) (Fig. 2).

Incidence of MAP in sheep

A total of 472 serum samples collected from different sheep farms were tested by ELISA. The sheep were either 6 months or 1 year old, and the detection rates of sheep in these two stages were similar. A total of 17.86% (35/196) and 18.48% (51/276) were separately tested positive for MAP in the two age groups.

Histopathological assessment

The heart, spleen, lung, kidney, intestinal, and mesenteric lymph nodes of the sheep were stained with hematoxylin and eosin (H & E) to detect MAP infection. The myocardial fibers were swollen, thickened, and partially broken, and the transverse striations were not obvious. Moreover, there were scattered calcifications of different sizes (Fig. 3A). The endothelial cells of glomerular capillaries were partially necrotic, with red stained silk reticular fibrin and a small amount of red blood cells distributed between

them; a small amount of red blood cells was also seen in the renal capsule. In addition, the epithelial cells of renal tubules were swollen and partially necrotic, and protein tubules appeared in most of the lumens (Fig. 3B). Many spleen lymphocytes were necrotic and bleeding seriously, especially around the splenic nodules, forming a red halo (Fig. 3C). The intestinal mucosa was thickened; many inflammatory cells were distributed in the lamina propria (Fig. 3D), and some lymphocytes in mesenteric lymph nodes were necrotic and scattered in granulomas of different sizes. Moreover, epithelioid cells and lymphocytes can be seen in granulomas (Fig. 3E). The pulmonary interstitium widened, and many inflammatory cells were distributed. Most of the alveoli were dilated, and the alveolar wall was ruptured (Fig. 3F).

Discussion

JD is one of the most economically important diseases affecting ruminants worldwide, and it is a growing concern in the sheep and goat industries (14). Interestingly, evidence suggests that MAP is a zoonotic pathogen that poses a challenge to public health (3). Sheep farming and industrial wool spinning have been developed in Bayannaoer. Therefore, MAP could act as a threat to workers in these industries and public health security. In 2011, the flocks in this region suffered from intermittent diarrhea and eventually died of exhaustion. Most of them were adult sheep, and the main symptoms were similar to those reported in previous studies—elevated body temperature, dark green and thick feces (15, 16). The course of the disease was 10–30 days; the sick sheep gradually started losing weight and the eyelids became white after the development of the disease. This brought huge economic losses to local herdsman; therefore, it is essential to control the spread of this disease. In this study, we provided a detailed description of the clinical inspection and pathological changes, as well as used molecular biology technique and ELISA to diagnosis of the naturally occurring *paratuberculosis* cases.

We found that approximately 10% of sheep were infected in February and March; notably, most infections occurred in August and fewer infections were observed in winter. This cycle is almost the same every year. The main symptoms were emaciation and general weakness with intermittent diarrhea in infected sheep, which was consistent with the infection of *paratuberculosis*(17). *Ptb* was confirmed via PCR using DNA extracted from mesenteric lymph node tissues and feces. Serum samples of different sheep flocks were subjected to a MAP ELISA, and the seroprevalence was found to be 17.86% (35/196) and 18.48% (51/276) of sheep of different ages. This prevalence was lower than 19.5% in sheep in the Eastern Province of Saudi Arabia(18), however, the prevalence was higher than the seroprevalence of 11.9% in Tibetan sheep in China(12), and 3.25% in female sheep in Tunisia(19). These data show that *Ptb* may be a risk factor in Inner Mongolia; however, detailed statistical analysis is needed.

MAP causes chronic diarrheic intestinal infections, which are difficult to treat, in domestic animals, including ruminants (20). Several aminoglycosides are active against several species of mycobacteria, including MAP (21). In this study, gentamicin was used in combination with lincomycin to treat diseases associated with *Ptb*. We found that the symptoms in sheep were relieved after treatment for the first time; however, diarrhea occurred approximately 30 days after the first treatment. At this time, the use of therapeutic drugs was ineffective, and the sick sheep continued to suffer from diarrhea and then died of

exhaustion. The results demonstrated that some products effectively inhibited bacterial growth, which findings applicable to the veterinary field may become available (21–23). Further research may substantiate the efficacy of pharmaceuticals for the treatment or control of Ptb.

