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# First molecular investigation John's disease (paratuberculosis) in water buffalo in Iraq

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***Abstract*---**This study was conducted to exam 100 fecal samples and 50 tissue samples from (100 ) buffaloes above one years at different villages and townships in Al-Najaf governorate from (January 2020 to January 2021) to detect of specific insertion Sequence IS900 in Mycobacterium paratuberculosis (John's disease) nested PCR. Out of 150 (100 fecal and 50 tissue) samples tested by Nested PCR, 14(9.3%) were positive for IS900 specific gen, Significant difference at P<0.05 in molecular result were observed between tissues and fecal samples. The molecular results revealed that 6(6%) out of 100 fecal sample were positive, while 8(16%) of tissue sample were positive for IS900 specific gen. There was no significant difference at P<0.05 in relationship between age gender and location with paratuberculosis prevalence.

***Keywords*---**paratuberculosis, buffalo, nPCR, John's disease, IS900, Iraq.

## Introduction

John's disease has been found in cattle, sheep, goats, bison, camelids and cervids, bighorn sheep, Rocky Mountain goats, deer, and tule elk, among other domestic and wild ruminants. (Biswal *et al.*, 2020; Cunha *et al.*, 2020). Paratuberculosis or John's disease is infectious chronic granulomatous enteritis of bovines caused by bacterium specie known as *Mycobacterium avium subsp. Paratuberculosis*. (Dziedzinska and Slana .2017). *Mycobacterium avium subsp. paratuberculosis* (MAP) as a member of the *M. avium complex* grows extremely slowly, taking long incubation period about 16 weeks to produce visible colonies on culture media (Rathnaiah *et al.*, 2017).

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It is a chronic progressive bacterial disease of ruminants affecting mainly intestinal tract and regional lymph nodes (Dziedzinska and Slana .2017). Economic losses are associated with reduced milk yield, lower reproductive efficiency, premature culling and decreased cull cow values (Rasmussen *et al.*, 2021). Clinical symptoms of paratuberculosis include slowly progressive wasting and diarrhea, which are intermittent at first but become progressively more severe until they are constantly present and sever decreases milk production (Bates *et al.*, 2018; Rieger *et al.*, 2021). The prevalence of disease was reported by several studies in worldwide: In Iraq the disease prevalence in cattle were 54.54 % and 2.64 % (Abdulrasool ,2016), 10.32% (Al-Farwachi *et al.*2018). While disease prevalence in buffaloes were reported in Italy 13.3% (Lillini *et al.* 2002), 2% (Sezzi *et al.*, 2010) , 27% (Pesce *et al.* ,2014), 54.7%, Martucciello *et al.*,2021).

Several diagnostic methods used in diagnosis of diseases ;such as fecal smear, acid-fast stain, bacterial culture and polymerase chain reaction (PCR) tests are used as direct tests (Al-Rubiaii *et al.*,Rose *et al.*, 2018;Cheng *et al.*, 2020). Nested Polymerase chain reaction assay (Nested PCR) was performed for diagnostic and confirmative detection of *M.paratuberculosis* by specific insertion of targeting IS900 gen ,This assay was done according to method described by (Leite *et al.*, 2013; Sharifzadeh *et al,*2010). These studies reported incidence of *M. paratuberculosis* disease in cattle ,sheep Goat and camel in many countries, But the incidence of *mycobacterium paratuberculosis* disease in buffalo is currently unknown in Iraq.

**Martials and Methods**

**Animals**

One hundred (100) fecal samples were collected from clinically affected and healthy animals, which divided to (50) from farms at different areas of Al- Najaf governorate and (50) from slaughter house animals in Al Najaf governorate. Fecal samples were taken from rectum (using lubricated gloves)with finger scraping, the specimens were put in sterile cub for further processing and direct smear and kept in -20c for DNA extraction (Correa -Valencia *et al.*2017).

