*First report of paratuberculosis (Johne's disease) in livestock farms of river buffaloes (*Bubalus Bubalis*) in Nineveh, Iraq*

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River buffalo, *Bubalus bubalis*, *Mycobacterium avium* subsp. *paratuberculosis* (MAP), Paratuberculosis.

Summary
The present study was designed to investigate *Mycobacterium avium* subsp. *paratuberculosis* (MAP) in dairy buffalo herds from six different geographical areas in Nineveh, Iraq. A total of 87 individual faecal samples from river buffaloes, representing 12 dairy herds, were investigated for detection of MAP using cultural, Ziehl-Neelsen and MAP-specific PCR-based methods. Overall, MAP was detected at a higher frequency at herd-level (4/12; 33%) compared to the total individual faecal samples (14/87; 16%) with a cell density ranging from 10^1 to 10^3 CFU g^{-1}. A significantly ($p < 0.05$) higher frequency (9/17; 53%) of MAP was observed in faecal samples collected from clinically diseased as compared to healthy (5/70; 7%) buffaloes selected for the study. However, no statistically significant difference ($p \geq 0.05$) was observed in the frequency of MAP occurrence between clinical (9; 64%) and apparently healthy (5; 36%) cases. This report, which is the first MAP study based on data from Iraqi dairy buffalo herds suggests that MAP transmission is a significant health risk for grazing livestock. In conclusion, this study would help farm owners and regulatory authorities to realise the importance of developing and applying best farm management practices in order to prevent transmission of MAP to healthy animals and the environment. In addition, effective diagnostic tests should be taken into account when carrying out the screening tests.

Introduction

Buffaloes in Iraq are generally divided into two groups: the marshland group in the south and the river group located principally in the north (Jaayid and Dragh 2014). There are approximately 285,000 buffalo heads in Iraq, where about 46% of the population is in the south, 21% in the central region, 23% in the central Euphrates and 11% inhabit the north region (Al-Saedy and Al-Fartosi 2013, FAO 2016). Currently, about 5% of northern herds are located in the Nineveh Governorate distributed over six different geographical areas near the Tigris River (FAO 2016).

Mycobacterium avium subsp. *paratuberculosis* (MAP) is the causative agent of paratuberculosis or Johne's disease (JD). The disease is globally widespread. It may cause significant economic losses due to propagation of chronic granulomatous enteric disease of domestic livestock where clinical signs appear almost after two years of age (Radia *et al.* 2013, Ur-Rehman *et al.* 2018). In addition, MAP is suspected to be the cause of Crohn's disease (CD) in humans. However, there is still considerable debate concerning the role of MAP as a causative agent for CD in humans (Over *et al.* 2011). Paratuberculosis has been reported in different dairy buffalo herds

throughout Asian, African, Latin American and European countries (Lillini *et al.* 1999, Desio *et al.* 2013, Audarya *et al.* 2016, Shohanda *et al.* 2016, Ur-Rehman *et al.* 2018, Correa-Valencia *et al.* 2018, Rocha *et al.* 2018). For example, in Italy, the first report of serological-based detection of MAP from water buffaloes was published in 1999 (Lillini *et al.* 1999). A more recent study by Audarya and colleagues (Audarya *et al.* 2016) estimated the bio-incidence of MAP in native populations of buffaloes at farm- and herd-level in central and north India. In Punjab, Pakistan, Ur-Rehman and colleagues (Ur-Rehman *et al.* 2018) studied the prevalence of paratuberculosis, based on tuberculin and Ziehl-Neelsen tests, in four public buffalo farms. Similarly, MAP was also recovered from individual or pooled buffalo faecal or tissue samples in Colombia and Brazil where low (5%) to high (ranging from 19 to 22%) prevalence of MAP were reported (Correa-Valencia *et al.* 2018, Rocha *et al.* 2018).

To our knowledge, no data, concerning MAP detection at either animal- or herd-level among dairy buffaloes, are currently available in Iraq. Therefore, the aim of the present study was to isolate and identify MAP from faecal samples of 87 lactating buffaloes collected randomly from 12 buffalo herds in six different geographical areas in Nineveh, Iraq, by using conventional culture-based MAP isolation assay.