Conclusion

In conclusion, this study is the first to estimate the infection of Ptb in sheep in Bayannaoer, Inner Mongolia, China. Our results showed that MAP was prevalent in this region. This information will provide comprehensive view for the prevention and control of MAP infection in Inner Mongolia sheep.

Methods

Sampled animals and Sample collection

This study was carried out during October 2020, a total number of 472 sheep from 13 small to middle-sized sheep farms were sampled. In addition, the number of 196 sheep at 6-month-old and 276 sheep at 1 -year-old were selected respectively. Sheep owners were interviewed about all treatments, therapeutic agents, and incidence of sheep their sheep flocks.

The three sheep suspected to have JD were euthanized. Serum and tissues samples, including ileum and mesenteric lymph nodes samples, were collected. Three milliliters of blood were collected from the jugular vein of each animal using a vacutainer. Sera were collected in Eppendorf tubes and stored at –20 °C until use (18). Necropsy findings of the carcasses showed emaciated intestinal mucosa, enlarged lymph nodes, and occasional serous fat atrophy. The samples were separated into two parts, immediately placed in sterile plastic bags, placed in cooling boxes, and transported to the microbiology laboratory. Samples of the carcasses were subjected to tissue staining and DNA extraction.

PCR detection of MAP

DNA was extracted from the feces and mesenteric lymph node samples of sick sheep and subjected to PCR for MAP detection. The primers IS900 L1 (5'-CTTTCTTGAAGGGTGTTCGG-3') and L2 (5'-ACGTGACCTCGCCTCCAT-3'), AV1(5'-ATGTGGTTGCTGTGTTGGATGG-3') and AV2 (5'-CCGCCGCAATC-AACTCCAG-3') were used for the first round of PCR and nested PCR, respectively as previously described (24). The first-round polymerase chain reaction condition was as follows: 94°C for 2 min, followed by 30 cycles at 94°C for 30 s, 58°C for 30 s, and 72°C for 30 s, with a final elongation step at 72°C for 3 min. 10 ×The products of the first round of dilution were used as templates, PCR and other systems and reaction parameters were carried out according to the primary reaction.

ELISA assays

Serum samples were collected from sheep farms in Bayannaoer, with herd sizes ranging from 300 to 500. The samples were analyzed using a commercial indirect ELISA kit. The tests were performed according to the manufacturer's instructions (ID-VET, Montpellier, France). Sera with sample to positive (S/P) ratios $\leq 75\%$ were scored as MAP negative, while those with ratios $\geq 85\%$ were considered MAP positive. Moreover, $75\% < \text{S/P} \% < 85\%$ were scored as "suspect" and treated as negative for data analysis.

Histopathological examinations

Tissue samples from heart, kidney, spleen, lungs, ileum and mesenteric lymph nodes were fixed in 10% neutral buffered formalin, embedded in paraffin, sectioned at 5 μm in thickness and stained with hematoxylin and eosin (H & E).

Abbreviations

DNA: DeoxyriboNucleic Acid; ELISA: Enzyme linked immunosorbent, assay; JD: Johne's disease; MAP: Mycobacterium avium subspecies paratuberculosis; PCR: Polymerase Chain Reaction; Ptb: paratuberculosis

Declarations

Ethics approval and consent to participate

This study was approved by the Committee on Animal Ethics of Chongqing Academy of Animal Sciences. All protocols and procedures were carried out in accordance with relevant guidelines and regulations. The animals sampled in this study are owned by private sheep farmers. The sheep owners consent to use the animals of the study and with the supervision of qualified veterinarians.

Consent for publication

Not applicable.

