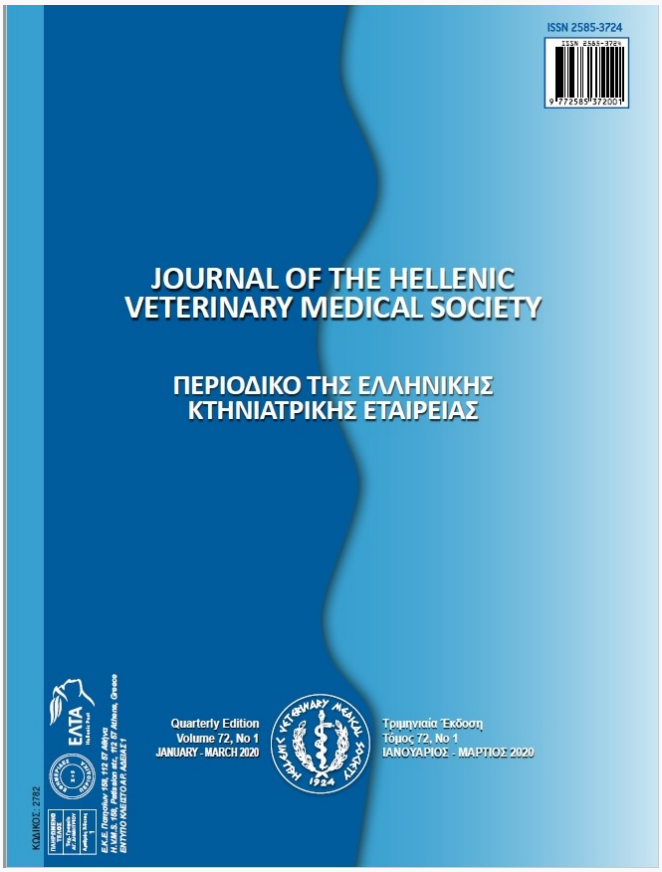
ePublishing

ΕΘΝΙΚΟ ΚΕΝΤΡΟ ΤΕΚΜΗΡΙΩΣΗΣ
EKT
NATIONAL DOCUMENTATION CENTRE

Journal of the Hellenic Veterinary Medical Society

Vol 72, No 1 (2021)



Detection of Mycobacterium avium subsp. paratuberculosis in liver of slaughtered cattle, sheep and goats by PCR and culture

M ZAREI, S TAJBAKHSH, M GHORBANPOUR, A YOUSEFVAND, S RAHIMI, M SHAHPOURI, A AMERI, N MOSAVARI

doi: 10.12681/jhvms.26751

Copyright © 2021, M ZAREI, S TAJBAKHSH, M GHORBANPOUR, A YOUSEFVAND, S RAHIMI, M SHAHPOURI, A AMERI, N MOSAVARI

This work is licensed under a Creative Commons Attribution-NonCommercial 4.0.

To cite this article:

ZAREI, M., TAJBAKHSH, S., GHORBANPOUR, M., YOUSEFVAND, A., RAHIMI, S., SHAHPOURI, M., AMERI, A., & MOSAVARI, N. (2021). Detection of Mycobacterium avium subsp. paratuberculosis in liver of slaughtered cattle, sheep and goats by PCR and culture. *Journal of the Hellenic Veterinary Medical Society*, *72*(1), 2683–2688. https://doi.org/10.12681/jhvms.26751

https://epublishing.ekt.gr | **e-Publisher:** EKT | **Downloaded at:** 27/08/2025 22:02:24

J HELLENIC VET MED SOC 2021, 72(1): 2683-2688
ΠΕΚΕ 2021, 72(1): 2683-2688

Research article
Ερευνητικό άρθρο

Detection of *Mycobacterium avium* subsp. *paratuberculosis* in liver of slaughtered cattle, sheep and goats by PCR and culture

M. Zarei[1,*], S. Tajbakhsh[1], M. Ghorbanpour[2], A. Yousefvand[1], S. Rahimi[1], M. Shahpouri[1], A. Ameri[1], N. Mosavari[3]

