ASSOCIATION OF POLYMORPHISMS IN SLC11A1 GENE WITH AUTOIMMUNITY CAUSED BY *Mycobacterium avium* subspecies *paratuberculosis*(MAP) IN CATTLE.

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ABSTRACT

Johne's disease is one of the main causes of economic losses in ruminants and a major health hazard in the developed and developing developed countries.In this study, PCR detection of insertion sequences IS900 of MAP in the buffy coat of cows ($n = 81$), of this 29 (35.8%) cow showed positive results. By Restriction fragment length polymorphism (PCR-RFLP),two single nucleotide polymorphisms (SNPs) of SLC11A1 gene were tested for finding their association with susceptibility to bovine Johens disease in Iraqi cattle. A total of 50 cows were tested, theirresult revealedthat at rs109453173 locus twoelectromorph'CC' (374 bp) and 'CG' (374, 293 and 81 bp).The rs109915208 locus also showed two electromorph, 'TT (344bp) and 'CT' (344, 215 and 129 bp) . The differences in the electromorph between IS900 positive and negative cows were found to be statistically significant (p = 0.0031). No significant difference in these electromorph at SNP locus rs109915208 between IS900 positive and negative cows. Out of two SNPs from SLC11A1 gene, rs109453173 had a significant association with the susceptibility to Johne's disease. The CC' electromorph observed at rs109453173 locus showed a significant association with the susceptibility to bovine paratuberculosis in cows. The OR of 'CC' in'IS900 positive versusIS900 negative cattle was7.8750, suggesting that cows having 'CC' electromorph were susceptible to Johne's disease compared to 'CG' electromorph .

INTRODUCTION

Mycobacterium falls under the category of 'Hazard group-III organisms' (1).Themost important mycobacterial infections incattle and buffalo includes Tuberculosis (TB)and Paratuberculosis (Johne's disease or JD)which are chronic and wasting diseases. BothTB and JD have zoonotic potential and areendemic in many countries causing severeeconomic losses due to morbidity, decreasein production and mortality**(2).**PCR-assays provide a rapid alternative for 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 found in 15-20 copies in the MAP genome (3, 4, 5).

An ideal approach to the control of the infectious diseases in animals is the development of genetic resistance. One of the candidate genes having role in resistance/susceptibility to infectious diseases is solute-like carrier family 11 A1 (SLC11A1) known as NRAMP1 (Natural resistance-associated macrophageprotein 1) (6,7). NRAMP1 is a member of the solute carrier (SLC11A1) family of ion transporter (8), which is an integral trans-membrane protein and expresses particularly on phagosome of macrophage (9). Genetic studies in mice have demonstrated SLC11A1 controls' innate resistance and susceptibility for *M. bovis* (10). The NRAMP1 gene mediates activity of macrophages against intracellular parasites during the early stages of infection (11). NRAMP1 affects the intraphagosomal microbial replication primarily by eliminating Mn2+ (12); or other divalent cations (Fe2, Mn2 , Co2 , etc.) from phagosomal interior (13,14) , which serve as essential cofactors for their survival by helping in many microbial metabolic processes. There are reports about association of SNPs in SLC11A1 to resistance/susceptibility to tuberculin reaction in humans as well as in animals (15, 16, 17). In previous study, an association of two polymorphic SNPs and one microsatellite marker pertaining to SLC11A1 genes with tuberculin reaction was observed (18). This study aimed to examining the association of the SLC11A1 polymorphisms in relation to the presence of MAP sinfection in cattle with subclinical paratuberculosis.

MATERIALS AND METHOD

Animals and samples

An apparently healthy cows (N = 81) from south of Iraq, was selected for case and control study for autoimmune disease (Johen's disease). These cows aged from 2 to 14years (two groups < 7 years verses ≥ 7 years). A minimum of 5 ml blood sample was collected from the jugular vein of each animal into tubes with anticoagulant (EDTA) by using 18 gauge needles. The EDTA-blood tubes were transported to the laboratory cold within 24 hours. .

