Occurrence of Paratuberculosis in Cattle Raised Under Small-Scale Dairy Production in Egypt: A Molecular Investigation

Sarah G. Yousef*, Ahmed Shehta1, Hend M. El Damaty4 and Hussein A. Elsheikh3
1Section of Infectious Diseases, Department of Animal Medicine, Faculty of Veterinary Medicine, Zagazig University, 44511-Zagazig, Sharkia, Egypt
2 Section of Internal Medicine, Department of Animal Medicine, Faculty of Veterinary Medicine, Zagazig University, 44511-Zagazig, Sharkia, Egypt
3Veterinary Hospital, Faculty of Veterinary Medicine, Zagazig University, 44511-Zagazig, Sharkia, Egypt
44511-Zagazig, Sharkia, Egypt

ABSTRACT

Johne’s disease (JD), chronic granulomatous disease caused by Mycobacterium avium subsp. paratuberculosis (MAP), is regarded as a potential public health issue and a significant threat to the dairy and agricultural food businesses. This study was conducted for the molecular investigation of MAP in cattle reared for small-scale dairy production in Sharkia Governorate, Egypt. Seventy-five fecal samples were collected from diarrheic and healthy dairy cows that came into contact with diarrheic ones for a period of time. The PCR targeting the insertion sequence IS900 gene revealed that 22.6% of examined cattle were positive for MAP. A rate was significantly higher in diseased older cattle than in young cattle with no symptoms of the disease. Furthermore, DNA sequencing and phylogenetic analysis revealed that our strains (ON816021 and ON816022) were 100% identical showed complete identity with MAP-C (M. paratuberculosis of cattle origin) as Japanese (CP066812) and German strain (CP022105). Overall, the findings highlight the potential of the IS900 gene-based molecular technique for MAP detection in small dairy cattle farms.

INTRODUCTION

Paratuberculosis (PTB) is a globally prevalent contagious disease affecting domestic, wild ruminants (Lombard, 2011) and some monogastric animals such as pigs and donkeys (Stief et al., 2012). It is caused by intracellular Mycobacterium avium subsp. paratuberculosis (MAP). The disease was first reported in Germany by Johnne and Frothingham (1895) and has been recorded in several European and Asian countries. Nonetheless, it was not until Twort and Ingram (1912) convincingly proved Koch’s postulates by growing MAP in the laboratory and reproducing the disease in experimentally infected cattle. Severe economic losses in the cattle industry due to PTB are represented by decreased productivity, reproductive failures, premature culling, and increased replacement costs (Selim and Gaede, 2015). The disease is characterized by a long subclinical stage, and clinically diseased animals suffer from cachexia, decreased milk production, and untreated profuse watery diarrhea (Harris and Barletta, 2001). MAP is intermittently shed through feces and milk and is then transmitted to susceptible animals by the fecal-oral route. Young calves are infected in early life via ingesting contaminated colostrum and milk (Abdellrazeq et al., 2014). Because it causes human inflammatory bowel disease (Chron’s disease), MAP has been studied not only for its economic importance, but also for its zoonotic potential (Fawzy et al., 2013). Several diagnostic aids have been developed for the detection of infection. Shin et al. (2007) employ the gold standard aerobic fecal culture of MAP, which require from two to five months, posing a substantial challenge for disease diagnosis and treatment. Serological assays are not suitable for early detection because the humoral immune response appears late after the disease is already established (Gamma et al., 2015). Molecular-based assays on fecal samples are more suitable as they are faster than the culture method (Douarre et al., 2010). Multiple genetically distinct sequences for MAP,
including IS900, F57element, and hsp X gene, have been identified. The IS900 gene is unique to MAP strains and has multiple copies in the MAP genome (Naser et al., 1998), so it has been used as a molecular target in several studies. Firstly, MAP strains have been classified genotypically into ovine (MAP-S) and bovine (MAP-C) strains. Recently, another molecular typing differentiates MAP into ovine (type I), bovine (type II), and intermediate (type III) strains (Bannantine et al., 2012). However, most JD studies in Egypt were limited to cattle used in large-scale dairy production. Therefore, the current study focused on molecularly investigating MAP in cattle reared on small dairy production. The animals underwent complete clinical examination, with a concrete floor and provided with milk-cooling equipment. The animals were reared on small backyard farms in Sharkia Governorate, Egypt, using PCR-based IS900 and sequencing analysis to differentiate current infection from other infected strains.

