Acta Scientiae Veterinariae, 2022. 50: 1889.

RESEARCH ARTICLE
Pub. 1889

ISSN 1679-9216

Mycobacterium avium subsp. *paratuberculosis* (MAP) in Goat Milk in the Semiarid Region of the Brazilian Northeast - Molecular Characterization

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ABSTRACT

***Background*:** Goat farming has been on the rise in Brazil in recent years. Overall, 93% of the national herd is concentrated in the Northeast, with the state of Paraíba being the largest goat milk producer in the country. Considering *Mycobacterium avium* subsp. *paratuberculosis* (MAP) as a sanitary issue for the development of animal farming with risks for human health and that is a notifiable disease, this research was structured with the objective of confirming the presence and performing a molecular characterization of MAP in goat milk destined for processing plants in the semiarid region of the Brazilian Northeast.

***Materials, Methods & Results*:** Samples from 179 production units and 5 collective bulk tanks and 4 samples of pasteurized goat milk were analyzed through Real-time polymerase chain reaction (qPCR). Genetic material (DNA) for MAP was found in the goat milk sample from 1 production unit (1/179). From this positive sample, 9 lactating goats were identified in the original property, 7 of which showed MAP DNA in milk samples (77.77%). The characterization of the nucleotide sequence detected in the positive sample has 99% identity with KJ173784.

***Discussion*:** One sample (1/179), from the production units, had MAP genetic material (DNA) detected using the molecular test. Samples from these production units represent the milk from all lactating goats from each producer. Therefore, it was possible to identify from which farm the samples originated, allowing individual animals to then be tested, with milk samples collected from 9 goats and MAP DNA detected in 7 of them (77.77%) via PCR. Control and/or prevention programs need this type of surveillance in reason that it allows the tracking of possible foci from milk samples collected from dairy products or cooling stations. The use of PCR to detect MAP foci via goat milk is thus advantageous because samples are obtained in a non-invasive manner, with faster results when compared to the culture technique. The low detection via PCR in goat milk may be related to factors such as the small amount of MAP eliminated and the intermittent excretion in asymptomatic animals, as also false-positive samples. Samples from the collective bulk tanks was negative. It is possible that the combination of milk from all the properties diluted the amount of MAP. This suggests that the sensitivity of the PCR can be improved if the samples are obtained from the pooled milk from the same property. In some regions of Brazil, for example, showed the frequency of Zona da Mata region of the state of Minas Gerais, Brazil, found 1.94% of positive samples (9/464) and 9.76% (4/41) of properties with at least 1 positive sample for MAP. Different results to what were found in the semiarid region of Paraíba, where climate and production characteristics are different. Goats are susceptible to 3 strains: type "S" (Sheep), "Bison type" and type "C" (Cattle). Previous contact with this species may explain the similarity between the strain found in goat milk and those detected from bovine samples. This must also be taken into consideration during diagnosis and upon implementation of control measures for paratuberculosis in goats. *Mycobacterium avium* subsp. *paratuberculosis* was recorded for the first time in goat milk in the semiarid region, which may reveal a potential biological risk to humans and suggests the need for active surveillance of the agent.

Keywords: paratuberculosis, Johne's disease, caprine, milk, PCR.

DOI: 10.22456/1679-9216.125071

Received: 10 June 2022 Accepted: 10 September 2022 Published: 24 October 2022

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INTRODUCTION

Goat farming has been on the rise in Brazil. The national herd has over 8.2 million heads, and the Northeast has 93% of the goats [10]. Paraíba has the fifth largest number of goats and the highest production of goat milk in the country, with 5.627.000 L/year [9,10]. However, failures in management and sanitation have been causing economic losses and providing the risk of transmission, such as paratuberculosis [5].

Also known as Johne's disease, paratuberculosis is an incurable infectious disease caused by *Mycobacterium avium* subsp. *paratuberculosis* (MAP) [17]. It has a worldwide distribution and affects various animal species, including domestic (mainly cattle, goats, and sheep) and wild ruminants [25]. *M. avium* subsp. *paratuberculosis* has a tropism for intestines, which differentiates it from other mycobacteria [12,26].

Infected goats excrete the bacteria (MAP) in milk and feces, enabling contagion through ingestion of contaminated water or food or to young animals when suckling [15]. Because the agent is eliminated via these routes, it is possible to diagnose MAP infection using milk in a non-invasive manner [18,23], through molecular and isolation techniques [20,23].

