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Prevalence and phylogenetic analysis of *Mycobacterium avium* subsp. paratuberculosis in cattle in Mosul city, Iraq

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Abstract

Mycobacterium avium subsp. *paratuberculosis* (MAP) is a globally distributed infection that causes chronic enteritis, fluctuating milk production, emaciation, and various degrees of diarrhea. It is also a public health concern. This task investigates the molecular prevalence of MAP in cows along with a phylogenetic analysis of this microorganism in Mosul City, Iraq. 176 fecal specimens were taken from healthy, diarrheic and emaciated cows of different ages and origins. The detection of gene IS900 of MAP revealed that 5.6% (10/176) of tested cows were confirmed using molecular technique. No significant prevalence between the cow's origin and a significantly higher prevalence of the MAP was recorded on cows over 5 years old and with symptoms. Furthermore, in this study, two sequences of *Mycobacterium avium* subsp. *paratuberculosis* were deposited in GenBank (PP976335.1 and PP976335.1); phylogenetically the study strains showed 100% identity near MAP strain in Saudi Arabia under the accession number (MN928512.1) and 99.85% identity with Spain, Germany and South Korea strain accession numbers (FJ775181.1, CP053068.1 and CP033909.1) respectively. Overall, this result showed that the IS900 gene may be used as a molecular tool to identify MAP in cows. In order to monitor, treat, and manage paratuberculosis in cows in Mosul, Iraq, further epidemiological research and isolation are needed.

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Introduction

The organism of MAP is a principal employee of Johne's ailment, which causes widespread gastro-enteric illness affecting various ruminants and can induce severe economic losses and trade limitations, especially in milk producer herds worldwide. Recently, it has received remarkable attention for participating in Crohn's sickness (CD) in humans (1-3). Although clinical disease is reported frequently in cattle, sheep and goats, it can strike a broad domain of hosts, including camelids, rabbits, red deer, hares, alpine ibex and river buffalo. In addition, the organism has been detected in humans with (CD) (4-6). The Mycobacterium is an obligate intracellular fastidious, slow growing gram-positive bacterium that can outrun in different environmental conditions (7). The MAP is a part of the Mycobacterium avium complex. This complex bacterium has unique pathogenicity and host range features (8). A few strains of MAP were identified, including the sheep strain Type I and Type III sub-lineages and cattle strain Type II (9). The MAP is dominantly transmitted in dairy cattle through positive animals' stool, milk, and colostrum intake. The outcomes of MAP in clinically infected cattle are characterized by emaciation, diarrhea, decreased milk production and death. At the same time, in subclinical infection, the animals intermittently secrete the bacteria in feces and milk, a source of infection to other cattle (10). Infected cattle also show reduced fertility, mastitis and susceptibility to infectious agents due to immunosuppression (11,12). In addition, there are cases of

infected cows with MAP without clear or even absence of clinical features. Because they continually shed the organism in their feces and spread the infection, it represented a challenge in constraining the infection (13). It has been clear that the clinical signs of MAP infection are generally apparent, and by the time the clinical manifestations become obvious, animal health usually deteriorates. The disease is regularly fatal (14). For the diagnosis of MAP from clinical animals and phenotyping, the IS900 restriction fragment length polymorphism (RFLP), multiplex PCR, real-time PCR, immunomagnetic separation-PCR (IMS-PCR), and pulsed-field gel electrophoresis (PFGE) were used by some authors. The molecular diagnosis is capable of the detection and quantifying of MAP DNA in various clinical samples. The PCR techniques are available, sensitive, rapid and cost-effective (15,16) and the ELISA assays (17,18). Generally, the diagnostic tests are influenced by the style of the gold standard, age, stage of ailment, and type of samples that have a role in diagnosis. Some studies used a single test, like fecal culture only. However, it lacks sensitivity and prolonged incubation, usually between 49 and 112 days on solid media (19,20).

This study aimed to determine MAP prevalence in cows with a phylogenetic analysis of detected bacteria in Mosul city, Iraq.

Materials and methods

Ethical approval

The College of Veterinary Medicine, University of Mosul, granted ethical permission for the UM.VET.2023.113 on October 15, 2023.

