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DIAGNOSTIC APPLICATION OF ELISA AND IS900 PCR USING BUFFY COAT AS A SOURCE SAMPLE FOR THE DETECTION OF *Mycobacterium avium* SUBSPECIES *Paratuberculosis* IN SUBCLINICAL CASES OF BOVINE PARATUBERCULOSIS.

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ABSTRACT

John's disease or paratuberculosis is a chronic mycobacterial infection that affects ruminants, adversely, leading to huge economic losses throughout the world. The estimation of sero-prevalence and molecular confirmation of this disease in the cattle population of south-Iraq were the objectives of this study. One of the diagnostic tools used to detect an antibody in plasma samples was the Enzyme Linked Immuno-Sorbent Assay, indirect-ELISA was used to diagnose and estimate the sero-prevalence of paratuberculosis in cattle. Out of 156 bovine plasma samples, 81 (51.9%) were positive and this MAP-seroprevalence is not significantly connected to age or breed of cows (P>0.05). In this study, a PCR-based detection of IS900, distinct insertion sequences of MAP from the buffy coat of seropositive cattle (n = 81) were used as a confirmative diagnosis. The positive PCR-based detection of IS900 was observed in animals having high S:P% ELISA values (n=29 :35.8%).

INTRODUCTION

Mycobacterium avium subsp. *paratuberculosis* (MAP) is etiological cause of Johne´s disease, a chronic inflammatory enteric disease affecting cattle, sheep, goats and other ruminants. Bovine JD has been recognized as a major disease causing high economic losses to cattle globally, India (1, 2), Mexico (3); Colombia (4); Pakistan (5,

6). A great individual variability of subclinical and clinical symptoms was detected. The infection is typically characterized by a long incubation period followed by chronic progressive diarrhea, decrease in milk production, edema, anemia, loss of weight leading to cachexia and finally to death (7,8). Initial intermittent and later persistent fecal excretion of MAP starts several months up to 2.5 years before the onset of clinical signs and leads to the contamination of both the infected animal and the environment. Following the previous way, the transmission of the infection mainly occurs following ingestion of MAP by offspring during the first month of life. Intrauterine transmission and direct excretion with milk are also described as additional routes of infection but their epidemiological importance is hardly to estimate (8, 9). Therefore, the essential precondition for eradication of paratuberculosis from herds is the identification and elimination of MAP-shedders. In control programs, ELISAs were widely used as screening assays for the detection of antibodies against MAP in blood or milk because of their advantages like easy performance, high capacity and low costs. Due to the late formation of antibodies, ELISAs are characterized by low sensitivity especially in animals without or with moderate shedding of MAP in their faeces (10, 11).

PCR-assays provide a rapid alternative and sensitive detection of MAP in clinical samples including blood. The insertion element 900 (IS900) is the mostly used target for identification and differentiation of MAP from other mycobacteria. The IS900 is 1.451 bp in length and is found in 15-20 copies in the MAP genome (12, 13, 14). The main goal of this study was paratuberculosis diagnosis through the objectives, estimation of sero-prevalence and molecular confirmation of this disease in the cattle population of south-Iraq.

MATERIALS AND METHODS

Animals and Samples

Apparently healthy cows (N = 156) from south of Iraq were selected for case and control study for autoimmune disease (John's disease). These cows aged from 2 to 14 years (two groups; < 7 years verses, ≥ 7 years). A minimum of 10 ml blood sample

was collected from the jugular vein of each animal into K2-EDTA-blood tubes by using 18 gauge needles. The K2-EDTA-blood tubes were transported to the laboratory cold within 24 hours.