Thus, the importance of further research on MAP for the refinement of diagnostic tests and development of efficient prevention, control, and eradication strategies is highlighted to ensure the health of production animals and food safety for humans. As such, the objective of this study was to confirm the presence and perform molecular characterization of MAP in goat milk destined for processing plants in the semiarid region of Northeastern Brazil.

MATERIALS AND METHODS

Area of the study and selection of municipalities

The study was performed in the intermediate region of Campina Grande, in the municipalities of Amparo, Prata, Sumé, and Monteiro (Figure 1), located in the semiarid region of the state of Paraíba, in Northeastern Brazil.

The area where this research was conducted has a semiarid climate, with an average temperature of 22°C and an altitude of 599 meters above sea level. It is a region known for the production of goat milk both in the state of Paraíba and Brazil. Among the municipalities included in the survey, Monteiro is the one with the largest number of goats in the state, with 27,060 animals; Sumé is in third place with 20,273 heads [10], showing goat farming is an important activity for the region.

Municipalities were selected because they had collection and/or processing plants that received goat milk from 460 producers (Table 1), which corresponds to approximately 37% of goat milk producers in the state of Paraíba that are registered in the Paraíba Milk Program [8].

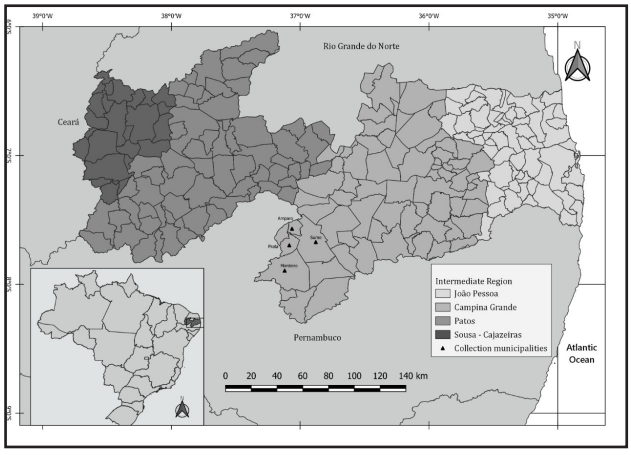

Figure 1. Geografical distribution of municipalities with goat milk processing plants where samples were collected from individual tanks from production units for molecular detection of MAP in the state of Paraíba, in Northeastern Brazil.

Table 1. Municipalities with active goat milk processing plants used by the Paraíba Milk Program, in the intermediate region of Campina Grande, Paraíba, Brazil.

Municipality	Plants/Milk receiving tanks	Producers
Amparo	1	93
Monteiro	2	207
Prata	1	46
Sumé	1	114
Total	5	460

Sample design

Samples were collected between the months of August and October 2019, considered to be a non-rainy period. This was accomplished in 3 stages: the 1st sample was obtained from each individual producer upon arrival at the processing plant, before being added to the collective bulk tank; the 2nd was obtained after milk from all producers was mixed into the collective

bulk tank; and the 3rd, after pasteurization of the milk. After analysis, samples were collected from individual lactating goats in the production unit that had a positive result. The minimum number of properties per visited municipality was calculated using a list of 460 producers from the municipalities which delivered goat milk to the processing plants, with 179 production units then randomly selected [24].

Field activities

A total of samples obtained were: 179 goat milk samples from production units, 5 from bulk tanks, 4 from pasteurized milk, and 9 from lactating goats. All producers who delivered milk to receiving tanks or processing plants on the day of sample collection had their milk samples analyzed. In all receiving units, goat milk was collected after weighing, in a single sample per individual tank. A total of 50 mL of in natura goat milk were collected in previously identified sterile, DNA-free Falcon tubes. Milk collection from individual goats was performed after cleaning and disinfection of the teats, discarding the first 3 jets and collecting 25 mL of milk from each teat directly into Falcon tubes. After collection, samples were stored under refrigeration in an isothermal box and sent to the Laboratory of Transmissible Diseases of the Federal University of Campina Grande (UFCG), Patos Campus, and kept frozen until the time of analysis.