[1] *Department of Food Hygiene, Faculty of Veterinary Medicine, Shahid Chamran University of Ahvaz, Ahvaz, Iran*

[2] *Department of Pathobiology, Faculty of Veterinary Medicine, Shahid Chamran University of Ahvaz, Ahvaz, Iran*

[3] *Department of Bacteriology, Razi Vaccine and Serum Research Institute, Hessarak, Karaj, Iran*

ABSTRACT: The presence of *Mycobacterium avium* subsp. *paratuberculosis* (*MAP*) in tissues other than intestines and associated lymph nodes is a potential public health concern. Therefore, the presence of *MAP* in the liver of slaughtered cattle, sheep and goats with unknown Johne's disease status was evaluated. Liver samples were collected randomly from 470 mature animals including 200 cattle, 135 sheep and 135 goats. The existence of *MAP* DNA from obtained liver samples was determined using PCR, thereafter PCR-confirmed samples were cultured. Based on liver PCR results, *IS900* genes were detected in 11/200 (5.5%), 3/135 (2.2%), 2/135 (1.48%) liver samples of cattle, sheep and goats, respectively. In cattle, sheep and goats, 6 (54.5%), 1 (33.3%) and 1 (50%) PCR-positive samples showed positive results in culture, respectively. Our results revealed that *MAP* can be detected and cultured from the liver of slaughtered cattle, sheep and goats with unknown Johne's disease status. Hence, given concerns about a potential zoonotic role for *MAP*, there is a crucial need to detect animals with *MAP* disseminated infection in the liver before slaughter.

Keywords: *Mycobacterium,* Paratuberculosis liver, Johne's disease, Public health, PCR

Corresponding Author:
M. Zarei, Department of Food Hygiene, Faculty of Veterinary Medicine, Shahid Chamran University of Ahvaz, Ahvaz, Iran
E-mail address: zarei@scu.ac.ir

Date of initial submission: 17-02-2020
Date of revised submission: 13-12-2020
Date of acceptance: 30-12-2020

INTRODUCTION

The possible role of *Mycobacterium avium* subsp. *paratuberculosis* (*MAP*) in human Crohn's disease, an inflammatory disease of the intestines, has been evaluated for many years (Behr and Kapur 2008; Bull et al. 2003; Uzoigwe et al. 2007). This organism is the causative agent of Johne's disease or paratuberculosis, which is primarily expressed in the gastrointestinal tract. Isolation of *MAP* from extra-intestinal tissues indicates that microbial dissemination via the haematogenous route may occur during the infection.

The role of *MAP* in human Crohn's disease is strongly supported by studies that demonstrate that human beings are exposed to *MAP* within the food, which thus might represent a mode of transmission. Amongst the food products, *MAP* has been already detected in milk and dairy products (Ellingson et al. 2005;Grant et al. 2001;Ikonomopoulos et al. 2005), meat and meat products (Alonso-Hearn et al. 2009; Reddacliff et al. 2010), and liver(Antognoli et al. 2008; Mutharia et al. 2010).

However, the presence of *MAP* in tissues other than intestines and associated lymph nodes; such as meat and liver; have been mostly reported in experimentally infected animals (Bower et al. 2011; Gwozdz et al. 2000) or animals that either showed overt clinical disease or were previously confirmed as fecal shedders by fecal culture (Antognoli et al. 2008; Mutharia et al. 2010).

The liver is a valuable edible organ with high nutritional value and a wide range of use in preparing different food products. Knowing the thermal resistance of *MAP* and the probability of survival of *MAP* in heated foodstuffs (Gao et al. 2002; McDonald et al. 2005; Mutharia et al. 2010), the presence of *MAP* in the liver of the slaughtered animals should be considered more.

The traditional gold standard testing method for *MAP* infection is a bacterial culture of the samples. However, this method has the drawbacks that it takes several weeks or months to yield a result with an estimated sensitivity between 30 and 50% (Whitlock et al. 2000) and unfavorable interlaboratory standardization (Juste et al. 2005). Furthermore, competing bacterial and fungal contamination often overgrow *MAP* cultures, making them unreadable (Stabel et al. 2002). Considering these hurdles, PCR has been widely used to detect *MAP* in suspected samples.