**MATERIALS AND METHODS**

**Study population**
Seventy-five Holstein dairy cows aged over two years were recruited in this study. Animals were reared on five small-scale cow-calf operations, with complaints of decreased milk production, body weight loss, and chronic diarrhea among older cows. Sharkia governorate is one of Egypt’s largest agricultural governorates, located in the Eastern Nile Delta, with a history of animal disease outbreaks. Furthermore, it has rural areas that rely primarily on agriculture and animal breeding in a small-scale system. These cattle have been raised in an open loose housing system with an earthen floor and separated lying and feeding areas. Forage was available all the time, and concentrates were accessed twice daily at milking time. Cows have been milked in a separate milking area with a concrete floor and provided with milk-cooling equipment. The animals underwent complete clinical investigations as described by Constable et al. (2017).

**Sample collection**
Seventy-five fecal samples were collected (one per animal) from dairy cattle in small backyard farms from September 2021 to March 2022. Of the fecal samples (n=75), 32 were taken from cows with untreated profuse watery diarrhea, 43 were taken from cows with no clinical symptoms of diarrhea but having contact with clinical cases of the previously mentioned symptoms. The samples collected using disposable plastic gloves in sterile cups and were labeled with the herd and animal identification number and the collection date. They were then transferred into an ice tank to the Animal Medicine Department laboratory, Zagazig University, where they were kept at -20°C for further analysis. Recruitment of dairy cows and the sampling process were performed with the permission of the small dairy backyard farm owners. Animal samples were taken for routine disease diagnosis as part of Egypt’s regular veterinary service organizations and following local and national standards.

**DNA extraction**
The QIAamp DNA Mini Kit (QIAGEN, Hilden, Germany) was used to extract DNA from fecal samples, as directed by the manufacturer. Briefly, 1.4 mL of ASL buffer was added to the samples (220 mg), incubated at 70 °C for 5 min and then centrifuged at 14000 rpm for 1 min to pellet the feces after homogenizing for 6 min with a QIAGEN Tissue Lyser. The supernatant (1.2 mL) was then added to one InhibitEx tablet, which was vortexed and placed at room temperature for a minute. After centrifuging the samples at 14000 rpm for 3 min, 200 µL of the supernatant was added to 15 µL of proteinase K and 200 µL of lysis buffer AL, and incubated at 70 °C for 10 min. After incubation, absolute ethanol (200µL) was added to the lysate.

**PCR amplification**
A conventional PCR assay targeting the IS900 of MAP was performed in a BiometraT3 thermo cycler. Primers (Metabion, Germany) as described by Khare et al. (2004) were used. 25 µL reaction containing 12.5 µL of Emerald Amp Max PCR master mix (Takara, Japan), 1 µL of each primer (P90: 5′-GAAGGTGTTCGGGCCGTC-3′ and P91: 5′-GAGGTCGTGCCCGTGAC-3′) at a 20 pmol concentration, 5.5 µL of water, and 5 µL of DNA template were utilized. The final products of PCR were separated on agarose gel (1.5%) using 5V/cm gradients (Applichem, Germany, GmbH) in 1x TBE buffer at room temperature.

**DNA sequencing and phylogeny**
A purified PCR output of the IS900 of MAP was sequenced in both directions on an Applied Biosystems 3130 sequencer, using a ready reaction Bigdye Terminator V3.1 cycle sequencing kit (Perkin-Elmer, Foster City, CA). Basic Local Alignment Search Tool (A BLAST® analysis) available at the National Center for Biotechnology Information was initially performed to establish the sequence identity within the GenBank database (Altschul et al., 1990). The phylogenetic tree has been constructed utilizing Laser gene DNAStar’s MegAlign Pro, version 12.1 (Thompson et al., 1994). The phylogeny was worked out according to Tamura et al. (2013) using maximum likelihood, neighbour joining and maximum parsimony in MEGA6.
Statistical analysis

To test the relationship between the MAP (dependent variable) and age (the independent variable) in the studied farms, Fisher exact test was conducted using GraphPad Prism software (v.5). The results were considered significant at $P$-value $\leq 0.05$.

RESULTS

The clinical manifestation of examined dairy cows

Thirty-two of the 75 examined cows showed decreased milk production and variable degrees of body weight loss represented by visible bony structures such as ribs, backs, hips, and pins by inspection. Afebrile non-fetid diarrhea with normal appetite was also expressed. Six cows showed persistent watery diarrhea, and twenty-six suffered from intermittent diarrhea with periods of normal fecal consistency (Table I).

