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# Bacteriological and Molecular investigation of Johne's disease in dairy cattle

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A B S T R A C T

Johne's disease (JD) caused by *Mycobacterium avium subsp. paratuberculosis* (MAP) represents a real threat to the agriculture and dairy food industries and believed to be a potential public health problem**.** No treatment for the disease exists and controlling the disease is difficult due to its long latent period. This study aimed to evaluate two diagnostic methods culture and PCR assay for detection of MAP in feces under the Egyptian circumstances. A total 200 fecal samples were collected from dairy cattle showed chronic diarrhea and have history of Johne's infection**.** Fecal culturing revealed 87 (43.5 %) positive from the total collected samples. In another side, the PCR targeting IS1311 revealed that 94 (47%) out of 200 of examined cattle were positive for MAP**.** Consequently, the both tests showed good agreement between them (Kappa value = 0.869). The sequence analysis of the obtained isolate showed that the Egyptian isolates (LC428286 and LC428285) have high similarity with MAP strain JII-1961 from Germany and MAP 316V strain of Netherlands reach up to 98%.

**Keywords**: Johne's disease, Cattle, PCR, MAP.

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## 1. INTRODUCTION

Johne's disease or paratuberculosis is a chronic enteric disease of livestock principally of ruminants. *Mycobacterium avium subsp. paratuberculosis* (MAP) is an acid fast gram positive slow growing bacterium (Selim et al., 2013). Johne's disease is of particular concern to the global dairy industry due to economic and production losses, the costs of testing and control measures, and a possible zoonotic risk with conflicting evidence of association of (MAP)

with human Crohn's disease (Lombard, 2011). Although Johne's disease could be found in most countries worldwide, very limited studies on the disease have been conducted in the Middle East and North African countries. Johne's disease was firstly reported in Egyptian cattle by Salem et al., (2005).

MAP is shed principally in the feces of affected animals, even without clinical signs (McGregor et al., 2015). Oral-fecal contamination is the most important mode of transmission of paratuberculosis (Fock-Chow-Tho et al., 2017)**.** Johne's disease is characterized by a long incubation period (1.5–2 years) (Eppleston et al., 2014). The clinical signs manifested by infected animals include chronic diarrhea, decreased milk production, emaciation and eventually death (Seyyedin et al., 2008).

Diagnosis of paratuberculosis is established by direct detection of causative agent using selective media (Selim et al., 2013) or by detection of

agents' genome using the PCR method (Selim and Gaede, 2015). Indirect methods are based on the detection of specific antibodies in blood sera or milk, or on the measuring of cellular immunity (Vidić et al., 2011).

The present study was aimed to detect MAP in feces bacteriologically with genetic characterization of the isolates using PCR and sequence analysis

## 2. MATERIALS AND METHODS

### *2.1 Ethic statement*

Fecal samples were collected under owner's consent, and the study was approved by the Internal Ethics Review Committee of Faculty of Veterinary Medicine, Benha University.

### *2.2 Samples collection and preparation*

A total 200 fecal samples were collected from dairy herd showed chronic diarrhea and have history of Johne's infection. The samples collected during 2016 from animals with age ranged between 2-10 years. The fecal samples were taken with a clean glove directly from the rectum and transported on ice to laboratory and kept -20 °C until further analysis by culture and PCR.

### *2.3 Fecal culture of MAP*

The fecal samples were thawed under 4 °C for 24 h prior to the decontamination procedure. The culture process of feces was performed as described previously by Fernández-Silva et al., (2011). Briefly, 3 g of feces were added to a 50 mL sterile tube containing 30 ml of a 0.75% X hexadecyl pyridinium chloride (HPC) weight/volume (w/v) solution. The suspension was thoroughly mixed by shaking and leaving stand in vertical position to allow sedimentation of large particles. Approximately 20 ml of the supernatant was transferred to sterile 50 ml Falcon tube, followed by mixing at 200 rpm for 30 min. Tubes were place in vertical position in the dark for 24 h at room temperature. Decontaminated pooled fecal samples were centrifuged at 900 x g during30 min, supernatant was discarded. 300 µl of the decontaminated pellet was inoculated into Herrold's egg yolk medium (HEYM), two with mycobactin J (Allied Monitor, Inc., Fayette, MO, USA) and two without. All medium was incubated for 18weeks at 37 °C and examined weekly for any growth. For sterility control, tubes containing HEYM without inoculums also were incubated at 37 °C. MAP growth was visually monitored for typical slow growth rate and colonial morphology according to previous descriptions (colonies developing after ≥ 3 weeks of incubation, initially round, smooth and white, then tending to heap up slightly and becoming dull light yellow with wrinkling of the surface (Correa-Valencia et al., 2017).