In the present study, the presence of *MAP* in the liver of slaughtered cattle, sheep and goats with unknownJohne's disease status was evaluated by PCR and culture method.

MATERIALS AND METHODS

Collection and preparation of liver samples

During period of six months, a slaughterhouse in the South-western part of Iran was visited once a week and liver samples were collected randomly from 470 mature animals including 200 cattle, 135 sheep and 135 goats. According to the results of the antemortem inspection which was performed by the slaughterhouse veterinarian, the livestock used in the present study for sampling had normal body conditions and also, they did not show any symptoms of Johne's disease. Immediately after opening the carcass, liver samples were taken with sterile single-use surgical blades and transferred to sterile plastic bags.

A pooled liver sample was prepared by cutting liver samples into small pieces and then, a two-gram piece of them was aseptically transferred to a sterile 50 ml falcon tube and gently crushed by a sterile glass rod. Then 18 ml of sterile physiological saline solution was added to the falcon and vortexed for 20 min. The homogenates were filtered through two layers of sterile gauze and centrifuged at 4000 rpm for 30 min. The supernatants were discarded, and the resulting pellets were resuspended in 1 ml of sterile physiological saline solution and divided into two microcentrifuge tubes.

Liver PCR

For DNA extraction, an aliquot of the resuspended pellet (500 µl) was transferred to a microcentrifuge tube and centrifuged at 12000 rpm for 5 min. The pellet was resuspended in 200 µl lysis buffer(100 mM Tris-HCl, pH 8.0; 200 mM NaCl; 0.1 % SDS; 1 % Triton X-100; 5 mM EDTA), stored at room temperature for 30 min and then heated at 100 °C for 10 min and centrifuged at 12000 rpm for 10 min. Subsequently, DNA was extracted with phenol:chloroform:isoamylalcohol (25:24:1), and finally precipitated with the one-tenth volume of sodium acetate (3 M, pH 5.2) and 2.5 volume of chilled absolute ethanol. The precipitated DNA was washed in 80% alcohol, dried and dissolved in 100 µl sterile distilled water. The extracted DNA was frozen until PCR analysis (Nolte et al., 1993).

PCR specific to *MAP* was performed by *IS900*

primers. The *IS900* primers (forward 5′-CCGCTA-ATTGAGAGATGCGATTGG-3′ and reverse 5′-AAT-CAACTCCAGCAGCGCGGCCTCG-3′) amplify a portion of an insertion sequence found 15 to 20 times throughout the *MAP* genome. Amplification condition was as follows: 5 min at 94 °C; 50 cycles of 1 min at 94 °C, 30 s at 65 °C, and 1 min at 72 °C; and a final 10-min extension at 72 °C. A sample was considered positive when an amplified product was observed at 229 bp (Ellingson et al. 1998; Miller et al. 1999; Ellingson et al. 2005).

Liver culture

The remaining aliquot of the resuspended pellet was decontaminated according to Giese and Ahrens (2000). Briefly, 1 ml of 4 % sodium hydroxide solution was added to the sample, stored at room temperature for 15 min and centrifuged at 12000 rpm for 5 min. The supernatant was discarded and the pellet was diluted in 1 ml of 5% oxalic acid solution containing 0.1 % malachite green. After 30 min storage at room temperature, centrifugation was performed and the supernatant discarded. The resulting pellet was diluted in 1 ml of Tryptic Soy Broth (TSB) containing amphotericin B (100 mg/l), chloramphenicol (50 mg/l), vancomycin (50 mg/l), nalidixic acid (50 mg/l). Samples were stored at 4 °C until the PCR results were revealed (Ellingson et al. 1998; Miller et al. 1999; Ellingson et al. 2005).Liver samples that were PCR positive, used formycobacterial culture.