Availability of data and materials

All data generated or analysed during this study are included in this published article.

Competing interests

The authors declare that they have no competing interest.

Funding

The study was supported by grants from performance incentive and guidance program of Chongqing scientific research institutions (cstc2019jxjl80016).

Authors' contributions

Yu Y D was the major contributor in writing the manuscript, participated to detection and statistical analysis. Shen K F and Fu L Z design the project. Shen K F and Zheng H carried out ELISA. Zhang S H, Xu G Y, Xu D F and Li B performed sample collection. All authors read and approved the final manuscript.

Acknowledgments

We thank Yatu Ji and Xin Li for their support during sample collection. The authors are grateful to the farmers that participated to the survey.

References

1. Stevenson K. Genetic diversity of *Mycobacterium avium* subspecies paratuberculosis and the influence of strain type on infection and pathogenesis: a review. *Vet Res.* 2015;46(1).
2. Barkema HW, Orsel K, Nielsen SS, Koets AP, Rutten V, Bannantine JP, et al. Knowledge gaps that hamper prevention and control of *Mycobacterium avium* subspecies paratuberculosis infection. *Transbound Emerg Dis.* 2018;65:125–48.
3. Kuenstner L, Kuenstner JT. *Mycobacterium avium* ssp. paratuberculosis in the Food Supply: A Public Health Issue. *Front Public Health.* 2021;9:647448.
4. Johansen MD, de Silva K, Plain KM, Whittington RJ, Purdie AC. *Mycobacterium avium* subspecies paratuberculosis is able to manipulate host lipid metabolism and accumulate cholesterol within macrophages. *Microb Pathog.* 2019;130:44–53.
5. Nielsen SS, Toft N. A review of prevalences of paratuberculosis in farmed animals in Europe. *Prev Vet Med.* 2009;88(1):1–14.
6. Whittington R, Donat K, Weber MF, Kelton D, Nielsen SS, Eisenberg S, et al. Control of paratuberculosis: who, why and how. A review of 48 countries. *BMC Vet Res.* 2019;15(1).
7. Windsor P. Managing control programs for ovine caseous lymphadenitis and paratuberculosis in Australia, and the need for persistent vaccination. *Vet Med,* 2014, 5: 11–22.
8. Sardaro R, Pieragostini E, Rubino G, Petazzi F. Impact of *Mycobacterium avium* subspecies paratuberculosis on profit efficiency in semi-extensive dairy sheep and goat farms of Apulia, southern Italy. *Prev Vet Med.* 2017;136:56–64.
9. Fiorentina P, Martino C, Mancini Y, De Iorio MG, Williams JL, Minozzi G. Using Omics Approaches in the Discovery of Biomarkers for Early Diagnosis of Johne's Disease in Sheep and Goats. *Animals.* 2021;11(7):1912.
10. Yue R, Liu C, Barrow P, Liu F, Cui Y, Yang L, et al. The isolation and molecular characterization of *Mycobacterium avium* subsp. paratuberculosis in Shandong province, China. *Gut Pathog.* 2016;8(1).
11. Liu X, Li J, Yang X, Wang D, Wang J, Wu J. The seroprevalence of *Mycobacterium avium* subspecies paratuberculosis in dairy cattle in Xinjiang, Northwest China. *Irish Vet J.* 2017;70(1).