**DNA extraction**

The DNA extracted from fecal sample by using fecal Quick-DNA™ Fecal/ Miniprep The DNA extracted from tissue by using G-spinTM Total DNA Extraction Kit / iNtRON Biotechnology (Korea) .The extracted fecal and tissue DNA were checked by using Nanodrop spectrophotometer (THERMO. USA), that check and measurement the purity through reading the absorbance in at (260 /280 nm) according Alejandro *et al.* (2020) nPCR amplification was performed with two sets of primers design in this study : outer oligonucleotide primers were IS900 1F :5´-GACGTCGGGTATGGCTTTCA and IS900-1R: AGCTCGACTGCGATGTCATC-3 (576bp). The inner oligonucleotide primers were IS900 2F:5´-AATGACGGTTACGGAGGTGG ISO900 and 2 R CGGGAATATAAAGCAGCCGC-3 (278bp). The target sequence was amplified in a 50 µl reaction volume containing 100 ng of genomic DNA, 0.2 mM dNTPs, 1X Taq buffer2 mM MgCl2, 100 ng of each primer and 1 unit of Taq DNA polymerase ((Bioneer. Korea).

**Statistical analysis**

Data analysis were performed according to ready program (SPSS) Copyright (2009). The Prevalence was determined with SPSS-version 10.1 wherein differences were calculated by Pearson's Chi-square test with $P < 0.05$.

**Results**

**Molecular results**

The quality of the extracted DNA was evaluated using a Nanodrop spectrophotometer, which reads the absorbance at (260/280 nm) to assess and evaluate the purity. A total of 100 fecal samples and 50 tissues sample of were tested for MAP using a nested PCR assay which enabled the detection of IS900 gene of the *M. avium* subsp. *paratuberculosis* and subsequent agarose gel analysis of the amplified products showed a single band of 278 bp. Figure (4-11). Out of 150 (100 fecal and 50 tissue) samples tested by Nested PCR, 14(9.3%) were positive for IS900 specific gen, Significant difference at $P<0.05$ in molecular result were observed between tissues and fecal samples. The molecular results revealed that 6(6%) out of 100 fecal sample were positive, while 8(16%) of tissue sample were positive for IS900 specific gen as in Table (4-9).

Table 4-9
Result of IS900 specific gen by Nested PCR in fecal and tissue samples

| Samples | Total no. | PCR + | PCR - |
|---|---|---|---|
| Fecal sample | 100 | 6 (6%) | 94 (94%) |
| Tissue sample | 50 | 8 (16%) | 42 (84%) |
| Total | 150 | 14 (9.3%) | 136 (90%) |
| $X^2$ | 3.93* | | |
| P value | 0.047 | | |

*Significant difference at $P<0.05$

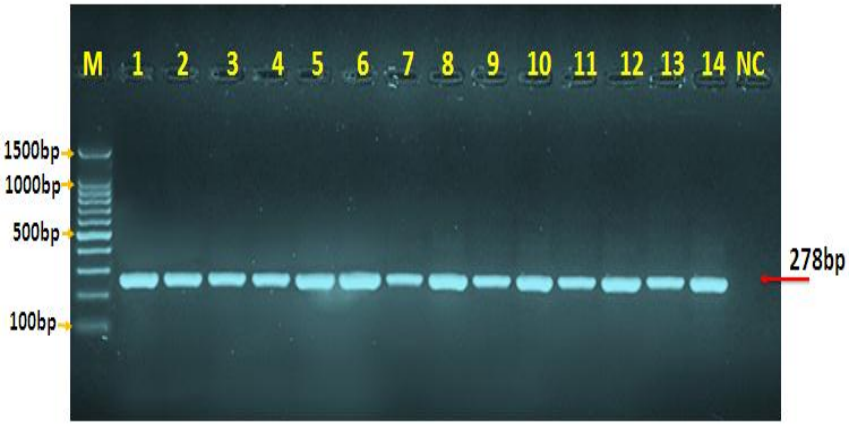


Figure 7. Agarose gel electrophoresis image that showed the Nested PCR product analysis of insertion sequence IS900 in *Mycobacterium avium* subsp. *paratuberculosis* from feces and intestinal tissue buffalo samples. Where M: marker (1500-100bp) Lanes (4-9) only Positive amplification of *Mycobacterium avium* subsp. *paratuberculosis* samples at (278bp) nested PCR product.

**Prevalence of MAP according to age groups**

The MAP was not detected in animals with age of 1-2 years by nPCR; the postive samples in animals with age >2-4 with by PCR were 5(16.1%). The animals with age >4-5 years were recorded high perecent about 4 (18.1%). Finally the animals with age mor than 5 years were apeared postive result as 2 (15.3%). There was no significant difference at P<0.05 in relationship between age and infection with *M.paratuberculosis* as in table (4-10).