Materials and methods

Animal observations and sample collection

Based on the dairy buffalo population, all six geographical areas (A-F) located in Nineveh were included in this study (Figure 1). During the entire study, the veterinarians recorded the health status of each animal including case history of animals in the herd and any symptoms related to the paratuberculosis and response to antibiotic treatment. Some of the observed symptoms recorded in interviews conducted with farm owners were similar to paratuberculosis. The farm owners responded to the clinical data questionnaire, describing symptoms such as emaciation, profuse diarrhea, decrease in milk production and early culling of animals, no response to antibiotic treatment as well as recurrent pregnancy and death.

A total of 87 lactating buffaloes, from 12 dairy herds, were selected from a total number of 451 animals based on their clinically diseased and apparently healthy (subclinical phase) status (Table I). Individual faecal samples were collected from January 2013 to April 2014 from a total of 19% of buffaloes in the selected studied herds. Individual faecal samples (ca. 100 g) were collected from dairy buffaloes (aged up to two year) using sterile disposable rectal examination gloves. Faecal samples were collected randomly from apparently healthy animals and from all other animals showing clinical signs of paratuberculosis. The faecal samples were kept at 4 °C in a cooler during transport and before processing. The samples were analysed within 48 h of their collection.

Cultural method

Individual faecal sample was culturally investigated for recovery and isolation of MAP as described by Heuvelink and colleagues (Heuvelink *et al.* 2017). In brief, each sample (ca. 100 g) was homogenized, and three grams were suspended in 30 ml 0.75% hexadecylpyridinium chloride (HPC) (Sigma-Aldrich, Deisenhofen, Germany) and shaken at 200 rpm for 30 min. The mixture was kept for 2 min at room temperature. The supernatant was transferred in a

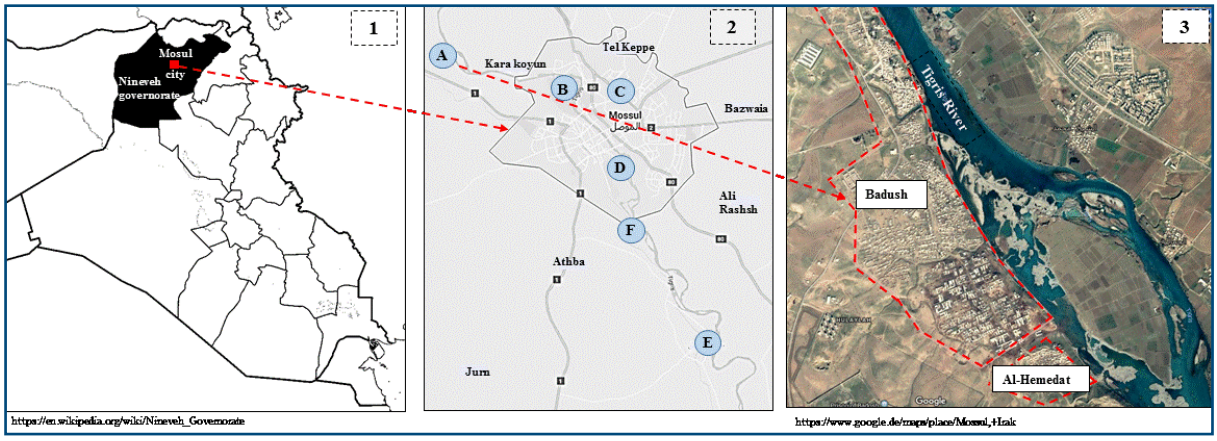

Figure 1. 1. *Map of Iraq, location of Nineveh Governorate and provincial capital Mosul city;* **2.** *Location of Mosul city and six geographical areas selected in the present study: A = Badush/Al-Hemedat; B = Haawee Kaniesah; C = Alrahmania; D = Danadan; E = Hammam al alil/ Kneitrah village; F = Albu Sayf;* **3.** *Location of Badush township and Al-Hemedat village, the largest area of buffalo population on the banks of the Tigris River.*

Table I. *Detection and prevalence of* Mycobacterium avium *subsp.* paratuberculosis *(MAP) based on geographical locations and herds.*

