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Efficacy of Different ELISA, Histopathology and PCR Assays for the Diagnosis of Ovine Brucellosis in Ram

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Diagnosis of ovine brucellosis infection inthe ram hasepididymitis and orchitis has not been established. The aim of this study,to investigate the diagnostic value of ELISA, Histopathology and PCR for ovine brucellosis in ram in Karbala city. 30 ram's serum and semen were collected from ram for serological and molecular assay, in addition three testicular tissueswere castrated from ram forhistopathological diagnosis of Ovine brucellosis. Sensitivity and specificity of ELISA and PCR were 80% ,70%; and 87.5%, 57.14%; respectivel,Palpable enlargement of the epididymis and testicular hypoplasia. Sensitivity by ELISA was higher in the ovine brucellosis detection than PCR, but specificity values were lower. Grossly visible lesions in the epididymis associated with Ram Brucellosis withPocket of inspissate material resulting from extravasation of sperm.

Keywords: ELISA, histopathology, PCR molecular tests, brucellosis,Ram.

The genus Brucella has many types and the main hosts farm animals, *Brucella melitensis* (sheep and goats), *Brucellaovis* (sheep), *Brucellaabortus* (cattle) and (pigs), generally, the main featuresofbrucellosis are reproductive failure, suchas in the female cause abortion still birth or birth of unthrifty newborn, and in the male cause epididymitis,orchitis and frequent sterility[1]. This facultative intracellular bacteria for that considered persistent infection and the infected animals shed the organism bymammary and reproductive secretions, Brucellosis is considered an important zoonosis disease and causing many signs in humans, and its one of the major diseases that cause economic losses in animal and a danger to humanhealth, many countries have programto control and eradicatebrucellosis from the domestic animalsand its considered the key to protect the human,

Control and prevention programs have twoprincipal methods: start with accurate diagnosis and vaccination of animals and slaughter ofinfected animals, usually onthe basis of a reaction to a molecular and serological tests[2].

Brucellosis has been annihilated fromcattle in different climatic regions of the world andis nearly eradicated in others. However, itis still diffuse and is an economicallysubstantial agricultural disease in manycountries. There are still many cases ofhuman brucellosis reported each year inregions where the disease has not been eradicated in livestock farming animals[3].

The epidemiological, clinical and pathological picture of *B. melitensis* is havesimilar

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to *B. abortus*infection incattle[4]. Only sheep are affected with *B.ovis* naturally and the ewe is less susceptible than the ram, there is bacteremia initially, with amild systemic reaction usually, and the bacteriumcan be isolated from differentedible parts of the slaughtered animal. Im Ram, the clinical signs of the disease results from infection, localizationand inflammatory processes in the epididymis.Inflammation in this part results in extravasation and spermstasis with a series ofimmunological stimulation which causing aspermatocele and therefore leads to reduced fertility. Not all rams that infected by brucellosis havesensible lesions in the epididymis: infectioncan also manifest in theseminal vesicles and the wall of the ampulla.The ejaculateof the infected ram is the way to shedthe organism[2].

However, many researchers have demonstrated the persistence of *Brucellaspp*. DNA in asymptomatic patients after the conclusion of therapy forlong periods of time[5].

The diagnosis by using molecular methods is more sensitive and faster than traditional methods, for brucellosis detection[6].

Various PCR assays were used to detection of Brucella DNA in clinical samples and consideredrapid (it takes a few hours), effective at all stages of the disease, and the specificity higher than serological tests,and sensitivity more than blood cultures. Identificationat the level of genus provided bythe use of different DNA targets (which is substantial to start antibioticsmedication) or at the level of organisms species(which is important for epidemiological andepizootological analysis). PCR a highly accurate screening test of clinical samples and improves analytical sensitivity. MultiplexPCR technique enables genotyping the bacterial pathogen and strain description[7].

Histologically, In the acute stage, there is inflammatory oedema in the dartosand scrotal fascia, exudates in the tunica vaginalis and early granulation tissue forming[8]. In the chronic stage, the tunics of the testes become fibrous,thickened and develop of chronic adhesions. There is circumscribed sclerosis in the epididymis and these granuloma may also extendedto the testicular tissues. In progressive stages they undergo granulomatous inflammation associated with caseous necrosis[9]. As the epididymis form the scrotal enlargementit is also atrophied,*B. ovis* can commonly be isolated from any of the organs of reproduction system, particularly the tail of epididymis, and rarely seenin thevisceral lymph nodesand internal organs,the *B. abortus* is characterized by edematous thick placenta, with fin, elevated yellow-white plaques in the intercotyledonary areas and varying degrees ovine focal cotyledonarynecrosis, In Iraq, brucellosis is still one of the endemic diseases and infects domestic animal species causing economiclosses and most their species can infect human., In the present study, our results have addressed these points and determined the feasibility ofELISA, Histopathology and PCR for the diagnosis of Ovine brucellosis in ram in karbala city,Iraq.

MATERIALS AND METHODS

Sampling

Our current study on animals is complete and extension of research[10] at the same area and the same timebut the study was allocated on the ramsand conducted on the incidence abortion in pregnant sheep and goat in some flocks of kerbala province with some of mortality rate 3% which suspect infection by brucellosis and the study dealt with 30rams have clinical signsand seropositive brucellosisby using enzyme linked immunosorbent assay (*Brucella ovis* Ab Test),The study was beginningfromNovember 2016 to January 2017 before the state-sponsored brucellosis vaccination in governmentally campaign with omitted any animal have biasedseropositivity.Fresh semen samples were directly collected from rams by testicular fine-needle aspiration with avoiding any contamination (such as urine, soil), theSemen was collectedinside the sterile container and then directly transmitted in to the ice box to veterinary microbiological laboratory University of kerbala for molecular investigation within one hour.