For mycobacterial culture, 250 µl of the above-mentioned TSB was streaked onto two slants of Lowenstein-Jensen medium supplemented with mycobactin J (2 mg/l), amphotericin B (100 mg/l), chloramphenicol (50 mg/l), vancomycin (50 mg/l), nalidixic acid (50 mg/l). Tubes were incubated at 37 °C in a slanted position with loose caps to allow the surface of the mediums to dry. Once the slant surfaces were dry, the tube caps were tightened. Cultures were examined every week for the first 6 weeks for signs of contamination. Contaminated samples were recultured. All cultures without signs of colony growth were held for 18 weeks before results were determined. Positive slants were rinsed with 500 µl of physiological saline solution. DNA extraction and PCR were performed on the slant rinses to confirm the results.

RESULTS

Based on liver PCR results, *IS900*genes were detected in 11/200 (5.5%), 3/135 (2.2%), 2/135 (1.48%) liver samples of cattle, sheep and goats, respectively. In cattle, sheep and goats, 6 (54.5%), 1 (33.3%) and 1 (50%) PCR-positive samples showed positive results in culture, respectively (Table 1).

Table 1. *MAP* culture results of PCR positive livers

		Liver culture	
	No of positive PCR results (Total samples)	**No of positive culture results (%)**	**No of negative culture results (%)**
Cattle	11 (200)	6 (54.5)	5 (45.5)
Sheep	3 (135)	1 (33.3)	2 (66.7)
Goat	2 (135)	1 (50)	1 (50)

DISCUSSION

The cultivation of *MAP* is laborious and time-consuming and does not always lead to bacterial growth; with 30 to 50 % sensitivity (Whitlock et al. 2000). Therefore, in the present study, PCR was used to determine the existence of *MAP* DNA in the liver samples and thereafter liver culture was performed on the PCR-positive samples. PCR targeting the *IS900* gene was considered specific for identification of *MAP* and has frequently been applied to confirm the presence of this organism in the diagnosis of Johne's disease (Ellingson et al. 1998, 2005).

In the present study, liver cultures were positive in 6/11, 1/3 and 1/2 liver samples with a positive result in PCRof cattle, sheep and goats, respectively. Difficulties in the cultivation of *MAP* on culture media (Whittington 2009), the type of culture media (de Juan et al. 2006), the presence of viable but non-culturable cells (Pribylova et al. 2011), and the decontamination die-off are the possible reasons for interpreting these results. According to Reddacliff et al. (2003),routine decontamination protocols in the laboratory were shown to decrease the number of organisms isolated per sample by about 2.7 log and 3.1 log for faeces and tissues, respectively. Hence, samples with low numbers of *MAP* that are subjected to decontamination may show negative culture results. Of course, it should be noted that in the absence of decontamination, competing bacterial and fungal

contamination often overgrow *MAP* cultures, making them unreadable (Stabelet al. 2002).

MAP culture from blood and extra-intestinal tissues in experimentally infected sheep has been evaluated by Bower et al. (2011). They reported disseminated infection in the liver and hepatic lymph nodes of 33.96 % of the 53 sheep which were infected following oral exposure to *MAP.* However*,* the bacterium was isolated from the blood of only 4 of these animals. In addition, they reported that disseminated infection can be detected more frequently from animals with a positive fecal culture result and animals with clinical disease and isolation of *MAP* from blood was difficult in the early stages of the disease while PCR was more effective. A lower lever of *MAP* isolation from the liver of experimentally infected sheep has been reported by Dukkipati et al. (2016). They isolated *MAP* from 9.1% of the livers of experimentally infected New Zealand Merino sheep.

In Denmark, *MAP* has been isolated from the livers of 6.7% and 2.2% of the cows aged 1.6-3.0 and 3.1-8.0 years, respectively (Hasonova et al. 2009). Reddacliff et al. (2010) isolated *MAP* from 59% of the muscle and 85% of peripheral lymph nodes of clinically infected sheep. Samples of liver, kidney, lymph nodes and muscle tissues from carcasses of five cows with advanced Johne's disease have been examined for the presence of viable *MAP* by Mutharia et al. (2010). They recovered viable *MAP* from 7 of 15 liver and mesenteric lymph nodes and 5 of 15 kidney and prescapular lymph node samples. According to Antognoli et al. (2008), 21 out of 40 dairy cows from four different livestock in the United States had *MAP* disseminated infection. They isolated *MAP* from 10 liver samples, 6 kidney samples, 2 heart muscle samples and 4 lung samples. In addition, they reported that 57% of the cows with disseminated infection had average to heavy body condition and no diarrhea and only 9 cows with disseminated infection showed evidence of diarrhea at the time of euthanasia.