### *2.4 Extraction of DNA from fecal samples*

Each fecal sample was homogenized for 5 min prior to DNA extraction procedure. DNA from individual fecal samples was extracted using QiAmp DNA Mini kit (Qiagen GmbH, Hilden, Germany) according to the manufacturer's instructions.

### *2.5 Detection of MAP-DNA using PCR assay*

The PCR amplification was carried out using specific pair of primers for target IS1311 previously described by Sevilla et al., (2005). Briefly, the isolated DNA was amplified using oligonucleotide primers M56 (Forward: 50-GCGTGA GGC TCT GTG AA-30) and M119(Reverse: 50-ATG ACG ACC GCT TGG GAGAC-30). The PCR reaction was carried in a 25 µl reaction volume. For a single reaction, the PCR master mix contained 1 µl of each primer (20 pmol/µl), 0.75 µl of each probe (10 pmol/µl), 12.5 µl of QuantiTect probe (Qiagen, Hilden, Germany), and 4.75 µl of RNase-free water. Finally, 5.0 µl of DNA template was added.

The PCR thermo cycling was done using (T3 Biometra, Germany) as follows: 94 °C for 15 min, followed by 40 cycles of 95 °C for 15 s, 56 °C for 30 s, and 72 °C for 2 min. Final extension was completed at 72 °C for 10 min. Amplicons of the expected size (608 bp) were visualized with ethidium bromide on 1.5% agarose gel.

*2.6 Sequence and phylogenetic analysis*

The purified PCR products were sequenced directly using the ABI PRISM® BigDye TM Terminators v3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA, USA). The sequences were edited and alignment was done with Bio- Edit software. Also, the phylogenetic analysis for of obtained sequence were performed by Mega7 software using the neighbor-joining tree method with 1000 bootstrap replicates.

*2.7 Statistical analysis*

The kappa values were calculated to evaluate the agreement between the bacterial culture and PCR (http://vassarstats.net/kappa.html) according to Viera and Garrett, (2005).

## 3. RESULTS

*3.1 Identification of MAP by HEYM agar and conventional PCR*

The PCR amplification using species specific primers revealed that 94 out of 200 of examined cattle were positive for *MAP*, with 608 bp amplified amplicon as shown in Fig 1

The results of culture and PCR targeting IS1311 showed 84 positive and 103 negative cases among examined cattle (Table 1). Although, the PCR provided ten cases which were negative with culture and culture provided three cases which were negative by PCR. Consequently, the both tests showed good agreement between them (Kappa value = 0.869, 95% CI: 80.03–93.77).

*3.2 Sequence and phylogenetic analysis of IS1311 gene*

The obtained sequence of IS1311 gene of the MAP strain was deposit in GenBank under accession number (LC428286 and LC428285). This sequence was aligned with the IS1311 gene sequences of other MAP strains accessible in GenBank. The sequence analysis of the obtained isolate showed that the Egyptian isolates have high similarity with MAP strain JII-1961 from Germany and MAP 316V strain of Netherlands reach up to 98%. Furthermore, heterogeneity appeared between the two isolates over five substitutions as in Fig 2.

The phylogenetic analysis of analyzed isolate showed that the Egyptian MAP strains in the current study present in one clade with MAP strain JII-1961 from Germany but it distinct from *Mycobacterium avium subsp. hominisuis* and *Mycobacterium avium subsp. avium* as shown in Fig 3.