Table 4-10
Distributions of the positive results of PCR according to age groups

| Age of animals (year) | Animals no. | PCR |
|---|---|---|
| 1- 2 | 34 | 0 (0%) |
| >2-4 | 31 | 5 (16.1%) |
| >4-5 | 22 | 4 (18.1%) |
| >5 | 13 | 2 (15.3.0%) |
| Total | 100 | 11 (11%) |
| $X^2$ | 6.45* | |
| P value | 0.092 | |

*No significant difference at P<0.05

**Prevalence of MAP according to gender of animal**

Out of 90 female animals 10 (11%) were infected with paratberculosis and Out of 10 male 1(10%) animal was infected with paratberculosis with no significant difference at P<0.05 as showed in Table (4-11).

Table 4-11
Prevalence of Paratuberculosis according to gender of animals

| Animals sex | Number | Percent of infection |
|---|---|---|
| Male | 10 | 1 (10.0%) |
| Female | 90 | 10 (11.1%) |
| Total | 100 | 11 (11%) |
| $X^2$ | | 0* |
| P value | | 1 |

*No significant difference at P<0.05

**Prevalence of MAP according the region**

The slaughter house animals reported high percent of MAP about 8(16%). While, the other regions were 2 (11.7%) Am Kashem, Al Najaf 1 (9.0%), and Map was not detected in Al abasia, Al-Azamia, Al-Mahajear and Mashkab with no significant difference at P<0.05 in geographical distribution of infection as mentioned in Table (4-12).

Table 4-12
Prevalence of MAP according regions

| Region | Samples No. | PCR | % |
|---|---|---|---|
| Am Kashem | 17 | 2 | 11.7 |
| Mashkab | 7 | 0 | 0 |
| Al-Azamia | 5 | 0 | 0 |
| Najaf | 11 | 1 | 9.0 |
| Al abasia | 7 | 0 | 0 |
| Al Mahajear | 3 | 0 | 0 |
| slaughter house animals | 50 | 8 | 16 |
| Total | 100 | 11 | 11(100) |
| $X^2$ | 4.04* | | |
| P value | 0.670 | | |

*No significant difference at P<0.05

**Discussion**

The molecular results revealed that 6(6%) out of 100 fecal sample were positive and out of 50 tissue samples of slaughtered buffaloes ,8(16%) were positive for IS900 specific gen by Nested PCR, Our finding was similar to that of Pedro *et al.*(2018) , who investigation 1 (9.09%) buffaloes fecal samples were positive by PCR in Brazil. The results present were lower than many studies as Lillini *et al.* (2002) in Italy , Rose *et al.*(2018 ) in Philippines and Abdellrazeq *et al.*(2014) in Egyptian ,the positive result of IS900 specific gen in fecal samples were 13.3% , (14.3%) and (52.94%) respectively. Moreover, fecal samples continue various PCR inhibitors led to lower the sensitivity of IS900-targeted PCR when few amounts of target DNA is used for the assay (Thornton and Passen,2004).

This high prevalence is attributed to large buffalo rearing, which means there is no sanitary or zootechnical management or, at the very least, animal segregation by age, which encourages MAP transmission in young animals because infection occurs in the first days of life (Radostits *et al.*,2017). In the present investigation, 8(16%) tissue sample of slaughtered buffaloes were positive for IS900 specific gen ,that resemble with previously, molecular studies by De Moraes Pereira *et al.* (2020) ,13% were detected IS900 specific gen in buffaloes tissue in Brazil and Farhan *et al.*(2009) in Pakistan have reported that , 12.8% intestinal and 12.4% MLN were positive by PCR for MAP in buffaloes. Our molecular results of tissue samples were contrast with previous reports , that reported high percent of positive IS900 specific gen in tissue buffaloes ,(70%) and (30%) in India and in Brazil respictively (Sivakumar *et al.*,2005; Pedro Paulo *et al.*,2018). The infection rate might be appears lower than actual prevalence because iceberg Phenomenon in affected herd and these rate also effected by the size of the and the diagnostic methods applied (Magombedze *et al.*, 2013; OIE, 2018). The incidence probably elevated when a certain population of animals was in poor health or had inadequate nutritional management in the herd (Sivakumar et al.2006).