Isolation of peripheral leukocytes (buffy coat)

The collected blood was processed by centrifugation at 3000 × g for 10 minutes at room temperature .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 (19).

DNA extraction

DNA was extracted from isolated leukocytes by using GeneaidgSYNC™DNA Extraction (Korea)as per recommended protocols. The concentration of DNA was determined using NanoodropQuawell (USA) .

Polymerase chain reaction

PCR employing IS900 gene specific primers of *Mycobacterium avium* subsp.*parartuberculosis*(MAP) was used for diagnosis of Map DNA.The primers (BA5: 5'-CTG GCTACC AAA CTC CCG A-3', BA6:5'-GAA CTC AGC GCC CAG GAT-3') (314 bp) were adopted from the IS900 sequence of MAP(20). The isolated DNA was amplified in 50 µl reaction mixture containing PCR buffer, mM MgCl2, dNTPs, Taq polymerase (Promega / USA), 1 µM of primers (BA5 and BA6) 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.

Restrictionfragment length polymorphism (PCR-RFLP).

In the present study, polymorphisms at two single nucleotide polymorphisms (SNPs) of SLC11A1 gene were investigated by restriction fragment length polymorphism (PCR-RFLP) for finding their association with susceptibilityto bovine paratuberculrosis in Iraqi cattle. A total of 50IS900 positive and negative cattle buffy coat (25 for each) were tested.

Primers forthe two SNPs (rs109915208 and rs109453173) andprimer for SLC11A1 microsatellitemarkerreported byBaqir(18).Thedetails of primers and restriction enzymes are tabulated inTable (1).Concerned amplicons were amplified under the optimizedPCR condition. The polymerase chain reaction (PCR)product are resolved in 1.5% agarose gel and visualized underUV light after staining with red safe nucleic acidstain. The Restrictionenzyme digestion was made at the optimized conditions andthe restriction-digested products were resolved in 3– 5%agarose gel and visualized under UV light after staining withred safenucleic acid.

Table:(1). Details of SNPs and microsatellite marker from Slc11A1 gene.

SNP	Primer Sequence (5′–3′)	AT*(°C)	RE**	Fragments
rs109915208	TGGACTGGAGGGTAAGAACG AGGGAGGAATGCAGGTAGATG	59	Bpu10I	344,215, 129
rs109453173	ATCTCCTTCCTACTGCCCG CACAAACTGTCCCGCGTAG	58	PstI	374, 293, 81

*Annealingtemperature.

**Restriction endonuclease.

Statistical analysis

The data obtained from the IS900 gene PCR and PCR-RFLP were analyzed by Fisher's exact test and OD ratio.The limit of significance being set at 5%.Statistical analysis is done by using SPSS softwareversion 11.

RESULTS

Detection of MAP by IS900 PCR

The results of IS900 PCR revealed that amplifiedproduct IS900 sequence (314 bp)wasdetected in29 (35.8%) out of 81 cow peripheral leukocytes (buffy coat) samples of which 19 (23.5%) were at age group ≤ 7 year and 10(12.3%) were at age group >7year.There was no significant difference in the detection rate of MAP genome between the two age groups (P=0.6361). (Table 2; Figure 1). In (table 3) 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).