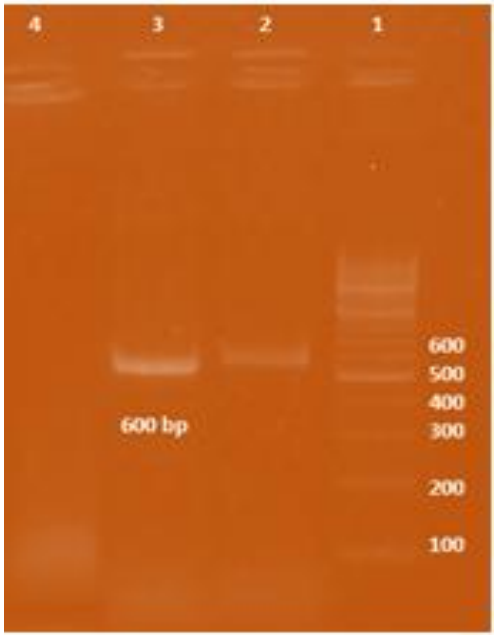


Figure 1: Results of PCR of gene IS1311, Lane 1 DNA ladder 100 bp, lanes 2-3: positive cases at 608 bp, lane 4: negative control.

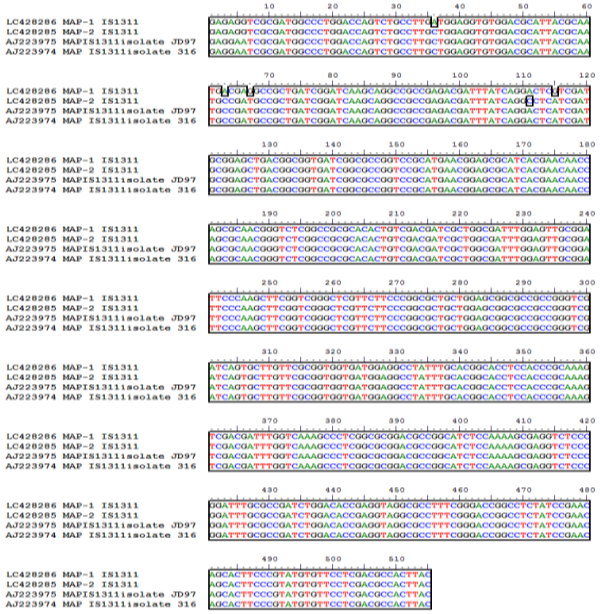


Figure 2: Nucleotide sequence alignment of Egyptian MAP isolates based on IS1311 gene with MAP isolates from Genbank database