Extraction of genetic material

Deoxyribonucleic acid (DNA) genetic material was extracted from milk samples using a Dneasy Blood and Tissue Kit[1] (Qiagen), following the manufacturer's recommendations. Real-time polymerase chain reaction (qPCR) was performed as described before [1], using specific primers from the IS900 region of MAP, DF – 5' -GACGACTCGACCGCTAATTG-3' and DR – 5' -CCGTAACCGTCATTGTCCAG-3'. As a positive control, a culture of *M. paratuberculosis* isolated from cattle and as a negative control, ultrapure water was used.

The sequencing reaction was performed with the DF and DR primers [1] and Big Dye Terminator v3.1 sequencing kit[2] (Applied Biosystems). For capillary electrophoresis, a 3130 gene analyzer and POP-7 polymer were used [16]. Sequence alignment was performed with Seaview[4] software [7]. The sequences used in the dataset were obtained from GenBank[3] (National Center for Biotechnology Information, Bethesda, MD, USA) (http.//www.ncbi.nlm.nih.gov).

Phylogenetic analysis was performed using Seaview[4] software[4], Bio Neighbor-Joining method, and Jukes and Cantor model bootstrap with 1,000 repetitions. This was visualized using FigTree v1.4.4 (http://tree.bio.ed.ac.uk/)[5], including sequences available from Genbank.

RESULTS

Indices for goat milk production/day destined for processing plants demonstrate a mean production at peak lactation of 5,327 L, with a mean of 29.9 L per producer (Table 2). Therefore, the daily goat milk production in the state is 14,000 L [8,10].

Results for the detection of MAP DNA via qPCR are shown in Table 3. A sample from a production unit tested positive via the molecular test (1/179). Samples from bulk collective tanks and pasteurized milk were negative. Using the registration data provided by the processing units, the production unit with the positive result was identified. Of the 9 lactating goats on that property, 7 had MAP DNA in their milk (77.77%).

Using the BLAST tool http://www.ncbi.nlm.nih.gov/BLAST/, there was an approximately 99% identity with KJ173784 in the BLAST Tool and with other sequences, as shown in Figure 2.

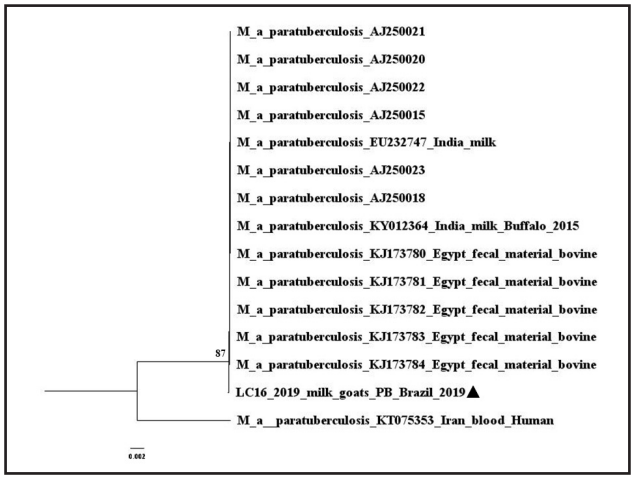

Figure 2. Phylogenetic tree for *Mycobacterium avium* subsp. *paratuberculosis* (MAP) established using the maximum likelihood method, where the sequence showed approximately 99% identity with KJ173784 in BLAST. Sample originated from a production unit in the intermediate region of Campina Grande, Paraíba, Brazil.

Table 2. Mean indices for daily goat milk production, considering peak lactation, destined for processing plants, given per receiving unit/ municipalities located in the semiarid region in the Northeast of Brazil.

Municipality	Collective bulk tank	Mean liters per producer
Monteiro	1,781.8	31.2
Amparo	1,137.6	23.7
Prata	1,497.6	31.2
Sumé	910	35
Total	5,327	29.9

Table 3. Number of samples from producers, collective samples, and pasteurized samples by municipalities and PCR results from goat milk destined for processing plants located in the semiarid region of Northeastern Brazil.

Municipality	Samples from Producers	PCR Results	Collective samples	PCR Results	Pasteurized Samples	PCR Results
Monteiro	57	1/57	2	0	1	0
Amparo	48	0	1	0	1	0
Prata	48	0	1	0	1	0
Sumé	26	0	1	0	1	0
Total	179	1/179	5	0/5	4	0

DISCUSSION

Of the samples originating from the production units, 1 sample (1/179) had MAP genetic material (DNA) detected using the molecular test. Samples from these production units represent the milk from all lactating goats from each producer. Therefore, it was possible to identify from which farm the samples originated, allowing individual animals to then be tested, with milk samples collected from 9 goats and MAP DNA detected in 7 of them (77.77%) via PCR.