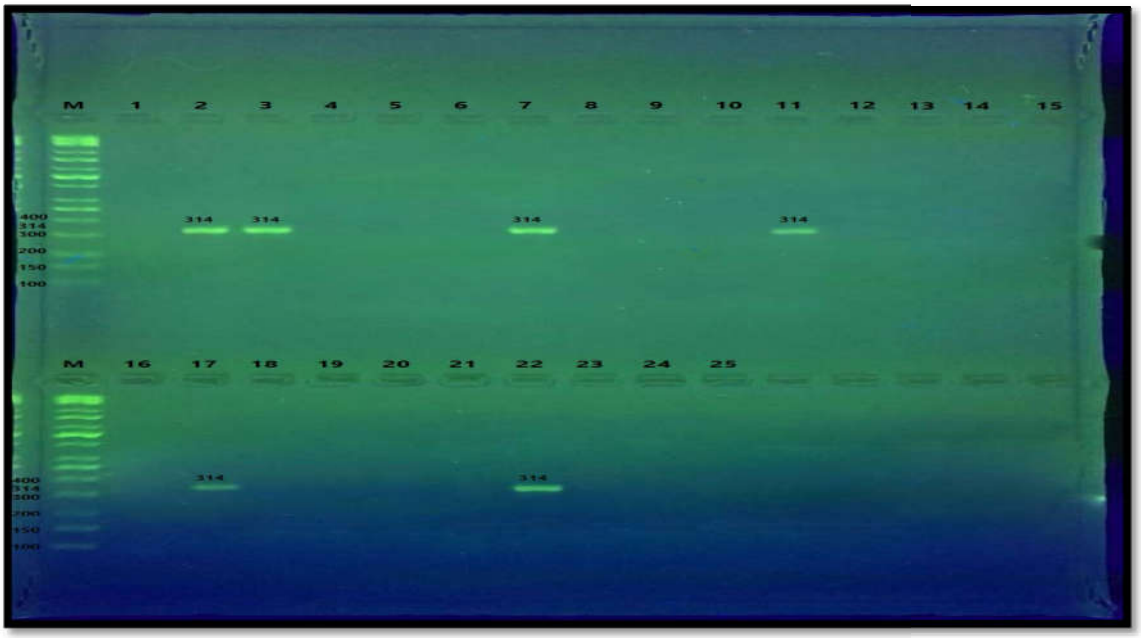

Figure 1: *Mycobacterium avium*subsp. *paratuberculosis*specific amplicons (314 bp) by PCR using IS*900* specific primers. Lane M:100 bp DNA ladder, Lane2,3,7,11,17,22: tested DNA samples.

Table :(2). Distribution of IS900PCR results according to cows age

Age group	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	

Table :(3). Distribution of IS900PCR results according to cows breed.

Cattle breed	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	

PCR-RFLP assay

The PCRRFLP was used todetect the polymorphism in two SNPs from SlC11A1 gene. At rs109453173 locus twoelectromorph‘CC’ (374 bp) and ‘CG’ (374, 293 and 81 bp) were observed.In addition rs109915208 locus showed two electromorph, ‘TT (344bp) and ‘CT’ (344, 215 and 129 bp) (Table 4; Figure2).The

Fisher's exact and OD ratio testsrevealed that out of two SNPsfrom SLC11A1 gene, rs109453173 had the significant association withthe susceptibility to MAP infection. At rs109453173 locus,the frequencies of 'CC' and 'CG' electromorph were 60 and40%,respectively, in IS900 positive, whereas CC and CG' electromorphwere present in16 and 56%IS900 negative cattle(Table 4).Thedifferences for the electromorphi between IS900 positive and negative cattlewere found to be statistically significant (p= 0.0031). While at SNP locus (rs109915208), the electromorph did not differ significantly in IS900 positive and negative cattle. The CC' electromorph observed at rs109453173 locus showed a significant association with the susceptibility to bovineparatuberculosis. OR was7.8750, suggestingthat animals having 'CC' electromorphwere susceptible forparatuberculosis compared to 'CG' electromorph.