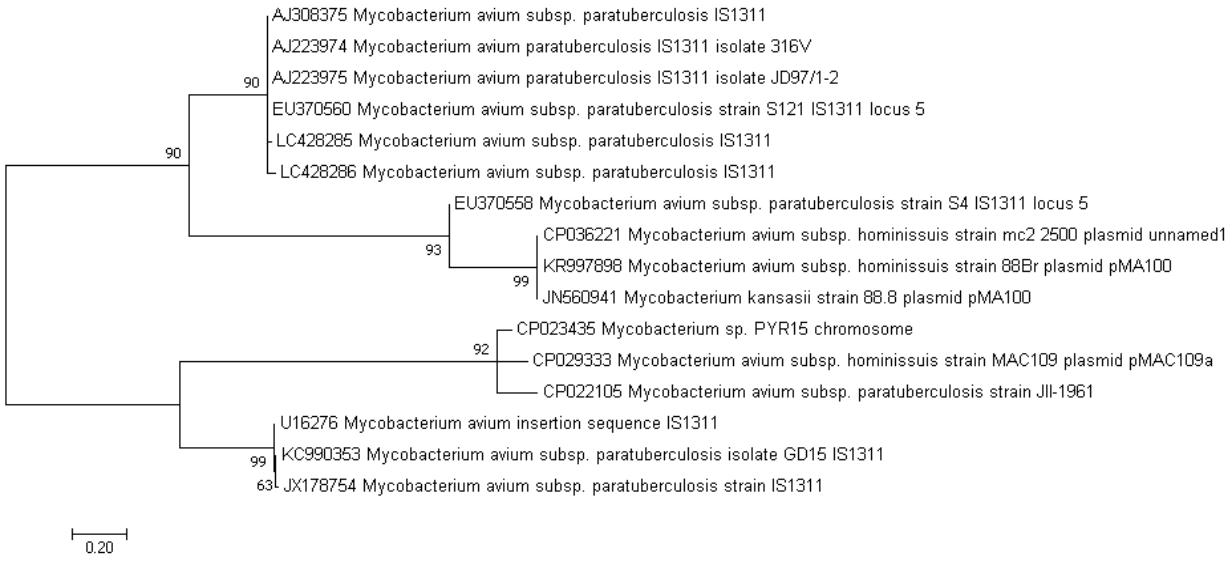


Figure 3: Neighbor-joining tree of IS1311 gene showing phylogenetic relationship of MAP isolate from Egypt in the present study and other MAP isolates and mycobacterium strains available from GenBank

## 4. DISCUSSION

MAP is one of the most important agents that cause several economic losses (Nielsen and Toft, 2009). Therefore, early diagnosis of Johne s disease (JD) is very important for the control of the disease in dairy herds (Seyyedin et al., 2008). In Egypt, MAP was mainly isolated from exotic cattle breeds (Holstein-Friesian) as they are usually reared in dairy farms due to their high milk yield, however, it was also isolated from native cattle and buffalo breeds (Amin et al., 2015; Salem et al., 2005).

Several studies considered the PCR assay as an alternative rapid and powerful tool to detect DNA of MAP directly in fecal samples (Selim et al., 2013). PCR assay was reported to be highly specific and sensitive diagnostic test; this supports its potential value for rapid and effective diagnosis of Johne's disease (Taddei et al., 2004). Fecal culturing revealed 43.5 % positive culture samples from collected samples. While 56.5% of samples were negative results. The obtained results are different from the result of previous studies carried in Egypt obtained by (Salem et al., 2005) who reported that 51% of fecal samples were positive culture and 49% were negative culture from cows that showed some clinical symptoms characteristic of JD, the negative results obtained from the clinically suspected animals may be attributed to misdiagnosis with malnutrition, bad water quality or with other chronic wasting diseases such as tuberculosis (TB), salmonellosis or fascioliasis.

Also, Abdellrazeq et al. (2014) reported that 68,8% of fecal samples were positive culture for MAP, While El-Hariri et al. (2014) were detected (16%) positive in examined fecal samples of dairy cattle.

On the other hand, although fecal culture is actually considered the "gold standard" for the diagnosis of bovine paratuberculosis for its good specificity, its extensive use in laboratory diagnosis of the diseases has been hampered due to the long incubation period (Paolicchi et al., 2003). The long generation time of MAP requires up to 20 weeks to produce visible colonies on solid medium composed mostly of Herrold's egg yolk (HEY) agar with mycobactin (Kim et al., 2004). This creates management problems for producers who need to make quick decisions for the purchase of replacement animals or to cull infected animals from their herd. However, the culturing method remains the gold standard despite its logistical and practical limitations (Douarre et al., 2010).

In recent years, advances have been made in the improvement of methods for the detection of MAP DNA by PCR (Fang et al., 2002; Khare et al., 2004; Selim et al., 2013). The PCR can detect

around 10000 organisms/g and can even as reported by Selim and Gaede, (2015) detect the presence of only one organism.

The PCR amplification using species specific primers revealed that 94 out of 200 (47%) of examined cattle were positive for MAP. The same result was obtained by Salem et al., (2005), as direct examination of fecal samples with PCR revealed positive results in 47%. In contrast, Amin et al. (2015) reported that the prevalence rate of MAP-infected cows to be 22.5% by the PCR assay. Also, Dina et al. (2014) revealed that 42.66% of faecal samples were PCR positive. This difference may be attributed to difference in method of detection, the number of samples, the environmental conditions of the farms and the animal age.