12. Ma J, Tian A, Zheng W, Zou Y, Zhang Y, Yang Z. First report of bovine viral diarrhea virus and *Mycobacterium avium* subspecies paratuberculosis infection in Tibetan sheep (*Ovis aries*) in Tibetan Plateau, China. *Trop Anim Health Pro.* 2019;51(3):719–22.
13. Cheng Z, Liu M, Wang P, Liu P, Chen M, Zhang J, et al. Characteristics and Epidemiological Investigation of Paratuberculosis in Dairy Cattle in Tai'an, China. *Biomed Res Int.* 2020;2020:1–7.
14. Windsor PA. Paratuberculosis in sheep and goats. *Vet Microbiol.* 2015;181(1-2):161–9.
15. Eslami M, Shafiei M, Ghasemian A, Valizadeh S, Al Marzoqi AH, Shokouhi Mostafavi SK, et al. *Mycobacterium avium* paratuberculosis and *Mycobacterium avium* complex and related subspecies as causative agents of zoonotic and occupational diseases. *J Cell Physiol.* 2019;234(8):12415–21.
16. Aitken JM, Phan K, Bodman SE, Sharma S, Watt A, George PM, et al. A *Mycobacterium* species for Crohn's disease? *Pathology.* 2021.
17. Carrigan MJ, Seaman JT. The pathology of Johne's disease in sheep. *Aust Vet J.* 1990;67(2):47–50.
18. Elsohaby I, Fayez M, Alkafafy M, Refaat M, Al-Marri T, Alaql FA, et al. Serological and Molecular Characterization of *Mycobacterium avium* Subsp. paratuberculosis (MAP) from Sheep, Goats, Cattle and Camels in the Eastern Province, Saudi Arabia. *Animals.* 2021;11(2):323.
19. Khamassi Khbou M, Romdhane R, Sassi L, Amami A, Rekik M, Benzarti MH. Seroprevalence of anti-*Mycobacterium avium* subsp. paratuberculosis antibodies in female sheep in Tunisia. *Vet Med Sci.* 2020;6(3):393–8.
20. Cirone KM, Lahiri P, Holani R, Tan YL, Arrazuria R, De Buck J, et al. Synthetic cathelicidin LL-37 reduces *Mycobacterium avium* subsp. paratuberculosis internalization and pro-inflammatory cytokines in macrophages. *Cell Tissue Res.* 2020;379(1):207–17.
21. Fecteau M, Whitlock RH. Treatment and Chemoprophylaxis for Paratuberculosis. *Vet Clin North Am Food Anim Pract.* 2011;27(3):547–57.
22. Wong SYY, Grant IR, Friedman M, Elliott CT, Situ C. Antibacterial Activities of Naturally Occurring Compounds against *Mycobacterium avium* subsp. paratuberculosis. *Appl Environ Microb.* 2008;74(19):5986–90.
23. Ali ZI, Saudi AM, Albrecht R, Talaat AM. The inhibitory effect of nisin on *Mycobacterium avium* ssp. paratuberculosis and its effect on mycobacterial cell wall. *J Dairy Sci.* 2019;102(6):4935–44.
24. Bull TJ, McMinn EJ, Sidi-Boumedine K, Skull A, Durkin D, Neild P, et al. Detection and verification of *Mycobacterium avium* subsp. paratuberculosis in fresh ileocolonic mucosal biopsy specimens from individuals with and without Crohn's disease. *J Clin Microbiol.* 2003;41(7):2915–23.

Figures

Figure 1

Symptoms and Necropsy of *Mycobacterium avium* subsp. *paratuberculosis* infected Sheep. (A) Sheep with signs of diarrhea and emaciation. (B) Sheep with runny noses. (C) Enlarged and edematous abomasum. (D) Mesenteric lymph node edema and liquefaction. (E) Intestinal mucosa exfoliation. (F) Mesenteric congestion.

Figure 2

PCR products from the IS900[L/AV] nested PCR. M: DNA marker 2000 bp. Lane 1-Lane3: Positive sample of feces; Lane 4-Lane3: Positive sample of mesenteric lymph node samples Lane 7: negative sample.

Figure 3

Pathological changes of the diseased sheep. (A) Myocardial fiber swelling, thickening and calcification. (B) Renal tubulointerstitial injuries. (C) Massive necrosis of spleen lymphocytes. (D) Thickening of the intestinal mucosa. (E) Necrosis of mesenteric lymph nodes. (F) Pathological changes of lung tissues.