In conclusion, the results of this study demonstrated that *MAP* can be detected and cultured from the liver of slaughtered cattle, sheep and goats with unknown Johne's disease status.PCR on liver tissue can be effective in preventing the entry of *MAP* infected livers into the human food supply chain. However, this method is not practical to use for all suspected livers at the slaughterhouse. Given concerns about the potential zoonotic role of *MAP*, it seems crucial to evaluate antemortem resources for the identification of animals with disseminated infection at slaughter and to determine which tissues are most frequently colonized by *MAP*. Having such information can lead to more effective ways to deal with human Crohn's disease.

ACKNOWLEDGMENTS

This study was supported by the research grant provided by Shahid Chamran University of Ahvaz, Iran.

CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

REFERENCES

Alonso-Hearn M, Molina E, Geijo M, Vazquez P, Sevilla I, Garrido JM, Juste RA (2009) Isolation of *Mycobacteriumavium* subsp *paratuberculosis* from muscle tissue of naturally infected cattle. Foodborne Pathog Dis 6:513-518.

Antognoli MC, Garry FB, Hirst HL, Lombard JE, Dennis MM, Gould DH, Salman MD (2008) Characterization of *Mycobacterium avium* subspecies *paratuberculosis* disseminated infection in dairy cattle and its association with antemortem test results. Vet. Microbiol 127:300-308.

Behr MA, Kapur V (2008) The evidence for Mycobacterium paratuberculosis in Crohn's disease. Curr Opin Gastroenterol24:17-21.

Bower KL, Begg DJ, Whittington RJ (2011) Culture of *Mycobacterium avium subspecies paratuberculosis* (*MAP*) from blood and extra-intestinal tissues in experimentally infected sheep. Vet. Microbiol 147:127-132.

Bull TJ, Mcminn EJ, Sidi-Boumedine K, Skull A, Durkin D, Neild P, Rhodes G, Pickup R, Hermon-Taylor J (2003) Detection and verification of *Mycobacterium avium* subsp. *paratuberculosis* in fresh ileocolonic mucosal biopsy specimens fromindividuals with and without Crohn's disease. J Clin Microbiol 41:2915-2923.

de Juan L, Alvarez J, Romero B, Bezos J, Castellanos E, Aranaz A, Mateos A, Dominguez L (2006) Comparison of four different culture media for isolation and growth of type II and type I/III *Mycobacterium avium* subsp. *paratuberculosis* strains isolated from cattle and goats. Appl Environ Microbiol 72(9):5927-5932.

Dukkipati VSR, Ridler AL, Thompson KG, Buddle BM, Hedgespeth BA, Price-Carterb M, Begg DJ, Whittington RJ, Gicquel B et al (2016) Experimental infection of New Zealand Merino sheep with a suspension of *Mycobacterium avium subspecies paratuberculosis* (*Map*) strain Telford: Kinetics of the immune response, histopathology and Map culture. Vet. Microbio 195:136-143.

Ellingson JL, Anderson JL, Koziczkowski JJ, Radcliff RP, Sloan SJ, Allen SE, Sullivan NM (2005) Detection of viable *Mycobacterium avium* subsp. *paratuberculosis* in retail pasteurized whole milk by two culture methods and PCR. J Food Prot 68(5):966-972.

Ellingson JL, Bolin CA, Stabel JR (1998) Identification of a gene unique to *Mycobacterium avium* subspecies *paratuberculosis* and application to diagnosis of paratuberculosis. Mol Cell Probes 12:133-142.

Gao A, Mutharia L, Chen S, Rahn K, Odumeru J (2002) Effect of pasteurization on survival of *Mycobacterium paratuberculosis* in milk. J Dairy Sci 85:3198-3205.