Table I. The clinical picture of diseased dairy cows.

| Clinical signs                                | Clinically diseased cows (n=32) |
|-----------------------------------------------|---------------------------------|
| Normal rectal temperature (38.8±0.4°C)        | 32 100                          |
| Intermittent diarrhea                         | 26 81.8                         |
| Persistent watery diarrhea                    | 6 18.75                         |
| Rough hair coat                               | 14 43.75                        |
| Decreased milk production                     | 32 100                          |
| Visible bony structures (ribs, back, hips, pins) | 28 87.5                        |

Table II. Total number of examined cattle and positive PCR results.

| Dairy farm | Total No. | No. of diseased cattle | No. of apparently healthy cattle | No. of positive samples (%) |
|------------|-----------|------------------------|---------------------------------|-----------------------------|
| 1          | 17        | 8                      | 9                               | 7 (41.2%)                   |
| 2          | 14        | 5                      | 9                               | 0 (0.00)                    |
| 3          | 16        | 3                      | 13                              | 0 (0.00)                    |
| 4          | 18        | 10                     | 8                               | 7 (38.8%)                   |
| 5          | 10        | 6                      | 4                               | 3 (30%)                     |
| Total      | 75        | 32                     | 43                              | 17 (22.6%)                  |

MAP infection rate by conventional PCR

PCR amplification using a specific primer targeting the IS900 gene with a 402 bp fragment revealed that (17/75; 22.6%) cattle tested positive for MAP infection. MAP infection was restricted to three of five examined backyard farms (Table II). All MAP positive cattle suffered from diarrhea whereas the symptomless animals were negative for MAP. According to animal age, the highest rate of the disease was in the (3-6 years) age group with a percentage of 38.2%, followed by age group over 6 years with a rate of 14.3%. On the contrary, MAP infection was not recorded in cattle younger than three years old (Table III). Table IV summarizes the recent reports about the prevalence of paratuberculosis in different countries.

Table III. Relation between ages of examined cattle and MAP infection.

| Age (years) | Total No. | No. of diseased animals | Positive (%) | P-value |
|-------------|-----------|-------------------------|--------------|---------|
| 2-3         | 13        | 1                       | 0 (0.00)     | 0.047*  |
| 3-6         | 34        | 20                      | 13 (38.2)    |         |
| > 6         | 28        | 11                      | 4 (14.3)     |         |
| Total       | 75        | 32                      | 17 (22.6%)   |         |
*The results were significant at $P$-value $<0.05$

Table IV. The prevalence rate of PTB in different countries.

| Country     | Year | Prevalence (%) | Reference                           |
|-------------|------|----------------|-------------------------------------|
| Egypt       | 2017 | 13.8           | Salem et al. (2019)                 |
| Saudi Arabia| 2019 | 30.3           | Elsohaby et al. (2021)              |
| Iraq        | 2020 | 6.0            | Al-Anbagi and Salman (2022)         |
| Sudan       | 2020 | 6.3            | Elmazgoub et al. (2020)             |
| Pakistan    | 2021 | 39.6           | Anwarullah et al. (2021)            |

The IS900 gene sequencing

The obtained sequences of IS900 MAP strains of two PCR products from two distinct farms were deposited in GenBank under accession numbers ON816021 and ON816022. The nucleotide sequencing showed complete homology between the studied isolates, and both of them showed 100% identity with MAP strains from Germany (CP022105 and CP042454) and Japan (CP066812). Meanwhile, 99.4% similarity when these isolates were compared with other Egyptian strains (KJ173783, KJ173782 and JQ937280). Regarding the phylogenetic analysis, our MAP strains (GenBank ON816021 and ON816022) were present in one clade with other Egyptian strains (KJ173783, KJ173782, and JQ937280), German (GenBank CP022105.1 and CP042454.1), and Japanese strains (GenBank CP066812.1) as shown in is shown in Figure 1.
Diseases also reported a low prevalence was recorded in Kafr Elsheikh, Gharbia, Menufia, and Qalubya study, recorded that the total infection rate was 13.8% contrary, Selim (2006) 23%, and Elsohaby et al. (2019), in their epidemiological study, recorded that the total infection rate was 13.8% in Kafr Elsheikh, Gharbia, Menufia, and Qalubya Governorates. Also, a low prevalence was recorded by Gupta et al. (2012) in Indian cattle (15.14%) and by Pruvot et al. (2014) in Canadian cattle, which was 0.8%. A lower prevalence (6%) was recorded in Iraq by Al-Anbagi and Salman (2022) and in Sudan (6.3%) by Elmagzoub et al. (2020). This variance may be due to different management conditions, the clinical stage of examined animals in each study, and detection methods. MAP infection is confined to clinically diseased cattle in this study and not recorded in contact healthy animals. This may be due to the limited sensitivity of diagnostic tests before the emergence of clinical signs, as concluded by Corne1i et al. (2021). On the contrary, Salem et al. (2005) concluded that 29% of examined Egyptian and German apparently healthy cattle were positive for MAP infection.