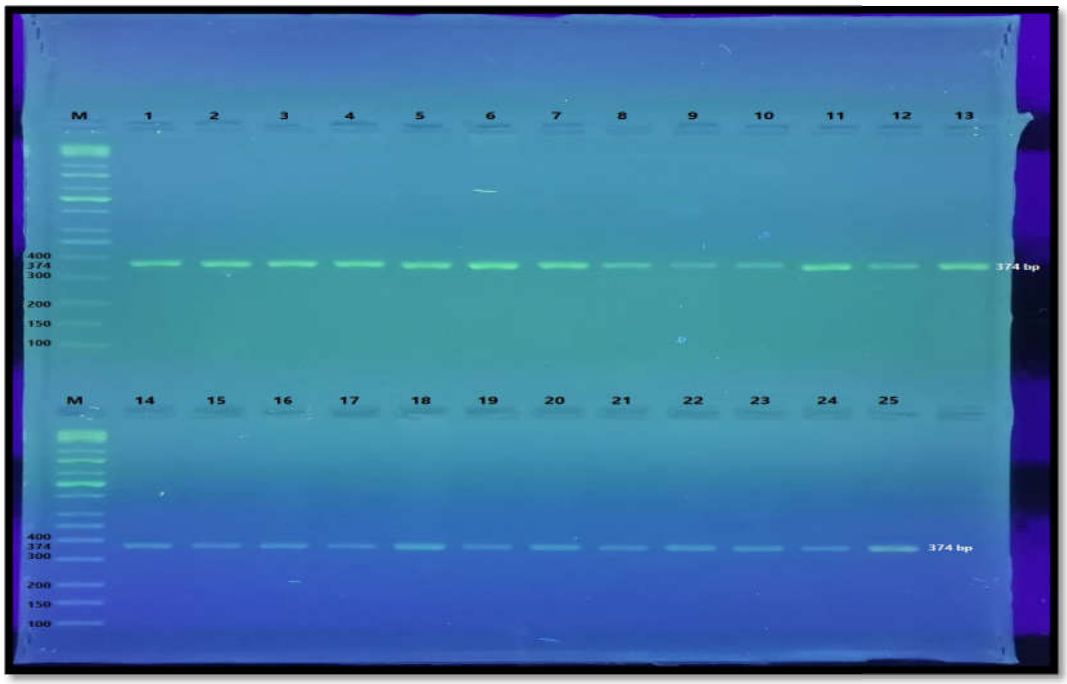

Figure 2: PCR product (374 bp) of rs109453173 from SlC11A1 gene. M: DNA ladder (100 bp). Lane 1-25 tested DNA samples (IS900 positive and negativecattle)