Isolation of peripheral leukocytes (buffy coat)

The K2- EDTA-blood tubes was processed by centrifugation at 3000 × g for 10 minutes at room temperature. A volume of 1 mL of plasma was transferred to a new sterile tube and stored at −20°C for future testing for anti-MAP IgG, The leukocyte containing buffy coat layer was carefully transferred to a new sterile tube. Leukocytes were then mixed with two volumes of red blood cell lysis buffer (Roche Applied Sciences, IN, USA). The hemolyzed samples were then centrifuged at 2500 × g rpm for 5 minutes at room temperature. The supernatant was discarded and the leukocyte pellet was stored at −20°C for further use in PCR analysis (15).

DNA Extraction

DNA was extracted from isolated leukocytes by using Geneaid gSYNC™DNA Extraction (Korea) as per recommended protocols. The concentration of DNA was determined using (Nanoodrop Quawell USA) .

ELISA Methodology

The IDvet.310.rue ELISA (France) was performed according to the manufacturer's instructions. Briefly, plasma samples, including positive and negative controls, were diluted (1:12) in a Dilution r and incubated for 45 minutes at room temperature (RT). The previously diluted samples and controls (100 µl) were dispensed to each well of an ELISA plate pre-coated with antigen, mixed with gentle tapping on the sides and incubated for overnight at RT. The plates were hand-washed three times with wash buffer, and a peroxidase-labelled monoclonal anti-ruminant IgG conjugate (100 µl) diluted with dilution buffer (1:25) was added to each well and incubated for 30 min at RT. The plates were washed as above and 100 µl of substrate was added and incubated for 15 min in dark. The chromogenic reaction was stopped by adding 100 µl of stop solution, and the optical density (OD) values were read at 450 nm using a plate reader (Wellkang Ltd., London, UK). The ELISA results were interpreted as the signal of the test sample as a proportion of the positive control, corrected for the negative control (sample-to-positive; SP %), according to the formula: **SP% =**

[(OD450 nm of Test sera _ OD450 nm of Negative control) / (OD450 nm of Positive control _ OD450 nm of Negative control)] × 100. SP% cut point for test plasma ≥70% were considered positive, according to the manufacturer's instructions. Test sera with an SP% (60%< S/P% <70%)were considered as suspect and re-tested. The re-test values were used for analysis. Test sera with an SP% ≤60% were considered negative.

Detection of MAP DNA by PCR

M. avium subsp. *Paratuberculosis infection* further identified by the presence of the IS*900* elements in the peripheral leukocytes of 81 seropositive cows. PCR employing IS900 gene specific primers of *Mycobacterium avium subsp. parartuberculosis* (MAP) was used for diagnosis of Map DNA .The primers (MAP5: 5'-CTG GCTACC AAA CTC CCG A-3', BA6:5'-GAA CTC AGC GCC CAG GAT-3') (314 bp) were designed from the IS900 sequence of MAP (16). The solated DNA was amplified in 50 µl reaction mixture containing PCR buffer, mM MgCl2, dNTPs, Taq polymerase (Promega / USA), 1 µM of primers (MAP5 and MAP6) and 1 µl of purified genomic DNA solution. The PCR conditions consisted of initial denaturation at 94°C for 4 min, 40 cycles each of denaturation at 94°C for 1 min, annealing at 58°C for 1 min and synthesis at 72°C for 1 min, and final elongation at 72°C for 4 min. The PCR product was analyzed on 2% agarose gel.

Statistical analysis

The data obtained from the IS900 gene PCR and ELISA were analysed by Fisher's exact test (SPSS software version 11).

RESULTS

S/P percentage and status of paratuberculosis disease

Table 1 , display the concentration of anti- MAP antibodies in different categories of S/P ratios (Strong positive (SP), Positive (P), Low Positive (LP) , Negative (N) with respect to status of MAP infection in 'tested cattle plasma. The positive category appeared in higher percentage (33.3%) of tested plasma samples followed by Strong positive (18.6%) category**.**

Table1: S/P percentage and status of paratuberculosis disease according to ELISA results of 156 plasma samples

S/P (%)	Number	Percentage(%)	Status of paratuberculosis disease
≤ 60	75	48.1	Negative
≥60 to <70	52	33.3	Positive
≥ 70	29	18.6	Strong positive

Influence of cows age on ELISA

Table (2) display the influence of age on ELISA results of sero prevalence among the cows screened by indirect ELISA. The higher seropositive prevalence **(31.4**%) was observed in the age group ≤7 compared with age group > 7 years (**20.5**%). However the difference between the two tested age groups is considered to be not statistically significant between the two tested age groups (P=0.4195).