This type of surveillance is important in control and/or prevention programs since it allows the tracking of possible foci from milk samples collected from dairy products or cooling stations. The use of PCR to detect MAP foci via goat milk is thus advantageous because samples are obtained in a non-invasive manner, with faster results when compared to the culture technique [2,23]

However, some studies have shown conflicting results regarding the sensitivity of PCR to detect MAP in goat milk. A research evaluated PCR using samples collected directly from bulk tanks and found a sensitivity of 0.0% in dairy goat herds in Canada, suggesting that a modified ELISA test would perform better for use in processing units and control programs [2]. Study demonstrated that PCR had a high sensitivity when detecting MAP in bulk tank samples from 3 MAP-seropositive goat herds [4].

The low detection via PCR in goat milk may be related to factors such as the small amount of MAP eliminated and the intermittent excretion in asymptomatic animals [2,6,14,20]. On the other hand, false-positive samples may also be observed, especially on farms with deficient management and low degree of technification, due to the possibility of ascending contamination via the teat canal, where milk would be contaminated by MAP originating from the environment and not from an active infection in the animal [19]. However, in this research, the collection of milk samples from individual goats in the production unit identified as positive followed careful hygiene of the udder and teats before collection, which reduces the risk of contaminating milk samples with MAP from the environment.

In the Zona da Mata region of the state of Minas Gerais, Brazil, a research using conventional PCR on individual goat milk samples from commercial herds with a minimum of 50 animals, found 1.94% of positive samples (9/464) and 9.76% (4/41) of properties with at least 1 positive sample for MAP [21]. Climate and production characteristics are different from those observed in the semiarid region of Paraíba, where this

research was performed. This may have interfered in the divergent results between these 2 studies.

In a seroepidemiological study in the state of Paraíba found 6.97% and 0.82% of properties and goats respectively seropositive, and observed a significant association between the absence of technification and seropositivity by ELISA [5]. It is therefore possible that systemic management flaws and absence of a surveillance program targeting paratuberculosis are contributing to maintaining MAP in properties in the studied region, where prior research had already identified high frequencies of positive goats and properties via serological tests using ELISA [13].

As observed in Table 3, the test performed on samples from the collective bulk tanks was negative, even after mixing milk from all production units, including the sample from the positive property. The low sensitivity of PCR testing for collective bulk tanks was reported [2], it is possible that the combination of milk from goats from all the properties diluted the amount of MAP to a level not detectable by the test. This suggests that the sensitivity of the PCR can be improved if the samples are obtained from the pooled milk from the same property instead of from the collective bulk tank.

As verified in the phylogenetic analysis (Figure 2), the characterization of the nucleotide sequence of the MAP DNA detected in the positive sample has 99% similarity with strains identified in cattle. Goats have been found to be susceptible to the three strains of MAP previously described: type "S" (Sheep), type "C" (Cattle), and "Bison type" [4,6,11,20,21].

In Brazil, a study reported the isolation of the MAP type "C" strain in dairy goats from the Zona da Mata region of Minas Gerais [21]. This strain has been isolated from a variety of domestic and wild hosts, including non-ruminants, however, it is the predominant type observed in bovine [3,22]. Mixed caprine and bovine herds are common in the studied region [5], which may favor circulation and maintenance of MAP type "C" among these 2 host species. This situation may explain the similarity between the strain found in goat milk and those detected from bovine samples (Figure 1). Therefore, this must also be taken into consideration during diagnosis and upon implementation of control measures for paratuberculosis in goats [21].

CONCLUSION

The presence of *Mycobacterium avium* subsp. *paratuberculosis* was recorded for the first time in goat milk in the semiarid region, which may reveal a potential biological risk to humans and suggests the need for active surveillance of the agent.

MANUFACTURERS

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***Funding*.** This research was financially supported by the PROAP/PPGCSA/UFCG.

***Acknowledgments*.** The authors would like to thank the Transmissible Disease Research Group, Federal University of Campina Grande (UFCG), Patos, PB, Brazil.

***Ethical approval*.** This Project was submitted to and approved by the Ethics Committee in the Use of Animals (CEUA) of the Center for Health and Rural Technology (CSTR) of the Federal University of Campina Grande (UFCG) under protocol number CEUA/CSTR n. 099/2019.