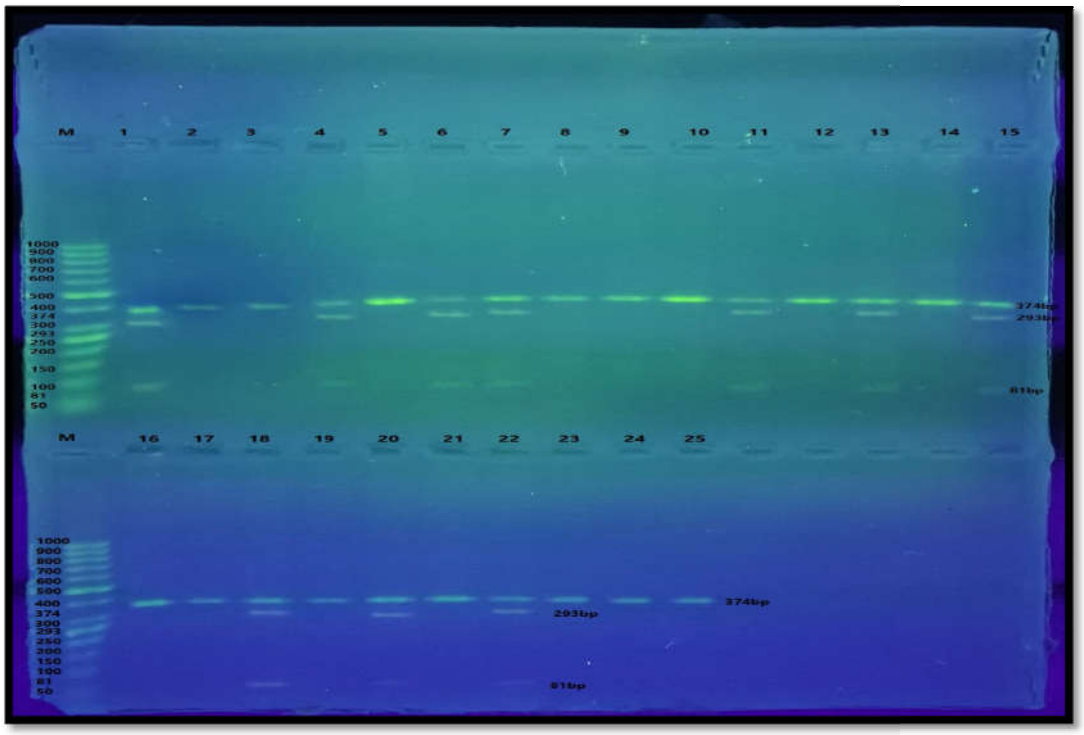

Figure:(3). PCR-RFLP profile of SNPs -rs109453173 from SlC11A1 geneinIs900 positive and negative cattle

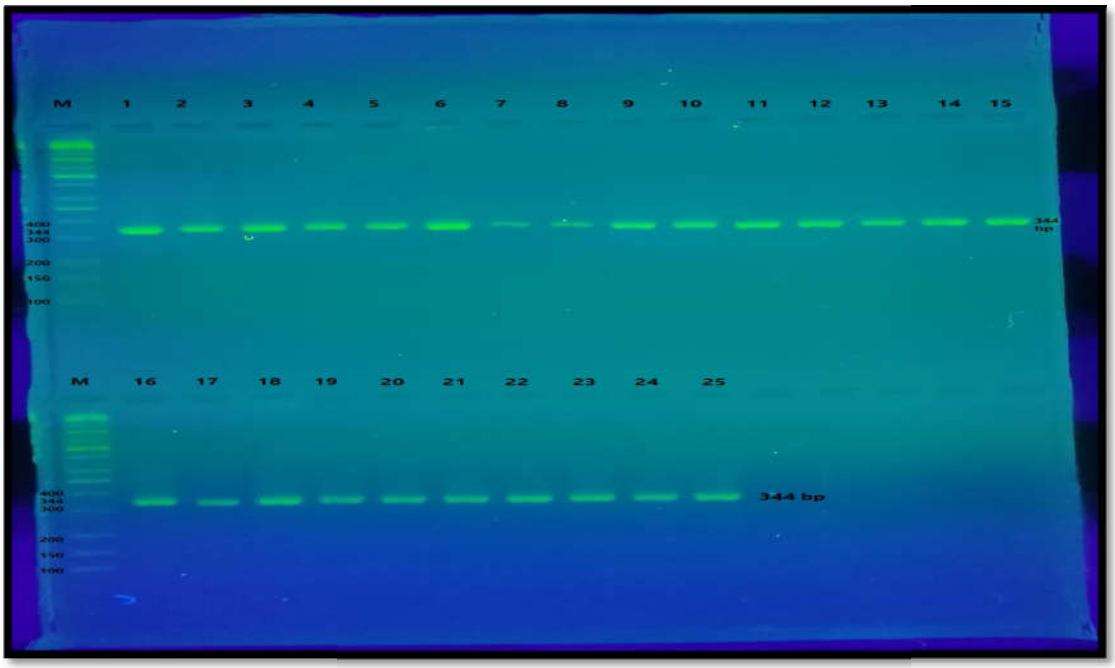

Figure:(4).PCR product (344 bp) of SNPsrs109915208 from SlC11A1 gene. M: DNA ladder (100 bp). Lane 1-25 tested DNA samples (Is900 positive and negative cattle)