Area, animals and sampling

Based on previous literature (21) and the sample size formula (22), according to the formula, the number of cows required in this study was 176 cows. For this study, 176 cows were obtained from different farms belonging to private owners in multiple districts in Mosul city. Animals ages were ≤ 5 years (n= 133) and > 5 years old (n= 43), Local breed (n= 127) and imported breed (n= 49), and some farms consist of interspecies rearing, i.e. cow and buffaloes or cows with sheep or all three species. The clinical symptoms were recorded by inspection and owner's data questionnaire. Furthermore, the animal's health status varied from normal, emaciated, intermittent, transient or chronic diarrhea and fluctuating milk production. Between November 2023 and May 2024, 176 feces (50 grams) were collected from each animal through disposable antiseptic conservators, placed in sterile cups, signalled, and transported in clean, dryish, cool bags to the laboratory at the Mosul Veterinary Medicine College. They were kept at 4°C and analyzed within a day of their collection (23).

DNA extraction

To abstract DNA from all fecal specimens, the QIAamp® Fast DNA Stool Mini Kit (QIAGEN, Hilden, Germany) was used, as mentioned in the menu instructions provided by the manufacturer. Its purity was measured using a NanoDrop spectrophotometer (24).

Amplification of genomic DNA

The amplification of genomic DNA utilizing cPCR reaction was performed to amplify and distinguish specific MAP IS900 markers using a thermal cycler (T100 BioRad, USA) with private primers, IS900F: 5'-GAAGGGTGTTCGGGGCCGTC GCTTAGG-3' and IS900R: 5'-GGCGTTGAGGTCGATCGCCCACGTGAC-3' in expected size of 413bp (25,26). The PCR reaction mixtures with a total reaction volume of 20µl, comprising 7.5µl of 2X-Taq Master Mix, 1µl from each primer, 5 µl of DNA, and 5.5µl of PCR-grade water (27,28) and thermal cycler were done with initial denaturation at 94˚C for 1 min, followed by 35 cycles of denaturation at 94˚C for 1 min, annealing at 55˚C for 1 min, extension at 72˚C for 10 min and final extension at 72˚C for 5 min, according to Rezig *et al*. (29). Positive PCR products from tested cows were sent to Macrogen Company (South Korea) for sequencing. The Neighbor-joining (NJ) and MEGA11 software tools (30) were used to create phylogenetic trees, and sequences of MAP (AP024266) in Japan were employed as an outgroup.

Statistical analysis

In order to set the status of MAP in cows, descriptive statistics were used to describe the data from the current study in Excel 2010 for Windows 10. Fischer's exact test and the chi-square were used to examine the odds ratio for (age, origin, and status). Significant data were those with $P<0.05$ value using Epi-Info™.

Results

Molecular results

The current effort revealed that the overall prevalence of MAP in cows in the study city, utilizing PCR reaction for 176 extracted fecal samples targeting the IS900 gene, was 10/176 (5.6%) with a band of approximately 413bp (Figure 1).

The results of our investigation also indicate that the MAP was detected in both the healthy apparent cows 2/120 (1.6%) and those with symptoms of diarrhea, emaciation, and fluctuant milk yield 8/56 (14.2%). The findings of the present study showed given distinction ($P <0.05$) of MAP relying on model age, with higher prevalence in animals age >5 years (odds ratio = 5.2297, Cl: 1.4014- 14.0680), $P = 0.007$ (Table 1). The outcome also revealed no appreciable variance ($P<0.05$) in the prevalence of MAP in

cows in terms of animal origin (odds ratio = 1.1180, Cl: 0.2772-4.5093), P=0.8 (Table 2). notably (P<0.05), the result indicates elevated prevalence in cows with symptoms (odds ratio = 9.8333, Cl: 2.0145-48.0003), P=0.00 contrast to Apparent healthy cows (Table 3).