MAP infection was detected in three out of five examined dairy farms. However, not all diarrheic cows were IS900 gene–MAP positive. Mitchell et al. (2015) concluded that most naturally infected cattle have modest and intermittent MAP organism fecal shedding patterns. Furthermore, the dilution of MAP organisms in large volumes of intestinal contents reduces the efficacy of fecal detection. Accordingly, poor management or the presence of other chronic enteric infections, such as salmonellosis, fascioliasis, or bovine viral diarrhea, could explain the failure to detect disease in two farms studied despite clinical diarrhea in cattle (Halim et al., 2019). Statistically, MAP infection has a significant association with the animal’s age. The highest infection rate was recorded in the 3-6 year age group (38.2%), followed by the age group over six years (14.3%). These results followed Selim et al. (2019), who recorded the highest infection rate in animals older than three years (19.6%). In contrast, MAP infection was not recorded in cattle younger than three years old. This is attributed to the chronic nature of the disease and the appearance of clinical signs in older animals, which are accompanied by high shedding levels (Garry, 2011). However, this does not negate the presence of infection in those animals. Still, a probable subclinical disease without or with a low shedding level of MAP organisms may be present and needs more confirmative tools for diagnosis. Therefore, representative samples of the MAP positive cases (n = 2) from distinct farms were subjected to DNA sequencing. The two strains were 100% identical to each other. However, their similarity percentage was 99.4% with different Egyptian strains (KJ173783 and KJ173782) from Alexandria and Ismailia, respectively (Abdel-Moghny et al., 2015). Furthermore, the partial sequence of the IS900 gene (402 bp) showed complete homology with the MAP K-10 reference genome (AE016958). These findings corroborated those of Amin et al. (2015), who reported that the Egyptian isolates they examined had more than 98% of their sequences in alignment with the MAP K-10
reference genome. In addition, the phylogenetic analysis of the available sequences on GenBank revealed that our strains (ON816021 and ON816022) showed complete identity with MAP-C (*M. paratuberculosis* of cattle origin) as Japanese (CP066812.1) and German strain (CP022105.1). However, they also showed complete homology with other MAP-S (*M. paratuberculosis* of sheep origin), such as the German (CP042454) and Spanish strains (FJ775181 and FJ775182). These results may be, to some extent, acceptable when compared with Elsohaby *et al.* (2021), who identified the S-type in three cattle herds in Saudi Arabia. In contrast, Amin *et al.* (2015) concluded that all examined Egyptian MAP isolates were subordinated to the C-type. Unfortunately, this study has some limitations regarding genotyping of the isolated MAP strains. Nevertheless, the phylogenetic tree shows that our strains are in one clade with the (CP005928) strain isolated from the breast milk of a Chron’s disease patient with high identity with MAP-C-type strains. This is corroborated by Bannantine *et al.* (2014) and Wu *et al.* (2006), who recorded human MAP strains clustered with bovine strains.

**CONCLUSION**

This study provides a preliminary epidemiological picture of Johne’s disease in cattle raised in Sharkia Governorate’s small-scale dairy production. Young cattle are a significant source of infection because they carry the disease without showing symptoms of illness. As a result, additional diagnostic testing should be performed to detect MAP infection in this age group. Although the sample size was insufficient to make definitive conclusions about the genotypic characterization of MAP strains in small-scale dairy production, future studies are warranted.

**ACKNOWLEDGMENT**

We sincerely appreciate small-scale dairy farmers’ collaboration; the study would not have been possible without their participation.

**IRB approval**

Animal samples were taken for routine disease diagnosis as part of Egypt’s regular veterinary service organizations and following local and national standards.