Herd code	No. of buffalo tested/ total no. of buffalo in herd	Geographical location	Number (n) of buffalo selected for sampling and clinical data	No. (%) of MAP positive
BU01/13	11/80*	A	(1), 3 years old, aborted, no clinical symptoms	0
			(2), 3-4 years old, continuous diarrohea, emaciation	
			(8), clinically healthy (no clinical symptoms of paratuberculosis)	
BU02/13	7/49	B	(1), 4 years old, suffering from diarrhea, recurrent abortion, no response to the therapy	0
			(1), 3 years old, continuous diarrohea, soft feces	
			(5), clinically healthy	
BU03/13	19/40*	A	(1), 6 years old, watery shooting diarrhea, emaciation, decreased milk production	0
			(18), clinically healthy	
BU04/13	2/13	A	(1), 5 years old, continuous diarrhea, emaciation, decreased milk production, no response to the therapy	1§ (8)
			(1), clinically healthy	1† (8)
BU05/13	7/23	B	(3), 4-5 years old, long-tern continuous diarrhea, decreased milk production	3§ (13)
			(4), clinically healthy	2† (9)
BU06/13	8/35	B	(8), clinically healthy	0
BU07/13	6/41	A	(2), 4-5 years old, continuous diarrhea, no response to the therapy	2§ (5)
			(4), clinically healthy	1† (2)
BU08/13	4/16	C	(4), clinically healthy	0
BU09/13	10/82*	D	(10), clinically healthy	0
BU10/14	8/40	D	(3), 3-4 years old, long-term continuous diarrhea, decreased milk production	3§ (8)
			(5), clinically healthy	1† (3)
BU11/14	4/19	E	(1), 2 years old, intermittent diarrhea, soft faecal consistency	0
			(3), 4-5 year old, clinically healthy	
BU12/14	1/13	F	(1), 4 years old, continuous diarrhea, emaciation	0

*Samples tested using individual and composite culture-based methods; A = Badush/Al-Hemedat; B = Haawee Kaniesah; C = Alrahmania; D = Danadan; E = Hammam al alil/ Kneitrah village; F = Albu Sayf; §MAP positive samples from buffalo showed clinical symptoms of paratuberculosis; †MAP positive samples from buffalo with no clinical symptoms (subclinical paratuberculosis)

new sterile tube and incubated at room temperature for 18-20 h followed by centrifugation at 2,500 × g for 15 min. The supernatant was decanted, and pellet was resuspended in sterile distilled water (total volume ca. 800 µl) where 0.2 ml suspension was inoculated on each of four tubes of modified Löwenstein-Jensen medium containing mycobactin J (Centraal Veterinair Instituut van Wageningen UR, Lelystad, Netherlands). The tubes were incubated at 37 °C and evaluated for growth every 4-week intervals for 16 weeks. In parallel, a MAP negative faecal sample was artificially spiked with MAP ATCC 19698 reference strain using a known (10^1 to 10^4 CFU) number of cells and used as a positive control. Based on colony morphology (1-2 mm small and whitish) similar to control mycobacterial colonies and Ziehl-Neelsen (ZN) (Merck, Darmstadt, Germany) staining reaction, positive colonies of acid fast, rod-shaped bacilli were further subcultured on fresh tubes of modified Löwenstein-Jensen medium, incubated at 37 °C for 4-12 weeks, and confirmed using MAP-specific PCR assay.

PCR method

For identifying MAP isolates, DNA was extracted from purified colonies of MAP and further confirmed using MAP-specific PCR primers and protocol targeting the IS900 gene in accordance with the method described by Heuvelink and colleagues (Heuvelink *et al.* 2017).

Data analysis

The Chi-square Contingency and Fisher's Exact t-test were applied to analyse data to test for significant differences in the detection and prevalence of MAP across sampling locations and herds as well as between clinical and healthy animals. $P < 0.05$ value was considered as significant.

Results

The faecal samples collected randomly from lactating buffaloes were initially divided into two groups: 1) apparently healthy (subclinical phase) buffaloes without symptoms of paratuberculosis; and 2) buffaloes with clinical like paratuberculosis symptoms including continuous or intermittent diarrhoea with or without emaciation and dehydration, weight loss, decreased milk production,

variable faecal consistency ranging from normal to soft and watery, fever and abortion. Some buffaloes suffered from infertility. The data of clinical and apparently healthy (subclinical phase) buffaloes were recorded during faecal sample collection by veterinarians and are summarised in Table I.