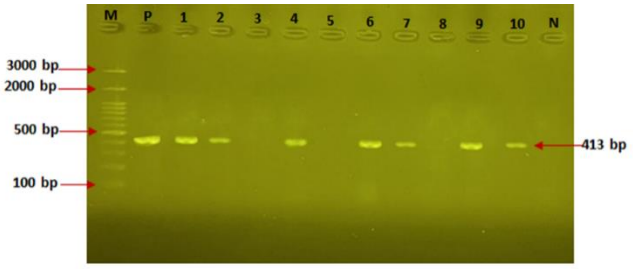

Figure 1: Gel image: lane M) Mark 100-3000bp DNA ladder; Lane 1,2,4,6,7,9,10) Conventional PCR technique detected MAP targeting IS900 gene in approximately band size 413bp; Lane N) negative control.

In this work, and from analyzing 176 fecal samples by PCR technique, ten obtained sequences of the MAP were detected in Mosul city participate 100% similarities, and Two of the sequences assigned the accession number (PP976335.1 and PP976335.1) (Figure 2).

Through paralleled the acquired local sequence (PP976335.1 and PP976335.1) of the IS900 gene of MAP and the analysis of the phylogenetic tree after 1000 replications using MEGA11 software with obtainable information in GenBank, results indicate the study sequence was neatly attached with those sequence of Saudi Arabia (MN928512.1) (100% identity), and with Spain (FJ775181.1), Germany (CP053068.1), South Korea (CP033909.1) were (99.85% identity) (Table 4 and Figure 3).

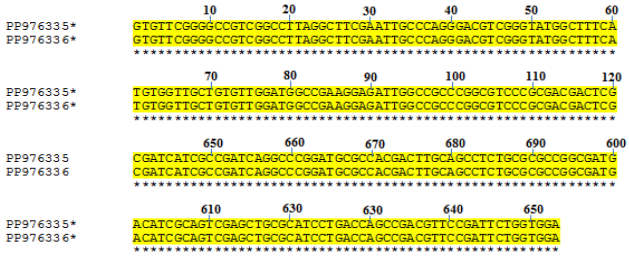

Figure 2: Alignment between local sequences of the IS900 gene of MAP showed an alignment score of 100, using multiple sequence alignment- CLUSTALW.

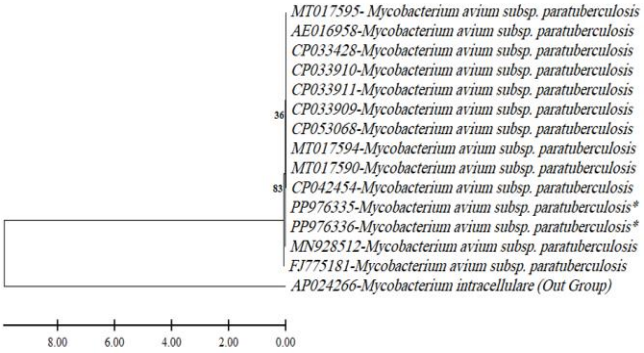

Figure 3: Phylogenic tree of MAP from Iraq (*). Partial DNA sequences of concatenated partial IS900 gene were used as input.

Table 1: The odds ratio of MAP in cows associated with the animal's age

Age	No. tested	+ve (%)	OR	CI	P
≤ 5 years	133	4 (3 %) [a]	1		
>5 years	43	6 (13,95 %) [b]	5.2297	1.4014- 14.0680	0.007

OR: Odds ratio, CL: Confidence of interval, letter a b c means significant, P: P value.

Table 2: The odds ratio of MAP in cows associated with the animal's origin

Origen	No. tested	+ve (%)	OR	CI	P
Imported	49	3 (6.12 %) [a]	1		
Local	127	7 (5.51 %) [a]	1.1180	0.2772-4.5093	0.8

OR: Odds ratio, CL: Confidence of interval, letter a b c means significant, P: P value.

Table 3: The odds ratio of MAP in cows associated with the animal's health status

Health status	No. tested	+ve (%)	OR	CI	P
Apparent healthy	120	2 (1.6 %) [a]	1		
With symptoms	56	8 (14.28 %) [b]	9.8333	2.0145-48.0003	0/00

OR: Odds ratio, CL: Confidence of interval, letter a b c means significant, P: P value.