In the present study, the PCR provided 10 positive cases which were negative with culture, these results come in accordance with Abraham et al., (2014), they reported 8 positive cases which were negative with culture. This results could be explained by the probability that the positive cultures / negative PCR- animals were mixed cultures, i.e., contained *Mycobacterium* spp. other than MAP. Also, culture provided three cases which were negative by PCR. This may be attributed to the presence of light MAP shedders or to the presence of PCR inhibitors in feces (Collins et al., 1993; Whipple et al., 1992).

Although that difference between PCR and culture. the both tests showed good agreement between them (Kappa value = 0.869, 95% CI: 80.03–93.77) in this study. The results obtained agree with Chevallier et al., (2003) who mentioned that the culturing and PCR methods were equivalent in sensitivity.

The sequence analysis of isolated MAP strain from in the present study showed high identity between obtained MAP strains and other MAP strains from Germany and Netherland. These results provided that MAP is circulating among dairy cattle in Egypt and the source of infection may be come with imported cattle from these countries.

## 5. CONCLUSION

The present study succeeded to isolate and identify MAP from Egyptian dairy cattle. The results confirm the epizoology of JD in Egypt and encourage the decision makers to start creating a program to control it.

**Acknowledgement**

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## 6. REFERENCES

Abdellrazeq, G., El-Naggar, M., Khaliel, S., Gamal-Eldin, A., 2014. Detection of Mycobacterium avium subsp. paratuberculosis from cattle and buffaloes in Egypt using traditional culture, serological and molecular based methods. Veterinary World 7.

Abraham, A., Thomas, N., Joseph, S., Raghavan, K., 2014. Comparitive study on the efficacy of ELISA and IS900 PCR for the diagnosis of paratuberculosis in goats. Biotechnology in Animal Husbandry 30, 661-667.

Amin, A.S., Hsu, C.-Y., Darwish, S.F., Ghosh, P., AbdEl-Fatah, E.M., Behour, T.S., Talaat, A.M., 2015. Ecology and genomic features of infection with Mycobacterium avium subspecies paratuberculosis in Egypt. Microbiology 161, 807-818.

Chevallier, B., Versmisse, Y., Blanchard, B., 2003. Development of a PCR test to detect Mycobacterium avium subsp. paratuberculosis in bovine feces, Proceedings of the 7th International Colloquium on Paratuberculosis. Madison, WI, USA: IAP, pp. 247-250.

Collins, D., Hilbink, F., West, D., Hosie, B., Cooke, M., De Lisle, G., 1993. Investigation of Mycobacterium paratuberculosis in sheep by faecal culture, DNA characterisation and the polymerase chain reaction. Veterinary Record 133, 599-600.

Correa-Valencia, N.M., Ramírez, N.F., Bülte, M., Fernández-Silva, J.A., 2017. Fecal culture and two fecal-PCR methods for

the diagnosis of Mycobacterium avium subsp. paratuberculosis in a seropositive herd. Revista Colombiana de Ciencias Pecuarias 30, 101-115.

Douarre, P.E., Cashman, W., Buckley, J., Coffey, A., O'Mahony, J.M., 2010. Isolation and detection of Mycobacterium avium subsp. paratuberculosis (MAP) from cattle in Ireland using both traditional culture and molecular based methods. Gut pathogens 2, 11.

El-Hariri, M., Soliman, R., Samir, A., 2014. Surveillance of Johne's diseases bacterium, Mycobacterium avium subspecies Paratuberculosis in dairy cattle in Egypt using three laboratory diagnostic techniques. International Journal of Veterinary Science 3, 33-36.

Eppleston, J., Begg, D.J., Dhand, N., Watt, B., Whittington, R.J., 2014. Environmental survival of Mycobacterium avium subsp. paratuberculosis in different climatic zones of eastern Australia. Applied and environmental microbiology, AEM. 03630-03613.

Fang, Y., Wu, W.-H., Pepper, J.L., Larsen, J.L., Marras, S.A., Nelson, E.A., Epperson, W.B., Christopher-Hennings, J., 2002. Comparison of real-time, quantitative PCR with molecular beacons to nested PCR and culture methods for detection of Mycobacterium avium subsp. paratuberculosis in bovine fecal samples. Journal of clinical microbiology 40, 287-291.