The results revealed that of the 87 total faecal samples, 14 (16%) were culturally MAP positive. These positive samples were from four (33%) different herds located in three geographical study locations (areas A, B and D) (Figure 1, Table I). However, there was no significant ($p > 0.05$) difference found in the frequency of MAP occurrence amongst the three different geographical locations and herds. Of the total 14 MAP positive samples, nine (64%) were from animals showing typical symptoms of paratuberculosis. Therefore, they were considered as clinical paratuberculosis cases. The remaining five (36%) MAP positive samples collected from apparently healthy were instead classified as subclinical paratuberculosis animals. However, no statistically significant difference ($p \geq 0.05$) was observed in the frequency of MAP occurrence between clinical (9; 64%) and apparently healthy (5; 36%) cases. A significantly ($p < 0.05$) higher frequency (9/17; 53%) of MAP was observed in faecal samples collected from clinically diseased as compared to apparently healthy (5/70; 7%) buffaloes selected for the study. Similarly, a statistically significant association between herds and clinically positive as well as herds and apparently healthy (subclinical phase) buffaloes was observed. The distribution of MAP across sampling locations, among herds and between clinically diseased and healthy cases is summarised in Table II.

The typical MAP colonies grown on four tubes of modified Löwenstein-Jensen agar of each sample were enumerated and the MAP cells calculated as colony-forming units (CFU) per g of faeces as described by Heuvelink and colleagues (Heuvelink *et al.* 2017). The MAP colonies showed irregular distribution of cell number in four tubes (CFU/tube) where no growth was observed in some tubes although the sample was MAP positive. The cell density was typically low (+) (1-10 CFU g^{-1}) in five samples that were from apparently healthy low shedder buffaloes showing no clinical symptoms (subclinical paratuberculosis) compared to the moderate cell density (++) (10-100 CFU g^{-1}) of eight samples showing clinical symptoms. Only one sample, showing clinical symptoms of paratuberculosis, had high cell density (+++) (≥ 100 CFU g^{-1}).

Discussion

To our knowledge, this is the first study which was performed to detect MAP in dairy buffalo herds distributed in six different geographical locations of the Nineveh, Iraq. The first study of paratuberculosis in Iraq was reported in 1967 among Awassi sheep (Rollinson and Bashir 1967) and first isolation of MAP from dairy cattle was in 2003 (Abdulrasool 2003). However, other sero-prevalence studies showed MAP in 10% of local Iraqi goat breeds, 1% of dairy cattle and 8% of sheep (Al-Kass 2009, Ahmed 2010, Al-Aalim 2010, Abdulrasool and Mahdi 2016, Al-Farwachi *et al.* 2018). India, which has approximately 61% of the total world

Table II. *Detection and distribution of* Mycobacterium avium *subsp.* paratuberculosis *(MAP) investigated in 12 Iraqi dairy buffalo herds.*

Herd Code	Geographical location	Total number of buffalo in herd	No. of buffalo selected for sampling	No. of samples from buffalo with clinical symptoms of paratuberculosis	No. of samples from healthy buffalo	No. of MAP positive (diseased vs. healthy)
BU01/13	A	80*	11	3	8	0
BU02/13	B	49	7	2	5	0
BU03/13	A	40*	19	1	18	0
BU04/13	A	13	2	1	1	2 (1§; 1†)
BU05/13	B	23	7	3	4	5 (3§; 2†)
BU06/13	B	35	8	0	8	0
BU07/13	A	41	6	2	4	3 (2§; 1†)
BU08/13	C	16	4	0	4	0
BU09/13	D	82*	10	0	10	0
BU10/14	D	40	8	3	5	4 (3§; 1†)
BU11/14	E	19	4	1	3	0
BU12/14	F	13	1	1	0	0
Total	6	451	87	17	70	14 (9§; 5†)

* Samples tested using individual and composite culture-based methods; § MAP positive samples from buffalo showed clinical symptoms of paratuberculosis;
† MAP positive samples from healthy buffalo showed no clinical symptoms (subclinical paratuberculosis).