Table 4: Homology of MAP based on partial IS900 according to BLASTn in GenBank of NCBI

Accession number	Query Cover %	Identic Number %	GenBank Accession Number	Country
PP976335.1	100	100	MN928512.1	Saudi Arabia
	100	99.85	MT017595.1	Saudi Arabia
	100	99.85	MT017590.1	Saudi Arabia
	100	99.85	FJ775181.1	Spain
	99	99.85	MT017594.1	Saudi Arabia
	99	99.85	CP053068.1	Germany
PP976336.1	99	99.85	CP033909.1	South Korea
	99	99.85	CP033911.1	South Korea
	99	99.85	CP033910.1	South Korea
	99	99.85	CP033428.1	South Korea
	99	99.85	CP042454.1	Germany
	99	99.85	AE016958.1	USA

Discussion

Johne's illness involves small and large ruminants, resulting in disturbances such as emaciation and diarrhea and is responsible for considerable losses and menace to dairy farms' production (31,32). To our knowledge, this is the former phylogenetic MAP data in Mosul city cows. The current study showed that the gross spread of MAP in cows through PCR reaction was 5.6%. This result may be similar and/or lower or higher than earlier literature that reported MAP prevalence in Mosul city and other governorates in Iraq and other countries using various samples, the same or different ruminants, and laboratory methods. Al-Farwachi *et al*. (33) revealed 10.32% seroprevalence in cattle. Ahmed, (34) indicates 7.6% seroprevalence in sheep. Ahmed *et al.* (21) also showed 6% positivity to MAP in raw cow's milk by PCR. In the Al-Najaf governorate, Angabi and Salman, (35) reported that 9.3% were positive for IS900-specific gen in buffaloes. In another study in dairy buffalo herds, Hassan *et al.* (36) found a distribution of 16%. In Iran was 6% seroprevalence (37). In Jordan, it was 26% (38). In Saudi Arabia, the IS900 gene of MAP amplified from fecal samples was 26.8% sheep, 27.6% goats, 30.3% cattle and 15.0% camels (32). In Turkey, it was 12.24%, 13.61% and 28.57%, respectively (39). In Spain, 8.1% of sheep and 20% of goats (40). The prevalence of MAP disease could differ between areas; the reasons could be attributed to indigent detection style, subclinical animals, rise in fecal spill, perpendicular transmission, and lack of owner's consciousness as well as foodborne transmission, population age, diagnostics tool (41), sample types, spacious cattle business, and free population locomotion. This finding is consistent with research (42-46).

The results restore search results have confirmed the MAP in a cow's feces pattern using PCR, the technique gets the IS900 gene sequence. This data indicates that molecular techniques are swift, qualitative and cost-efficient. Our success is parallel with prior works (32,43-49). It is known that one of the target genes used to detect MAP is the IS900 region, described initially by Green *et al*. (50). Subsequently, it enables the diagnosis of disease even in the initial infection. The specificity and sensitivity of PCR have enhanced up to detecting 1 CFU of MAP in samples (51,52). Moreover, PCR recognized the causative in feces with a sensitivity of 70% -100% (17,53,54) and a specificity of 100% (51). The prior endeavours have targeted the IS900 gene and revealed an intense sensitivity grade (55).

Regarding animals' age, a higher prevalence was recorded in animals aged >5 years (odds ratio = 5.2297). The present study's findings showed agreement with previous documents (46-57). The cows could be reared for a longer time for yielding. For this instance, cows live sufficiently to shed and show symptoms of paratuberculosis. Our vision is near to the results of (56,58). In a study, Ben Romdhane *et al*. (59) announced that the tendency with age had a spectacular outcome on herd transmission. Moreover, it could contribute to the long-term figure of the MAP and occurrence of manifestation in elderly animals, coinciding with pronounced shedding of the organism (60). However, attention must not ignore the existence of exposure in younger ages and the subclinical diseased animals without or with lower shedding of MAP, giving priority to the use of optimal tools for diagnosis (61).

This study revealed no considerable variance of MAP among cows of dissimilar origin, which may be based on the worldwide distribution of this disease. Furthermore, free animals can acquire MAP from infected animals via several routes, such as milk and colostrum and by fecal-oral route to animals of all ages. In addition, cattle movement also plays a major role in the transmission of MAP. This information matches the evidence of (48,62,63). In a study, Stevenson, (64) reported that uterine conveyance of MAP can occur, and the organism can be detected in the saliva, suggesting a probable additional approach to transmission. It has been revealed that John's disease is endemic throughout the world, with remarkable losses, mainly in dairy farms (48).