Giese SB, Ahrens P (2000) Detection of *Mycobacterium avium* subsp. *paratuberculosis* in milk from clinically affected cows by PCR and culture. Vet. Microbiol **77**:291-297.

Grant IR, Rowe MT, Dundee L, Hitchings E (2001)*Mycobacterium avium* ssp. *paratuberculosis*: its incidence, heat resistance and detection in milk and dairy products. Int J Dairy Technol 54:2-13.

Gwozdz JM, Thompson KG, Murray A, West DM, Manktelow BW (2000) Use of the polymerase chain reaction assay for the detection of *Mycobacterium avium* subspecies *paratuberculosis* in blood and liver biopsies from experimentally infected sheep. Aust Vet J 78:622-624.

Hasonova L, Trcka I, Babak V, Rozsypalova Z, Pribylova R, Pavlik I (2009) Distribution of *Mycobacterium avium* subsp. *paratuberculosis* in tissues of naturally infected cattle as affected by age. Vet Med 54(6):257-269.

Ikonomopoulos J, Pavlik I, Bartos M, Svastova P, Ayele WY, Roubal, P, Lukas J, Cook N, Gazouli M (2005) Detection of *Mycobacterium avium* subsp. *Paratuberculosis* in retail cheeses from Greece and the Czech Republic.Appl Environ Microbiol 71:8934-8936.

McDonald WL, O'Riley KJ, Schroen CJ, Condrom RJ (2005) Heat inactivation of *Mycobacterium avium* subsp. *paratuberculosis* in milk. Appl Environ Microbiol 71:1785-1789.

Miller JM, Jenny AL, Ellingson JL (1999) Polymerase chain reaction identification of *Mycobacterium avium* in formalinfixed, paraffin-embedded animal tissues. J Vet Diagn Invest 11:436-440.

Mutharia LM, Klassen MD, Fairles J, Barbut S, Gill CO (2010)*Mycobacterium avium* subsp. *paratuberculosis* in muscle, lymphatic and organ tissues from cows with advanced Johne's disease. Int J Food Microbiol 136:340-344.

Nolte FS, Metchock B, McGowan Jr JE, Edwards A, Okwumabua O, Thurmond C, Mitchell PS, Plikaytis B, Shinnick T (1993). Direct Detection of *Mycobacteriumtuberculosis* in Sputum by Polymerase Chain Reaction and DNA Hybridization. J Clin Microbiol 31(7):1777-1782.

Pribylova R, Slana I, Kralik P, Kralova A, Babak V, Pavlik I (2011) Correlation of *Mycobacterium avium* subsp. *paratuberculosis* counts in gastrointestinal tract, muscles of the diaphragm and the masseter of dairy cattle and potential risk for consumers. Int J Food Microbiol 151:314-318.

Reddacliff LA, Marsh IB, Fell SA, Austin SL, Whittington RJ (2010) Isolation of *Mycobacterium avium* subspecies *paratuberculosis* from muscle and peripheral lymph nodes using acid-pepsin digest prior to BACTEC culture.Vet. Microbiol145:122-128.

Reddacliff LA, Vadali A, Whittington RJ (2003) The effect of decontamination protocols on the numbers of sheep strain *Mycobacterium avium* subsp. *paratuberculosis* isolated from tissues and faeces. Vet. Microbiol95:271-282.

Stabel JR, Wells SJ, Wagner BA (2002) Relationships between fecal culture, ELISA, and bulk tank milk test results for Johne's disease in US dairy herds. J Dairy Sci 85:525-531.

Uzoigwe JC, Khaitsa ML, Gibbs PS (2007) Epidemiological evidence for *Mycobacterium avium* subspecies *paratuberculosis* as a cause of Crohn's disease. Epidemiol Infect 135(7):1057-1068.

Whitlock RH, Wells SJ, Sweeney RW, van Tiem J (2000) ELISA and fecal culture for paratuberculosis (Johne's disease): Sensitivity and specificity of each method. Vet. Microbiol77:387-398.

Whittington RJ (2009) Factors affecting isolation and identification of *Mycobacterium avium* subsp. *paratuberculosis* from fecal and tissue samples in a liquid culture system. J. Clin. Microbiol 47:614-622.