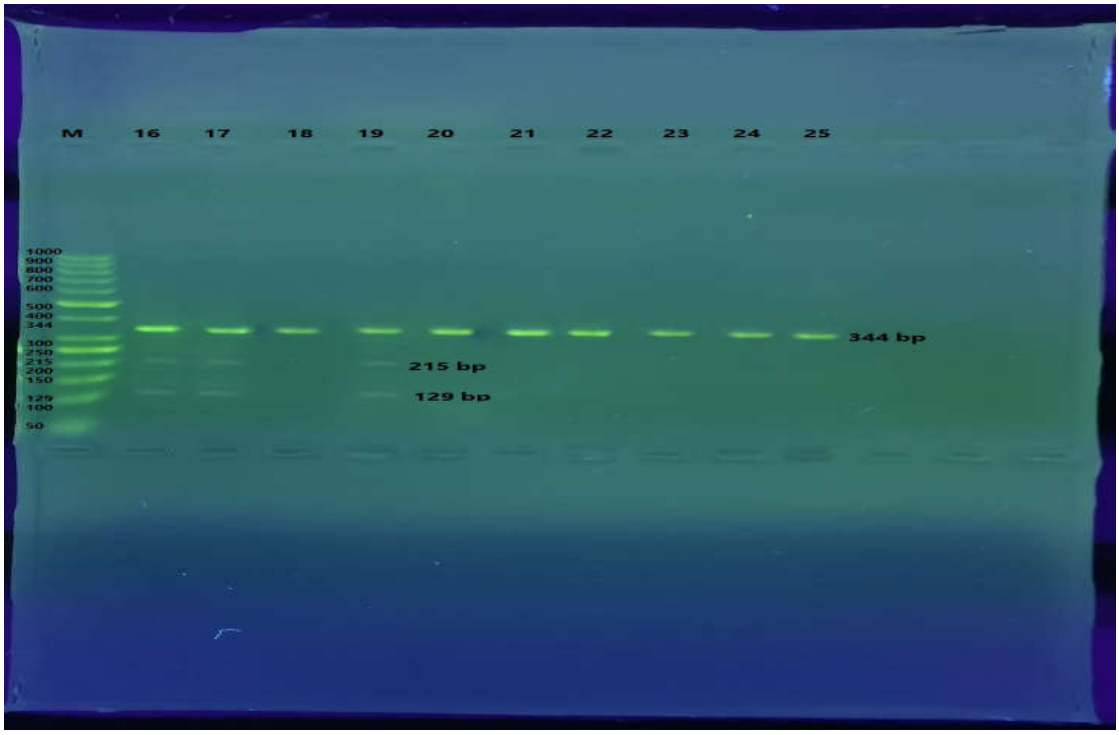

Figure :(5). PCR-RFLP profile of SNPs rs109915208from SlC11A1 gene inIs900 positive and negative cattle

Table :(4) Electro morph frequencies of SNPs - rs109453173 from SlC11A1 gene and their association with susceptibility to the bovine paratuberculosis

Gene	Electro morph	, RE fragments bp	Is900 positive Cattle n.(%)	Is900 negative cattle n.(%)	Odds ratio (95%CI)	P-Value
rs109453173	CC	374	15(60)	4(16)	7.8750 (2.071 to 29.940)	0.0031
	CG	374,293,81	10(40)	14(56)	0.5238 (0.170 to 1.612)	0.3961
rs109915208	TT	344,	15(60)	12(48)	1.6250 (0.5298 to 4.9837)	0.5709
	CT	344, 215, 129	10(40)	13(52)	0.6154 (0.2007 to 1.8873)	0.5709

DISCUSSION

Molecular methods, especially PCR, real-time PCR and multiplex PCR are the most promising methods for the rapid and specific diagnosis of many bacteria in different clinical samples(21,22,23,24)..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(25, 26, 27).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 diagnosis of infection. Because of the extremely slow progression of Johne'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. Thisobservation was not correlated with the exceptionally long incubation period of Johne'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%)(28)mentioned that prevalence of MAP in cattle was 11.45% and revealed the risk of MAP infection in older animals. Out of 81,29 buffy coat samples were confirmed positive for MAP infection by IS900. An ideal approach to the control of the infectious diseases in animals is the development of genetic resistance**.**In the present study, polymorphisms at two single nucleotide polymorphisms (SNPs) SLC11A1 gene were investigated for finding their association with susceptibility to autoimmune bovine disease (Johne's disease) in Iraqi cattle