**Ethics statement**

Recruitment of dairy cows and the sampling process were performed with the permission of the small dairy backyard farm owners. The study was approved by the committee of Animal Welfare and Research Ethics, Faculty of Veterinary Medicine, Zagazig University, Egypt.

**Statement of conflict of interest**

The authors have declared no conflict of interests.

**REFERENCES**

Abdel-Moghny, A.F., Helmy, N.M. and Arafa, A.A., 2015. Studies on *Mycobacterium avium* subspecies *paratuberculosis* isolated from Egyptian dairy farms. *Assiut. Vet. Med. J.*, 61: 71-91. https://doi.org/10.21608/avmj.2015.170215

Abdellrazeg, G.S., Elnaggar, M.M., Khaliel, S.A. and Gamal-Eldin, A.E., 2014. Detection of *Mycobacterium avium* subsp. *paratuberculosis* from cattle and buffaloes in Egypt using traditional culture, serological and molecular based methods. *Vet. World*, 7: 586-593. https://doi.org/10.14202/vetworld.2014.586-593

Al-Anbagi, N. and Salman, S., 2022. First molecular investigation John’s disease (paratuberculosis) in water buffalo in Iraq. *Int. J. Hlth. Sci.*, 6: 2403-2414. https://doi.org/10.53730/ijhs.v6nS3.6052

Altschul, S.F., Gish, W., Miller, W., Myers, E.W. and Lipman, D.J., 1990. Basic local alignment search tool. *J. mol. Biol.*, 215: 403-410. https://doi.org/10.1016/S0022-2836(05)80360-2

Amin, A.S., Hsu, C.Y., Darwish, S.F., Ghosh, P., Abd El-Fatah, E.M., Behour, T.S. and Talaat, A.M., 2015. Ecology and genomic features of infection with *Mycobacterium avium* subspecies *paratuberculosis* in Egypt. *Microbiology*, 161: 807-818. https://doi.org/10.1099/mic.0.000051

Anwarullah, M., Durrani, A., Ijaz, M., Anjum, A., Usman, M., Iqbal, M., Ali, M., Anjum, A., Ashraf, Q. and Rafique, G., 2021. Retrospective study on the association of risk factors of Johne’s disease along with physiological biomarker in large ruminants of Punjab, Pakistan. *Pakistan J. Zool.*, 54: 641-645. https://doi.org/10.17582/journal.pjz/20191021161043

Bannantine, J.P., Li, L., Mwangi, M., Cote, R., Raygoza Garay, J.A. and Kapur, V., 2014. Complete genome sequence of *Mycobacterium avium* subsp. *paratuberculosis*, isolated from human breast milk. *Genome Announc.*, 2: e01252-13. https://doi.org/10.1128/genomeA.01252-13

Bannantine, J.P., Wu, C.W., Hsu, C., Zhou, S., Schwartz, D.C., Bayles, D.O., Paustian, M.L., Alt, D.P., Sreevatsan, S., Kapur, V. and Talaat, A.M., 2012. Genome sequencing of ovine isolates of *Mycobacterium avium* subspecies *paratuberculosis*
offers insights into host association. BMC Genom., 12: 13-89. https://doi.org/10.1186/1471-2164-13-89

Clark, D., Koziczkowski, J., Radcliff, R., Carlson, R. and Ellingson, J., 2008. Detection of Mycobacterium avium subspecies paratuberculosis: comparing fecal culture versus serum enzyme-linked immunosorbent assay and direct fecal polymerase chain reaction. J. Dairy Sci., 91: 2620–2627. https://doi.org/10.3168/jds.2007-0902

Constable, P.D., Hinchcliff, K.W., Done, S.H. and Gruenberg, W., 2017. A textbook of diseases of cattle, horses, sheep, pigs, and goats. Vol 1. 11th ed. Elsevier, 3251 Riverport lane, St. Louis, Missouri 63043.

Corneli, S., Di Paolo, A., Vitale, N., Torricelli, M., Petrucci, L., Sebastiani, C., Ciullo, M., Curcio, L., Biagetti, M., Papa, P., Costarelli, S., Cagiola, M., Dondo, A. and Mazzone, P., 2021. Early detection of Mycobacterium avium subsp. paratuberculosis infected cattle: Use of experimental Johns and innovative interferon-gamma test interpretative criteria. Front. Vet. Sci., 8: 638890. https://doi.org/10.3395/fvets.2021.638890

Donat, K., Schlotter, K., Erhardt, G. and Brandt, H.R., 2014. Prevalence of paratuberculosis in cattle and control measures within the herd influence the performance of ELISA tests. Vet. Rec., 174: 119.