***Declaration of interest*.** The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

REFERENCES

1 **Alburquerque P.P.F., Santos A.S., Souza Neto O.L., Kim P.C.P., Cavalcanti E.F.T.S.F., Oliveira J.M.B., Mota R.A. & Pinheiro Jr. J.W. 2017.** Detection of *Mycobacterium avium* subsp. *paratuberculosis* in bovine milk from the state of Pernambuco, Brazil. *Brazilian Journal of Microbiology*. 48: 113-117. DOI: 10.1016/j.bjm.2016.10.010.

2 **Bauman C.A., Jones-Bitton A., Jansen J., Kelton D. & Menzies P. 2019.** Evaluation of bulk tank milk PCR and bulk tank milk modified ELISA tests for the detection of paratuberculosis at the herd level in goat and sheep dairies in Ontario, Canada. *Journal of Dairy Science*. 102: 511-520. DOI: 10.3168/jds.2018-15020.

3 **Dimareli-Malli Z., Mazaraki K., Stevenson K., Tsakos P., Zdragas A., Giantzi V., Petridou E., Heron I. & Vafeas G. 2013.** Culture phenotypes and molecular characterization of *Mycobacterium avium* subsp. *paratuberculosis* isolates from small ruminants. *Research in Veterinary Science*. 95: 49-53.

4 **Favila-Humara L.C., Chávez-Gris G.G., Carrillo-Casas E.M. & Hernández-Castro R. 2010.** *Mycobacterium avium* subsp. *paratuberculosis* detection in individual and bulk tank milk samples from bovine herds and caprine flocks. *Foodborne Pathogens and Disease*. 7: 351-355. DOI: 10.1089/fpd.2009.0374.

5 **Freitas T.D., Azevedo S.S., Silva M.L.C.R., Garino Jr. F., Santos C.S.A.B., Clementino I.J., Amaral F.R. & Alves C.J. 2015.** Epidemiological characterization and risk factors associated with *Mycobacterium avium* subsp. *paratuberculosis* infection in dairy goats in the Brasilian semiarid region. *Semina: Ciências Agrárias*. 36: 267-276. DOI: 10.5433/1679-0359.2015v36n1p267.

6 **Galiero A., Turchi B., Pedonese F., Nuvoloni R., Cantile C., Colombani G., Forzan M., Cerri D., Bandecchi P. & Fratini F. 2017.** Serological, culture and molecular survey of *Mycobacterium avium* subsp. *paratuberculosis* in a goat flock in Tuscany. *Folia Microbiologica*. 62: 471-477. DOI: 10.1007/s12223-017-0518-7.

7 **Gouy M., Guindon S. & Gascuel O. 2010.** SeaView version 4: a multiplatform graphical user interface for sequence alignment and phylogenetic tree building. *Molecular Biology and Evolution*. 27(2): 221-224. DOI: 10.1093/molbev/msp259.

8 **Governo do Estado da Paraíba. 2015.** Secretaria de Desenvolvimento Humano. Agricultores familiares com pertinência ao programa leite da paraíba em setembro de 2015, 25p. Disponível em: <http://https://paraiba.pb.gov.br/diretas/secretaria-de-desenvolvimento-humano/fornecedores-paa-leite/>.

9 **Haenlein G.F.W. 2004.** Goat milk in human nutrition. *Small Ruminant Research*. 51: 155-163.

10 **Instituto Brasileiro de Geografia e Estatística (IBGE). 2019.** Censo agropecuário: resultados definitivos 2017. Disponível em: <https://censoagro2017.ibge.gov.br/templates/censo_agro/resultadosagro/pecuaria.html>.

11 **Kumar S., Singh S.V., Sevilla I., Singh A.V., Whittington R.J., Juste R.A., Sharma G., Singh P.K. & Sohal J.S. 2008.** Lacto-prevalence, genotyping of *Mycobacterium avium* subspecies *paratuberculosis* and evaluation of three diagnostic tests in milk of naturally infected goat herds. *Small Ruminant Research*. 74: 37-44. DOI: 10.1016/j.smallrumres.2007.03.005.

12 **Lombard J.E. 2011.** Epidemiology and economics of paratuberculosis. *Veterinary Clinics of North America: Food Animal Practice*. 27: 525-535.

13 **Medeiros J.M.A., Garino Jr. F., Almeida A.P., Lucena E.A. & Riet-Correa F. 2012.** Paratuberculose em caprinos e ovinos no Estado da Paraíba. *Pesquisa Veterinária Brasileira*. 32: 111-115. DOI: 10.1590/S0100-736X2012000200003.