Table 2: Distribution of ELISA results according to cow's age

Results	≤ 7 year	Percentage Taken for 156	>7year	Percentage Taken for 156	Total	P value
ELISA Positive	49	31.5	32	20.5	81	0.4195
ELISA Negative	40	25.6	35	22.4	75	
Total	89	57.1	67	42.9	156	

Influence of cows breed on ELISA results

The seroprevalence of paratuberculosis in Indigenous breed (32 %) was higher than cross breed cattle ELISA prevalence (19.9 %), however the difference between the two tested breeds with respect to ELISA positivity is considered to be not statistically significant (P=0.2010; Table3).

Table3: Distribution of ELISA results according to cows breed

Results	Indigenous breed	Percentage Taken for 156	Cross breed	Percentage Taken for 156	Total	P value
ELISA Positive	50	32	31	19.9	81	0.2010
ELISA Negative	60	38.5	15	9.6	75	
Total	110	70.5	46	29.5	156	

Detection of MAP by IS900 PCR

IS900 PCR confirmed the presence of MAP in tested samples by using BA5: BA6 primers. The amplified product IS900 sequence (314 bp) were detected in 29 (**35.8**%) out of 81 cow peripheral leukocytes (buffy coat) samples (Table 4; Figure,1).

IS900 PCR detected 29 (35.8%) positive, cows of which 19 (**23.5**%) were at age group **≤ 7 year** and 10(**12.3**%) were at age group **> 7year** (Table 4; Figure-1.) There was no significant difference in the detection rate of MAP genome between the two age groups(P=**0.6361**)**.**