**Prevalence of MAP according to age groups**

The prevalence of MAP infection according the age was variety ; the MAP no detected in animals with age of 1-2 years by PCR , the postive samples in animals with age 3-4 with by PCR were 4 (12.9%) ,the animals with age 4-5 years were recorded high perecent about 7 (31.8). Finally the animals with age mor than 5 years were apeared postive result as 3 (23.0%). In this study , We noticed the prevalence of Map increase at the age of 3 years and above, This result corresponds with previous studies , Shabana and Aljohani,(2020),who noticed the aged 3–5 years had the highest frequency ,also Mahdi *et al.*,2021 observed the average age of infected animals by MAP was similarly about 4 years. The age of infection mentioned by Radostits *et al.*(2017) who that most infection prevalence of disease occur between 3-5 years of age because long incubation period of MAP baciili may be reaches 2-6 years.

While our result of disagreement with Tuberquia-López *et al.* (2021) reported most MAP infection in average age of buffaloes was 4.7 years. While Rehman *et al.*,(2017) , showed that the animals above the age 5 were to have a greater prevalence. Statistically, no significant difference in the incidence rates according

to the age of the animal, although there is a variation in the rates of prevalence MAP based on the age in present study, but this finding resemble to Mahdi *et al.* (2021) showed that the MAP infection rate is not associated with gender, age and geographical location in Iran and Hussain *et al.,* (2018) reported that, there was no a significant relationship between age and MAP prevalence and age of buffalo in Pakistan.

Present result discrepancy with many reports mentioned that the age had been significantly related to infection by Cetinkaya *et al.* (1996), Woodbine *et al.* (2009), Weber *et al.* (2010), Fecteau *et al.* (2010), Attili *et al.* (2011) and Karimi *et al.* (2012). Furthermore, there is a strong association between age and MAP infection. (Nielsen and Ersbll, 2006). The clinical disease is most frequent among cattle 2-5 years old, although younger and older cattle (0-13 years old) can be affected (Nielsen and Toft, 2008). Clinical signs in most cases do not appear before 2 years of age and are most common in the 2 to 6 year-old age group (Windsor and Whittington 2010). This observation may be attributable to a variety of biases, including as selection, information, or measurement, confounders like husbandry and management, and the statistical approach used. Also might be due the long incubation period of disease and paratuberculosis consider a chronic disease therefore all age of animals will exposed to infection (Karthikeyan *et al.*,2019; Biswal *et al.*, 2020).

**Prevalence of MAP according to gender of animal**

The results of the prevalence of paratubeculosis based on sex of animals appeared, the female animals reported percent of prevalence of disease about 10 (11 %) whereas the male was reported about 1 (10.0%). These findings of this study was agreement with another study by Tuberquia-López *et al.*(2021) in dairy buffalo herd in Colombia . In a current study, we found that statistically no effect of gender on prevalence of MAP may be due to a very small male cohort (group) compared with females, although the prevalence of partuberculosis among female was higher than males. This result supported by McGregor *et al.*,(2015) and Shabana and Aljohani ( 2020) were mentioned no a significant relationship between at MAP prevalence and gender. The transmission of *Mycobacterium paratuberculosis* through feco-oral route is the most important route; in addition the shedding of pathogen through milk ,colostrum and semen from the diseased animals is also the source of infection of female ,male and newborn animal, therefore there no effect the gender on MAP infection prevalence . (Sweeney *et al.*, 2006; Gamberale *et al.*, 2019).

**Prevalence of MAP according the region**

The infection rates varied among different cities ranging from zero (0%) to (16%) in buffalo. The slaughter house animals was reported high percent of MAP about 8(16%),while the molecular result of the other regions was 2(11.7%) Am Kashem, Al Najaf 1 (9%) and zero for all Al abasia ,Al-Azamia ,Al-Mahajear and Mashkab. The prevalence of MAP at an abattoir in current study was (16%) ,which resemble to previous abattoir-based study in India reported a prevalence of MAP 12.4% in buffaloes (Khan *et al.*, 2010).In present study ,it is obvious that the slaughterhouse has a higher prevalence than the farm. It's

reasonable that farmers ship off their low-producing or untreatable animals, and those animals end up in abattoirs, where they're slaughtered, and therefore the slaughterhouse prevalence is greater.( Hussain *et al.,*2018). The present study showed , there was no statistically significant correlation between geographic location and MAP infection .these finding agreement with two studies In Pakistan also revealed that the prevalence of MAP infection in buffaloes is not related with location .( Rehman *et al.,* 2017; Hussain *et al.*, 2018) .We thought the animal management, such as herd size, health, nutrition, and stress, might explain the influence of geographical location on MAP infection rate.( Mahdi *et.al*,2020).

**Conflict of interest statement**

The authors declare that they have no competing interests.

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