buffalo population contributed significantly to the surveillance studies of MAP in dairy buffaloes, reporting a variable rate of frequency from 28.6 to 40.3% in different regions of India using serological screening methods (Singh *et al.* 2008, Audarya *et al.* 2016). Similarly, following the first study of Lillini and colleagues (Lillini *et al.* 1991), other serological-based studies estimated 74% rate of prevalence of Map in water buffaloes in Italian herds (Desio *et al.* 2013, Gamberale *et al.* 2014). Desio and colleagues (Desio *et al.* 2013) studied MAP in Mediterranean buffaloes showing clinical symptoms of paratuberculosis using cultural and serological assays. They suggested that paratuberculosis often occurs in buffaloes with mild symptoms, thus making difficult to clinically diagnose the disease in buffaloes. The accuracy and consistency of MAP studies appears to be affected by various factors such as the condition of the animal herds, stages of infection, herd size, type of sample, method of sampling and the investigation technique (Tavornpanich *et al.* 2004, Crossley *et al.* 2005). In general, low shedding of MAP cells (< 10 CFU g^{-1}) in faecal samples (individual or pooled samples) is mostly undetectable in the early and preclinical stages of the disease (Tavornpanich *et al.* 2004, McKenna *et al.* 2018). The results of the aforementioned study indicate that MAP was not detectable in the individual faecal samples from clinically diseased buffaloes due to possible low cell density (< 10 CFU g^{-1}) (Tavornpanich *et al.* 2004). Nevertheless, in the present study, the results from investigated individual faecal samples revealed the first reporting of detection of MAP in dairy buffaloes at a frequency of 16% at individual- and 33% at herd-level. MAP was isolated from buffaloes showing typical symptoms of the disease as well as from apparently healthy animals. The concentration of MAP cells in faecal samples (CFU g^{-1}) were variable depending on shedding of bacteria in the faeces and the stage of disease. Based on clinical symptoms and MAP density in the faeces, paratuberculosis infection in cattle can be categorised into three stages: i) silent: absence of symptoms and not shedding; ii) subclinical: absence of symptoms but shedding; iii) clinical and advanced clinical disease: abundance of symptoms and shedding (Crossley *et al.* 2005). These stages could have close resemblance in buffaloes, Sivakumer and colleagues (Sivakumer *et al.* 2006) reported that gross and histological lesions in the small intestine of buffaloes mostly showed the subclinical infection stage. Similar to our study results, Whitlock and colleagues (Whitlock *et al.* 2000) investigated cell density in ten dairy herd faecal samples using four Herrold's egg yolk medium (HEYM) tube assay where 71% samples, classified as low shedders, had < 10 CFU per tube, followed by 10-50 CFU per tube in 10% of samples classified as mid-shedders, and > 50 CFU per tube in 19% samples classified as high shedders, respectively. Similarly, Crossley and colleagues (Crossley *et al.* 2005) assessed cell density in Holstein cow faecal samples obtained from 93 dairy herds where the majority of dairy cows were observed as low shedders (< 10 CFU per tube, 51.3%) compared to moderate (10-50 CFU per tube, 17.9%) and high (> 50 CFU per tube, 30.8%) shedders. They reported that reduction of HYEM tubes to three and two tubes might reduce the sensitivity of fecal culture for MAP by approximately 6% to 12%, respectively. The study results indicate that there are possible causes of difference due to lack of application of control and multi-tube culture-based assay which may impact on isolation and detection sensitivity. Based on previous and present study results, it is suggested that the use of the four-tube HEYM or modified Löwenstein-Jensen culture strategy can substantially enhance the recovery and sensitivity of MAP detection particularly in apparently healthy low shedder cases when MAP cells are present at a low concentration.

Conclusions

The present study provides first individual- and herd-level based information on the prevalence of MAP in Iraqi dairy buffaloes in Nineveh Governorate. The study shows that MAP was prevalent at a low frequency in both apparently diseased (clinically positive) and healthy (subclinical phase) buffaloes across herds located at various geographical locations. Regular screening of farm animals should be carried out but it should consider numerous factors including spatio-temporal, animal population and health status. Finally, isolation (cultural-based) and identification (PCR-based) techniques that can substantially help to estimate the prevalence of MAP in livestock should be taken into account when carrying out the screening tests.

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Ethics statement

Faecal samples from animals were collected under supervision of veterinarians, and no experiment was performed on the animals.

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