Fernández-Silva, J.A., Abdulmawjood, A., Akineden, Ö., Bülte, M., 2011. Serological and molecular detection of *Mycobacterium avium subsp. paratuberculosis* in cattle of dairy herds in Colombia. Trop Anim Health Prod 43, 1501-1507.

Fock-Chow-Tho, D., Topp, E., Ibeagha-Awemu, E., Bissonnette, N., 2017. Comparison of commercial DNA extraction kits and quantitative PCR systems for better sensitivity in detecting the causative agent of paratuberculosis in dairy cow fecal samples. Journal of dairy science 100, 572-581.

Khare, S., Ficht, T.A., Santos, R.L., Romano, J., Ficht, A.R., Zhang, S., Grant, I.R., Libal, M., Hunter, D., 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. Journal of clinical microbiology 42, 1075-1081.

Kim, S.G., Kim, E.H., Lafferty, C.J., Miller, L.J., Koo, H.J., Stehman, S.M., Shin, S.J., 2004. Use of conventional and real-time polymerase chain reaction for confirmation of Mycobacterium avium subsp. paratuberculosis in a broth-based culture system ESP II. Journal of veterinary diagnostic investigation 16, 448-453.

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

McGregor, H., Abbott, K.A., Whittington, R.J., 2015. Effects of Mycobacterium avium subsp. paratuberculosis infection on serum biochemistry, body weight and wool growth in Merino sheep: a longitudinal study. Small Ruminant Research 125, 146-153.

Nielsen, S.S., Toft, N., 2009. A review of prevalences of paratuberculosis in farmed animals in Europe. Preventive veterinary medicine 88, 1-14.

Paolicchi, F.A., Zumarraga, M.J., Gioffre, A., Zamorano, P., Morsella, C., Verna, A., Cataldi, A., Alito, A., Romano, M., 2003. Application of different methods for the diagnosis of paratuberculosis in a dairy cattle herd in Argentina. Journal of Veterinary Medicine, Series B 50, 20-26.

Salem, M., Zeid, A., Hassan, D., El-Sayed, A., Zschoeck, M., 2005. Studies on Johne's

disease in Egyptian cattle. Journal of Veterinary Medicine, Series B 52, 134-137.

Selim, A., El-Haig, M., Galila, E.S., 2013. Direct detection of Mycobacterium avium subsp. Paratuberculosis in bovine milk by multiplex Real-time PCR. Biotechnology in animal husbandry 29, 513-525.

Selim, A., 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.

Sevilla, I., Singh, S., Garrido, J., Aduriz, G., Rodriguez, S., Geijo, M., Whittington, R., Saunders, V., Whitlock, R., Juste, R., 2005. Molecular typing of Mycobacterium avium subspecies paratuberculosis strains from different hosts and regions. Revue scientifique et technique-Office international des épizooties 24, 1061.

Seyyedin, M., Salehi, T., Tadjbakhsh, H., Najafi, M., Rabbani, M., 2008. Isolation and identification of Mycobacterium avium subsp. Paratuberculosis from milk, manure and fecal samples of holstein-friesian dairy cattle. Pakistan Journal of Biological Sciences 11, 2639.

Taddei, S., Robbi, C., Cesena, C., Rossi, I., Schiano, E., Arrigoni, N., Vicenzoni, G., Cavirani, S., 2004. Detection of Mycobacterium avium subsp. paratuberculosis in bovine fecal samples: comparison of three polymerase chain reaction—based diagnostic tests with a conventional culture method. Journal of veterinary diagnostic investigation 16, 503-508.

Vidić, B., Savić, S., Prica, N., 2011. Paratuberculosis in cattle: The outspread, diagnostics and control. Biotechnology in Animal Husbandry 27, 335-344.

Viera, A.J., Garrett, J.M., 2005. Understanding interobserver agreement: the kappa statistic. Fam Med 37, 360-363.

Whipple, D.L., Kapke, P.A., Andersen, P.R., 1992. Comparison of a commercial DNA probe test and three cultivation procedures for detection of Mycobacterium paratuberculosis in bovine feces. Journal of Veterinary Diagnostic Investigation 4, 23-27.