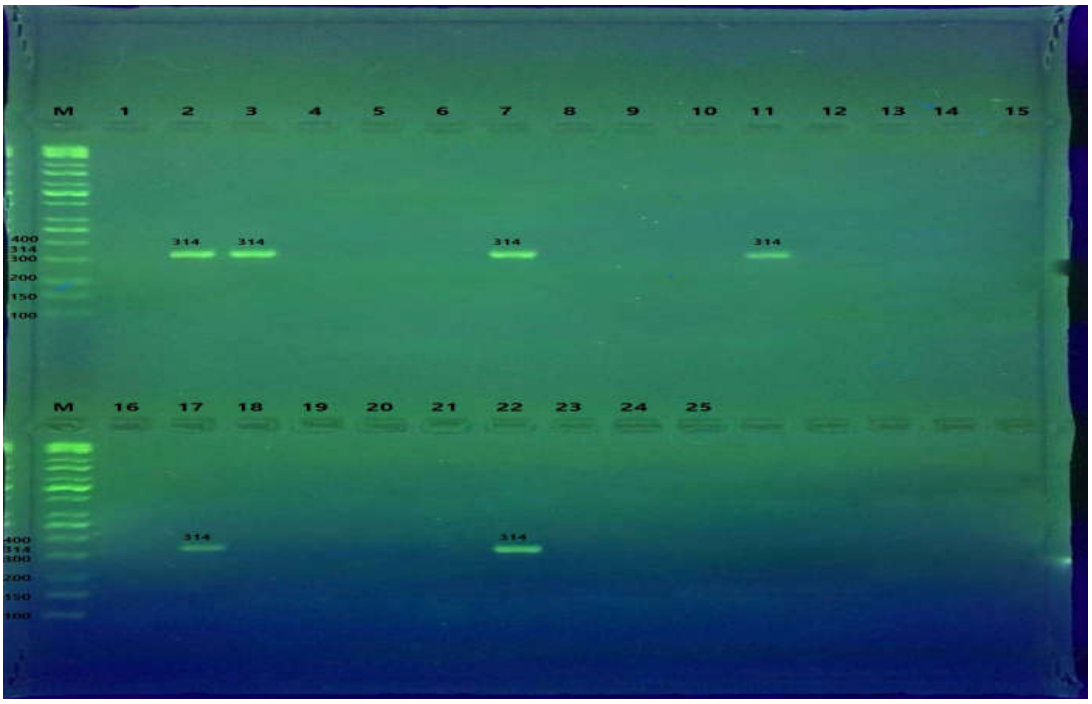

Figure 1: *Mycobacterium avium* subsp. *paratuberculosis* specific amplicons (314 bp) by PCR using IS*900* specific primers. Lane M: 100 bp DNA ladder, Lane 2, 3,7,11, 17, 22: tested DNA samples

In table (5) Indigenous breed showed higher percentage of IS900 PCR positivity(24.7%) compare to cross breed but this difference was not considered to be statistically significant (P>0.05).

Table4: Distribution of IS900PCR results according to seropositive cow's age

ELISA Positive	IS900 PCR positive	Percentage Taken for 81	IS900 PCR negative	Percentage Taken for 81	Total	P value
≤ 7 year	19	23.5	30	37	49	0.6361
>7year	10	12.3	22	27.2	32	
Total	29	35.8	52	64.2	81	

Table5: Distribution of IS900PCR results according to seropositive cows breed

ELISA Positive	IS900 PCR positive	Percentage Taken for 81	IS900 PCR negative	Percentage Taken for 81	Total	P value
Indigenous breed	20	24.7	30	19.9	50	0.3502
Cross breed	9	11.1	22	9.6	31	
Total	29	35.8	52	29.5	81	

DISCUSSION

Due to insidious nature of MAP infection in animals, prolonged incubation period and detection of disease only in adult animals, the control and eradication of Johne's disease has been difficult throughout the world. In early stages of the infection, where excretion of the bacilli is too low to be detected by culture or PCR, serological screening of anti-Map antibodies and subsequent confirmation by genetic test appear to be a logical option for detecting MAP infection in the livestock. an accurate estimation of the prevalence of *Map* in cattle is difficult since most infected animals are asymptomatic, the diagnosis in the early stages of disease is difficult and the animals with clinical signs of decreased milk production can be slaughtered without confirmation of *Map* infection (17). To date, only one Iraqi study (18) had tried to assess the prevalence of paratuberculosis in Iraq, namely in the cattle population, where this disease probably runs under-diagnosed. ELISA can detect specific antibodies in blood or milk with a high sensitivity, and antibodies generally develop late in the infection (19). Accordingly, an indirect ELISA for detection of antibodies against *Mycobacterium paratuberculosis* was performed on plasma samples obtained from 156 Iraqi apparently healthy cattle representing 110 local and 46 crossbreed. The present results revealed that seropositive ELISA results (51.9%) was observed in tested plasma.

In the present study, the sero-prevalence was found (51.9%) in south of Iaq which can be considered high when compared with the Abdulrasool 2016 who reported

2.64% sero prevalence in 6 animals out of 227 apparently healthy cows in south and middle of Iraq .Separately 50% herd level prevalence in European countries (20,21) other studies in different parts of the world have shown a varying range of cattle lower level sero-prevalence: as 15.14% in India (1) 5% in Mexico (3); 3.125% in India (2);8% in Colombia(4).; 5.88% in Pakistan. (5) and 2.31% in Bhutan (6). Many factors probably contribute to the differences in prevalences between present results and other countries results, such as animal characteristics management, climate, and environment effects.

As Iraq depends on importation of livestock and livestock products from many countries such as india where JD is prevalent in cattle, importation of large volumes of dairy and meat products may act as potential sources of MAP exposure for humans as reported by other studies in other parts of the world (22) There is obvious risk of spreading MAP in ruminant population and possibly also to human.