Douarre, P.E., Cashman, W., Buckley, J., Coffey, A. and O’Mahony, J.M., 2010. Isolation and detection of Mycobacterium avium subsp. paratuberculosis (Map) from cattle in Ireland using traditional culture and molecular based methods. Gut Pathog., 2: 11. https://doi.org/10.1186/1757-4749-2-11

Elmagzoub, W.A., Adam, N.M., Idris, S.M., Mukhtar, M.E., Abdelaziz, S.A., Okuni, J.B., Ojok, L., Abd El Wahed, A., Eltayeb, E., Gameel, A.A. and Eltom K.H., 2020. Seroprevalence of Mycobacterium avium subsp. paratuberculosis in dairy cattle in Khartoum state, Sudan. Vet. Sci., 7: 209. https://doi.org/10.3390/vetsci7040209

Elsohaby, I., Faye, M., Alkafafy, M., Refaat, M., Al-Marr, T., Alaql, F.A., Al Amer, A.S., Abdallah, A. and Elmoslemany, A., 2021. Serological and molecular characterization of Mycobacterium avium subsp. Paratuberculosis (MAP) from sheep, goats, cattle and camels in the Eastern Province, Saudi Arabia. Animals, 11: 323. https://doi.org/10.3390/an111020323

Fawzy, A., Prince, A., Hassan, A.A., Fayed, A., Zschöck, M., Naga, M., Omar, M., Salem, M. and El-Sayed, A., 2013. Epidemiological studies on Johne’s disease in ruminants and Crohn’s disease in humans in Egypt. Int. J. Vet. Sci. Med., 1: 79-86. https://doi.org/10.1016/j.jvsvm.2013.09.004

Ganusov, V.V., Klinkenberg, D., Bakker, D. and Koets, E.D., 2015. Evaluating contribution of the cellular and humoral immune responses to the control of shedding of Mycobacterium avium spp. paratuberculosis in cattle. Vet. Res., 46: 62. https://doi.org/10.1186/s13567-015-0204-1

Garry, F., 2011. Control of paratuberculosis in dairy herds. Vet. Clin. N. Am. Fd. Anim. Pract., 3: 599-607. https://doi.org/10.1016/j.cvfa.2011.07.006

Gupta, A., Rani, S.M., Agrawal, P. and Gupta, P.K., 2012. Seroprevalence of paratuberculosis (Johne’s disease) in cattle population of South-Western Bengalare using ELISA kit. Open J. Vet. Med., 2: 196-200. https://doi.org/10.4236/ojvmm.2012.24031

Halim, R., Selim, A., Galila, E., Khalil, F. and Ghanem A., 2019. Molecular and bacteriological investigation of Johne’s disease in dairy cattle. Benha Vet. Med. J., 36: 289-304. https://doi.org/10.21608/bvmj.2019.15663.1069

Harris, N.B. and Barletta, R.G., 2001. Mycobacterium avium subsp. paratuberculosis in veterinary medicine. Clin. Microbiol. Rev., 14: 489–512. https://doi.org/10.1128/CMR.14.3.489-512.2001

Johne, V.A. and Frothingham, L., 1895. Ein eigen tümlicher fall von Tuberculo se beim Rind. Dtsch. Z. Tiermed. Vergl. Path., 21: 438-455.

Khare, S., Ficht, T.A., Santos, R.L., Romano, J., Ficht, A.R, Zhang, S., Grant, I.R., Libal, M., Hunter, D. and Adams, L.G., 2004. Rapid and sensitive detection of Mycobacterium avium subsp. paratuberculosis in bovine milk and feces by a combination of immunomagnetic bead separation-conventional PCR and real-Time PCR. J. clin. Microbiol., 42: 1075–1081. https://doi.org/10.1128/JCM.42.3.1075-1081.2004

Lombard, J.E., 2011. Epidemiology and economics of paratuberculosis. Vet. Clin. N. Am. Fd. Anim. Pract., 27: 525–535. https://doi.org/10.1016/j.cvfa.2011.07.012

