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

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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 molecular screening. Clinically, 32 of 75 examined cattle showed variable degrees of body weight loss and diarrhea. 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.

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SGY, AS and HAE contributed to the conception and design of the study, formal analysis and methodology. SGY, AS, HME, and HAE writing original draft. SGY and HME edited the initial manuscript. All authors approved the final manuscript.

Key words
Cattle, Egypt, *IS900* gene, *Mycobacterium avium* subsp. *paratuberculosis*

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 Johne 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 (Ganusov *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,

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including *IS900*, *F57*element, 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 genetically 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 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 (the average number of cattle per herd was not more than 50) in Zagazig district, Sharkia Governorate, 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 was chosen due to a lack of available research data on the studied topic compared to other governorates in Egypt. Furthermore, it has rural villages that rely primarily on agriculture and animal breeding in a small-backyard 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 labouratory, 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′GAAGGGTGTTCGGGGC-CGTC-3′ and P91: 5′-GAGGTCGATCGCCCACGTGAC -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 ≤ 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)	
	Number of cows	%
Normal rectal temperature (38.8±0.4 Cº)	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	Elmagzoub *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.

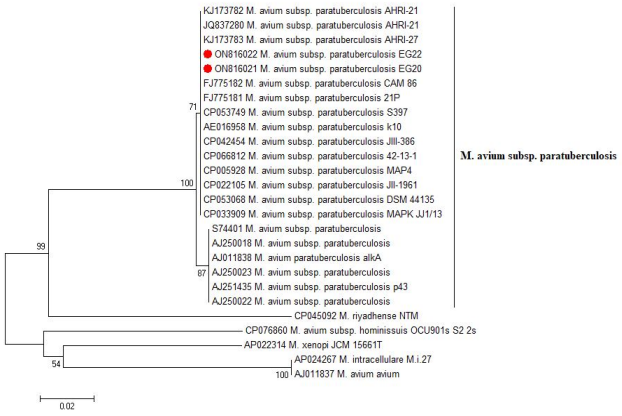

Fig. 1. Neighbor-joining tree of insertion sequence *IS900* gene of MAP showing the phylogenetic relationship of Egyptian isolates and other MAP isolates available from GenBank.

DISCUSSION

Paratuberculosis is a chronic debilitating disease that triggers substantial financial losses in the livestock industry. Most studies investigated the disease occurrence in large dairy herds in Egypt (Abdel Moghny *et al*., 2015; Amin *et al*., 2015; Selim *et al*., 2019). Meanwhile, the available epidemiological data concerning the disease in cattle under small-scale production are scarce. So, this study aimed for screen MAP infection in suspected dairy herds reared by small farmers with a history of enteric problems. Five small dairy herds in Sharkia Governorate with a history of chronic diarrhea in animals aged over two years were screened to increase the likelihood of molecular detection of MAP. The most prevalent signs in this study were body weight loss, decreased milk production, and diarrhea. Most clinically diseased cattle were three years old; this agreed with Salem *et al*. (2013), who reported that the symptoms of bovine PTB appear in in this age group.

The overall percentage of the disease using the molecular-based *IS900* method was 22.64%. Our findings are nearly similar to those of Clark *et al*. (2008) 29.6%, Wells *et al*. (2006) 23%, and Elsohaby *et al*. (2021) 30.3% in Saudi Arabia. Abdellrazeq *et al*. (2014) found high levels of MAP infection (52.94%) in the Egyptian Governorates of Gharbia, Menufia, El-Beheira, Alexandria, and Kafr Elsheikh. Also, a high prevalence was reported in Germany by Donat *et al*. (2014) (41.43%) and in Pakistan (39.6%) by Anwarullah *et al*. (2021). On the contrary, Selim *et al*. (2019), in their epidemiological study, recorded that the total infection rate was 13.8% in Kafr Elsheikh, Gharbia, Menufia, and Qualubya 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 Corneli *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. Mitchel *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.

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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.

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