Statistically, the current investigation indicates a higher prevalence in cows with symptoms than healthy cows. This data is the same as mentioned by Al-Farwachi *et al*. (33). It is known that Paratuberculosis is a wasting illness that occurs in large and small ruminants, causing varying degrees of diarrhea, mild to severe body weight loss, emaciation and milk-dropping. Infected animals usually spill MAP in feces and milk for a long time before the onset of signs and introduce the pathogen to susceptible hosts through the fecal-oral route. This manifestation is in concordance with (32,65-67). Two obtained local sequences (PP976335.1 and PP976335.1) obtained from cow's fecal samples in the present work of the IS900 gene of MAP were deposited in GenBank for the first time in Mosul city, Iraq, and the analysis of the phylogenetic tree using the MEGA11 software to the obtainable database in GenBank, results indicate that it was potential to display that the study sequence was neatly attached with those sequence of Saudi Arabia (MN928512.1) (100% identity), and with Spain (FJ775181.1), Germany (CP053068.1), South Korea (CP033909.1) were (99.85% identity). The phylogenetic characteristics of this study sequences seem to have an evolutionary link with the other *M. avium subsp paratuberculosis* sequences in the NCBI GenBank of different countries such as Saudi Arabia (32), Spain (28), Germany (68,69), South Korea (67), and USA (70), with high similarity (99.85%-100%) after 1000 replications using MEGA11 software (30). These data propose that the MAP is circulating among cows and their environment, which may presume the introduction of MAP infection in imported dairy cows. This probability is harmonious with the literature (71-73).

Conclusion

Data from the current investigation indicate that the MAP disease is circulating among cows in the study area; care must be taken to monitor and eliminate this infection. The present study also confirms the possibility of genotyping to recognize the origin of infection and highlight the movement, either through the export and/ or import of infected animals (small and large ruminants) between the regions.

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Conflict of interest

Team authors state no possible conflicts of interest in this work.

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الانتشار والتحليل الوراثي لجراثيم نظير السل في الأبقار في مدينة الموصل، العراق

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الخلاصة

جراثيم نظير السل، هي عدوى منتشرة عالميًا تسبب التهاب الأمعاء المزمن، وقلة إنتاج الحليب، والهزال ودرجات مختلفة من الإسهال، بالإضافة إلى مخاوفها على الصحة العامة. يهدف هذا العمل إلى دراسة الانتشار الجزيئي لجراثيم نظير السل في الأبقار إلى جانب التحليل الوراثي لهذه الجراثيم في مدينة الموصل، العراق. تم جمع ١٧٦ عينة براز من أبقار سليمة وأبقار تعاني من الإسهال والهزال من مختلف الأعمار والمنشأ. أشار الكشف عن الجين IS900 أن ٥,٦% (١٠/ ١٧٦) من الأبقار المفحوصة تم تأكيد إصابتها بجراثيم نظير السل. لا يوجد اختلاف معنوي في نسبة الانتشار بين منشئ الأبقار، مع تسجيل زيادة معنوية نسبة انتشار جراثيم نظير السل في الأبقار التي أعمار أكبر من ٥ سنوات وفي الأبقار التي أظهرت أعراض سريرية. فضلا عن، إيداع تسلسلين من جراثيم نظير السل في بنك الجينات PP976335.1 وPP976335.1، وأظهر التحليل الوراثي للعترات المسجلة في الدراسة تماثل ١٠٠% مع عترات جراثيم نظير السل في المملكة العربية السعودية برقم تسلسلي MN928512.1 وتماثل ٩٩,٨٥% مع عترات إسبانيا وألمانيا وكوريا الجنوبية برقم تسلسلي FJ775181.1 و CP053068.1 وCP033909.1 على التوالي. بشكل عام، أظهرت هذه النتائج أنه يمكن استخدام الجين IS900 في التقنيات الجزيئية للتعرف على جراثيم نظير السل في الأبقار. من أجل مراقبة وعلاج والتعامل مع مرض نظير السل في الأبقار في الموصل، العراق، وكذلك هناك حاجة إلى مزيد من البحوث الوبائية والعزل.