Mitchell, R.M., Schukken, Y., Koets, A., Weber, M., Bakkar, D., Stabel, J., Whitlock, R. and Louzoun, R., 2015. Differences in intermittent and continuous fecal shedding patterns between natural and experimental Mycobacterium avium sub species paratuberculosis infections in cattle. Vet. Res., 46: 66. https://doi.org/10.1186/s13567-015-0188-x

Naser, S., Gillespie, R., Naser, N. and El-Zaatari, F.,
1998. Effect of IS900 gene of *Mycobacterium paratuberculosis* on *Mycobacterium smegmatis*. *Curr. Microbiol.*, 37: 373–379. https://doi.org/10.1007/s002849900396

Pruvot, M., Kutz, S., Barkema, H.W., De Buck, J. and Orsel, K., 2014. Occurrence of *Mycobacterium avium* subspecies *paratuberculosis* and *Neospora caninum* in Alberta cow-calf operations. *Prev. Vet. Med.*, 117: 95–102. https://doi.org/10.1016/j.prevetmed.2014.09.003

Salem, M., Heydel, C., El-Sayed, A., Ahmed, S.A., Zschöck, M. and Baljer, G., 2013. *Mycobacterium avium* subspecies *paratuberculosis*: as insidious problem for ruminant industry. *Trop. Anim. Hlth. Prod.*, 45: 351-366. https://doi.org/10.1007/s11250-012-0274-2

Salem, M., Zeid, A.A., Hassan, D. and Zschoeck, M., 2005. Studies on Johne’s disease in Egyptian cattle. *J. Vet. Med. B.*, 52: 134-137. https://doi.org/10.1111/j.1439-0450.2005.00832.x

Selim, A., Ali A.F. and Ramadan, E., 2019. Prevalence and molecular epidemiology of Johne’s disease in Egyptian cattle. *Acta Trop.*, 195: 1-5. https://doi.org/10.1016/j.actatropica.2019.04.019

Selim, A., and Gaede, W., 2015. Comparative evaluation of PCR assay for direct detection of *Mycobacterium avium* subsp. *paratuberculosis* in ruminant. *Asian J. Anim. Vet. Adv.*, 10: 761–771. https://doi.org/10.3923/ajava.2015.761.771

Shin, S.J., Han, J.H., Manning, E.J. and Collins, M.T., 2007. Rapid and reliable method for quantification of *Mycobacterium paratuberculosis* by use of the BACTEC MGIT 960 system. *J. Clin. Microbiol.*, 45: 1941–1948. https://doi.org/10.1128/JCM.02616-06

Stief, B., Möbius, P., Türk, H., Hörügel, U., Arnold, C., and Pöhle, D., 2012. Paratuberculosis in a miniature donkey (*Equus asinus f. asinus*). *Berl. Munch. Tierarztl. Wochenschr.*, 125: 38–44.

Tamura, K., Stecher, G., Peterson, D., Filipski, A. and Kumar, S., 2013. MEGA6: Molecular evolutionary genetics analysis version 6.0. *Mol. Biol. Evol.*, 30: 2725–2729. https://doi.org/10.1093/molbev/mst197

Thompson, J.D., Higgins, D.G. and Gibson, T.J., 1994. CLUSTAL W: Improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. *Nucl. Acids Res.*, 22: 4673-4680. https://doi.org/10.1093/nar/22.22.4673

Twort, F.W. and Ingram, G.L.Y., 1912. A method for isolating and cultivating the *Mycobacterium enteritidis chronicae pseudotuberculosae bovis, Johne*, and some experiments on the preparation of a diagnostic vaccine for pseudotuberculous enteritis of bovines. *Proc. R. Soc. Lond.*, 84: 517–543. https://doi.org/10.1098/rspb.1912.0011

Wells, S.J., Collins, M.T., Faaber, K.S., Wees, C., Tavormpanich, S., Petrini, K.R., Collins, J.E., Cernichiaro, N. and Whitlock, R.H., 2006. Evaluation of a rapid fecal PCR test for detection of *Mycobacterium avium* subsp. *paratuberculosis* in dairy cattle. *Clin. Vaccine Immunol.*, 13: 1125–1130. https://doi.org/10.1128/CVI.00236-06

Wu, C.W., Glasner, J., Collins, M., Naser, S. and Talaat, A.M., 2006. Whole-genome plasticity among *Mycobacterium avium* subspecies: insights from comparative genomic hybridizations. *J. Bact.*, 188: 711-723. https://doi.org/10.1128/JB.188.2.711-723.2006