14 **Nebbia P., Robino P., Zoppi S. & Meneghi D. 2006.** Detection and excretion pattern of *Mycobacterium avium* subspecies *paratuberculosis* in milk of asymptomatic sheep and goats by Nested-PCR. *Small Ruminant Research*. 66: 116-120. DOI: 10.1016/j.smallrumres.2005.07.049.

15 **Patel A. & Shah N. 2011.** *Mycobacterium avium* subsp. *paratuberculosis* - incidences in milk and milk products, their isolation, enumeration, characterization, and role in human health. *Journal of Microbiology, Immunology and Infection*. 44: 473-479.

16 **Platt A.R., Woodhall R.W. & George Jr. A.L. 2007.** Improved DNA sequencing quality and efficiency using an optimized fast cycle sequencing protocol. *Biotechniques*. 43(1): 58-62. DOI: 10.2144/000112499.

17 **Rathnaiah G., Zinniel D.K., Bannantine J.P., Stabel J.P., Gröhn Y.T., Collins M. & Barletta R.G. 2017.** Pathogenesis, molecular genetics, and genomics of *Mycobacterium avium* subsp. *paratuberculosis*, the etiologic agent of Johne's disease. *Frontiers in Veterinary Science*. 4: 1-13. DOI: 10.3389/fvets.2017.00187.

18 **Roberto J.P.L., Limeira C.L., Barnabé N.N.C., Soares R.R., Silva M.L.C.R., Gomes A.A.B., Higino S.S.S., Azevedo S.S. & Alves C.J. 2021.** Antibody detection and molecular analysis for *Mycobacterium avium* subspecies *paratuberculosis* (MAP) in goat milk: Systematic review and meta-analysis. *Research in Veterinary Science*. 135: 72-77. DOI: 10.1016/j.rvsc.2021.01.004.

19 **Schwarz D.G.G., Lima M.C., Barros M., Valente F.L., Scatamburlo T.M., Rosado N. & Moreira M.A.S. 2017.** Short communication: Passive shedding of *Mycobacterium avium* ssp. *paratuberculosis* in commercial dairy goats in Brazil. *Journal of Dairy Science*. 100: 8426-8429. DOI: 10.3168/jds.2017-12918.

20 **Singh M., Gupta S., Chaubey K., Singh S. & Sohal J. 2019.** Profiling of *Mycobacterium avium* subspecies *paratuberculosis* in the milk of lactating goats using antigen-antibody based assays. *Comparative Immunology, Microbiology and Infectious Diseases*. 64: 53-60. DOI: 10.1016/j.cimid.2019.02.006.

21 **Souza M.C.C., Lima M.C., Braga I.F.E., Schwarz D.G.G., Rodrigues A.N.S., Sales E.B., Fonseca Jr. A.A. & Moreira M.A.S. 2016.** Molecular typing of *Mycobacterium avium* subsp. *paratuberculosis* (MAP) isolated from dairy

goats in Brazil. *Small Ruminant Research*. 140: 18-21. DOI: 10.1016/j.smallrumres.2016.05.009.

22 **Stevenson K. 2015.** Genetic diversity of *Mycobacterium avium* subspecies *paratuberculosis* and the influence of strain type on infection and pathogenesis: a review. *Veterinary Research*. 46: 1-13. Doi: 10.1186 /s13567-015-0203-2.

23 **Sukumar B., Gunaseelan L., Porteen K. & Prabu K. 2014.** Goat milk as a non-invasive sample for confirmation of *Mycobacterium avium* subspecies *paratuberculosis* by IS900 PCR. *Journal of Advanced Veterinary and Animal Research*. 1: 136-139. DOI: 10.5455/javar.2014.a25.

24 **Thrusfield M. & Brown H. 2018.** Surveys. In: Thrusfield M. (Ed). *Veterinary Epidemiology*. 4th edn. Oxford: Blackwell Science, pp.270-295.

25 **Windsor P.A. 2015.** Paratuberculosis in sheep and goats. *Veterinary Microbiology*. 181: 161-169. DOI: 10.1016/j.vetmic.2015.07.019.

26 **World Organization for Animal Health (OIE). 2018.** *Leptospirosis: Manual of diagnostic tests and vaccines for terrestrial animals*. Paris: World Organization for Animal Health, 598p.