In the present study seroprevalence of paratuberculosis in local breed (32 %)was higher than cross breed cattle seroprevalence (19.9 %), however the difference between the two tested breeds with respect to ELISA positivity is considered to be not statistically significant (P=0.2010) difference between the two tested breeds with respect to seropositivity. The finding of Benavides (5) .that MAP-seroprevalence is not connected to age, race, location and clinical status supported the present results, but it is linked to body condition.

Molecular methods, especially PCR, real-time PCR and multiplex PCR are the most promising methods for the rapid and specific diagnosis of JD (23) to date; studies have focused on the PCR-based detection of MAP from feces, milk or culture. IS900-PCR-based MAP detection directly from peripheral blood of animal was investigated by few studies (24, 25, 26). In this study, the presence of MAP was investigate in buffy coat of cattle .The introduction of IS900-dependent PCR has reduced the time and labor required for the Because of the extremely slow progression of John's disease, infected animals appear healthy, without shedding MAP in milk or feces, while harboring potential infection in phagocytic cells, such as macrophages. Such animals pose a real threat for the herd.. The present study, successfully detected MAP with the help of the IS900-PCR technique from peripheral

blood leucocytes of cattle. The prevalence of MAP in cattle was 35.8%, reflected in the risk of MAP infection in younger animals. This observation was not correlated with the exceptionally long incubation period of John's disease. The higher occurrence of MAP positive cases in apparently healthy cattle indicates the chances of either mixed infections or increased susceptibility to MAP infection in stressed animals.

In contrast with the present prevalence (35.8%) Bhide *et al* (24) mentioned that prevalence of MAP in cattle was 11.45% and revealed the risk of MAP infection in older animals. A total of 81 samples were found positive by indirect ELISA, twenty nine of these seropositive samples were confirmed positive for MAP infection by IS900.The current finding disagreed with Bhide *et al* .,2006 who reported that all (n=13) seropositive samples were confirmed positive for MAP infection by IS900. In conclusion, results of the present study indicated the utility of ELISA and conventional PCR assay in rapid, sensitive and specific detection of MAP in in cattle plasma and buffy coat.

تطبيق ELISA و IS900 PCR في تشخيص جرثومة *Mycobacterium avium Subspecies Paratuberculosis* باستخدام طبقة الخلايا البيضاء كمصدر لعينات الكشف عن الحالات تحت السريريه لمرض نضير السل البقري

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الخلاصة

مرض جونز او نظير السل هو مرض مزمن تسببه بكتريا نظير السل الطيري يصيب المجترات بشكل واسع مؤديا الى خسائر اقتصادية هائلة في ارجاء مختلفة من العالم. هدفت الدراسة هو تحديد او تقدير انتشار المرض في ابقار جنوب العراق سيرولوجيا وجزيئا. أحد الطرق التشخيصية التي استخدمت لتحديد مستوى الاجسام المناعية الخاصة بالبكتريا هواختبار الاليزا غير المباشر حيث استخدم لتشخيص وتقدير انتشار المرض سيرولوجيا في الابقار. ٨١ (٥١,٩%) من مجموع ١٥٦ عينة بلازما ابقار أظهرت نتيجة موجبة ل فحص الاليزا ولم تظهر النتائج ارتباط م بعمر او نوع الابقار(مستوى المعنوية اكبر من٠,٠٥) .كأختبار تأكيدي تم اجراء تفاعل البلمرة التسلسلي المعتمد على تحديد القطعة المميزة لبكتريا نظير السل الطيري ٩٠٠على عينات الحامض النووي المستخلصة من طبقة خلايا الدم البيضاء للأبقار التي أظهرت نتائج اليزا موجبة (عدد٨١).النتائج الموجبة لتفاعل البلمرة التسلسلي في الابقار ذات النسب المئوية السيرولوجية العالية لاختبار الاليزا(عدد ٢٩ نسبة ٣٥,٨ %) .

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