The present results revealedthat rs109453173 SNP from SlC11A1 gene showed significant association with the susceptibility to bovine paratuberculosis in cattle while rs109915208 SNP from SlC11A1 gene associated with paratubeculosis resistance. In contrast with the present results, (18) found that SlC11A1 genepolymorphisms at rs109453173 was associated with resistance to bovine tuberculosis and rs109915208 SNP from SlC11A1 gene associated with

paratubeculosissusbtibility. SlC11A1 gene polymorphisms at 823 C/T (exon 8) are associated with resistance to human tuberculosis(16) In human tuberculosis(16).In a study carried out in human, the G > C mutation of intron 4 of NRAMP1 gene was reported as a susceptible factor to paratuberculosis(17). Studies at 469 + 14 G/C (INT4), 1465–85 G/A and C274T polymorphisms of NRAMP1 in ethnic Russians revealed that none of the polymorphisms was associated with tuberculosis (15) In conclusion SNPs of SLC11A1gene had a significant role for the paratuberculosisresistance andsusbtibility.

ارتباط تعدد اشكال الجينSLC11A1 بالمناعة الذاتية الناتجة من جرثومة *Mycobacterium avium subspecies paratyberculosis*في الابقار

كوثر كاظم جبر،فوزيه علي عبدالله ، رشا منذر عثمان

فرع الاحياء المجهريه ،كلية الطب البيطري و جامعة البصره ،البصره ،العراق.

الخلاصة

مرض جونزJohne's هو أحد الأسباب الرئيسية للخسائر الاقتصادية في المجترات ومخاطر صحية كبيرة في العالم النامي والمتقدم.في هذه الدراسة تم استخدام تفاعل البلمرة التسلسلي (PCR)للكشف عن تتابعات الإدراج IS900لجرثومة*Mycobacterium avium subspecies paratuberculosis* في الخلايا البيضاء لواحد وثمانونبقرة،(35.8%) 29منها اظهرت نتائج موجبة. .بواسطة تقنيةPCR-RFLP فحصت اثنين من النيوكليوتيدات متعددة الأشكال (SNPs) للجين SLC11A1لأجل ايجاد ارتباطها مع القابلية للإصابة بمرض Johne's في الابقار العراقية.اختبرت ٥٠ بقرة ، وبينتالنتائج أن في الموقع rs109453173 اثنين من الاشكال المهاجرةكهربائيا هما(374) (electromorph 'CC' و"CG (374، 293 و 81bp) ، كما أظهر الموقع rs109915208 اثنين من الاشكال المهاجرةكهربائيا، (344bp) "TT (و CT("344 و 215 و 129bp).اختلاف الاشكال المهاجرة كهربائيا بين الابقار الموجبة و السالبةلوجودIS900 كان معنويا (p = 0.0031).لايوجد فرق احصائي معنوي بين هذه الاشكال في الموقع. بين الابقار الموجبة و السالبة لوجود IS900 rs109915208. من أصل اثنين من SNPsللجين SLC11A1 كان ارتباطrs109453173معنويا من الناحيهالإحصائيةمع القابليةللاصابةبمرض Johne's. اظهر إالشكل الكهربائي المهجرCC الذي تم ملاحظته في موقع rs109453173 وجود ارتباط معنوي مع القابلية للإصابة بمرض Johne's في الأبقار ، وكان مقدار

(OR7.875)في الابقار الموجبةIS900 مقابل الابقار 0 السالبةIS900 ، مما يشير إلى أن الأبقار التي لديها 'electromorph 'CC لها استعداد للإصابة بمرض Johne's جونز.

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