Animals

Thirty Rams were clinically investigatedby naked eye and checked macroscopically andmicroscopicallyin order to knowledge the testicular atrophy and epididymitis, three unilateral testicular of three infected rams were collected in a manner castration[11], the surgical and aspiration were execute in accordance with the ethical

standards fixed by the Research and Ethics Committee of the University of Kerbala, Veterinary Medicine College/Iraq.

Serum collection and analyzing

30 ram serums have been collectedby sterile syringe fromjugular vein of animal and these serums were collected in tubes without anticoagulant [12]. A commerciallyavailable *Brucella ovis* Ab Test kit(Chekit, IDEXX Laboratories, LasRozas) was used according to the manufacturer´s instruction, the optical density was read andanalyzed in relation to positive and negative controls to calculate a serum of ram/positive.ratio and cut-off values were interpreted as: e"50% positive;e"10% to <50% suspicious; and <10% negative, our data was reported that Serafrom suspicious ELISA is intermittent positive.

Sample handling

Our study had two limitations**:** Aliquots (0.5 ml) of the semencollection were used to extract bacterial DNA by using Wizard® DNA Clean-Up System, Promega, USA, according to the manufacture company. the other limitation were handling with three testis tissue collection immediately , all entire testis were cut and taken aseptically from ramsby castration ,immediately, taking the time delay into consideration that causing testicular autolysis, all steps in the process was performed optimallyfor routine histopathological examination[13].

Molecular study

The current study was used PCR with a oligonucleotide primers pair targeting the IS711 sequence (ISP1: 5'-GGTTGTTAAAGGAGAACAGC-3' and ISP2: 5'-GACGATAGCGTTTCAACTTG -3') designed from the nucleotide sequenceof the *Brucella ovis*(14, 15). the reaction was performed usingapproximately 10 pmol of each primer at 25µM, 1.25U of TaqPolymerase (Invitrogen), 25µL of a PCR master mix (PCR Supermix,Invitrogen), containing 200 mM each dNTP, , 1 mM MgCl2, A typical reaction will start with a five minute were denaturation at 95°C; fellowed by 35 cycles of (95°C for 35sec), annealing (62°C for 45 sec), andextension (72°C for 45 sec); and a finalprimer extension at 72°C for 6 minutes.

Histopathological study

Testicular tissue with gross visible lesions waskeptten percent neutral buffered formalin and operation for histopathological screeningusing standard protocol (16). The paraffinembeddedtissues were cut into sections of five micrometer thicknessand stained with haematoxylin and eosin (H & E) stain.

Statistical analysis

Our results were used online software (https://www.medcalc.org/calc/diagnostic_test.php) to determine the prevelance , specificity and sensitivity with a 95% confidence level for both test according to the formula equation

Prevalence of Disease= True of disease/ Total × 100.

Sensitivity: A/(A+B) × 100.

Specificity: D/(D+C) × 100.

A represented True positive, B: represented false negative, C: represented false positive, D: represented True negative, the gold standard test is PCR assay of *B. ovis*, usually from semen.

RESULTS AND DISCUSSION

Brucellosis is a chronic infectious bacterial disease caused by members of the genus Brucella. It is a disease of worldwide importance and affects a number of animal species. Brucella are obligate parasites, requiring an animal host for maintenance. Infections tend to localize to the genital tract and reticuloendothelial system with abortions in females and orchitis with epididymitis in males[17].

Brucella ovis Ab Test and PCR of semen were performedprior to vaccination by Karbala governmentally campaign, and the study were accelerated because occurrence of abortions, still births and seroprevalence of brucellosis. The most importing things for transmitted and introduced of disease of an infected ram are in the breeding season, they can lead to rapid spread of infection within the flock[18], or transmission occurs when an uninfected ram breeds a ewe recently bred by an infected ram. The ewe acts mainly as a mechanical vector for transmitting infection. Homosexual activity of rams is another means of spreading infection among rams[19].

The clinical features of sheep on these flocks were varies widely, So, it is reflecting to difficult in recognizing a disease that lack clinical symptoms (fever, appetite, normal size of testis), therefore, Our study confined just on the thirty rams

for those have visible signswhen rams develop epididymitis and testicular atrophy.

Table 1, showed two columns which indicate the actual condition of diseases of rams have brucellosis and none infected by brucellosis. Thirty rams were tested for brucellosis, 10 rams have diseases by using Elisa test, 20 rams are not diseased, So, prevalence was 33.3% , on the other hand the prevalence of molecular methods were 46.6%.The results showed that there were an increase in the number of brucellosis by using PCR rather than serological test,these results are due to work and efficiency ofBrucella ovis Ab Test,they are considered asrapid, sensitive and specific assay for detecting antibodies against *Brucella ovis* in serum and plasma of rams, it can also be used for other ovin brucellosis like *B. abortus*. However, there was fogginess about this test so that present most countries encourage that ELISA results should be confirmed by another test, and the routine use of vaccines against brucellosis is no longer allowed[20], Several studies of this test have been reported this assay as indict ELISA and used ABTS (2,2'-azino-bis-[3-ethylbenzothiazoline-6-sulphonic acid]) as chromogen(21and 22), these results different from[23] who decided that indirect ELISA is the best test to detect serum ram before their admission to artificial insemination units, and it's also recommended that all ram must be undergo serological pre-movement tests.

		ELISA test		Total Number
		Diseases NO.	None diseases NO.	
PCR assay	Positive	True positive	False positive	14
	NO.	8	6	
		A	B	
	Negative	False negative	True negative	16
	NO.	2	14	
		C	D	
Total		10	20	30

The sensitivity of ELISA Test was 80%,with 95% confident interval (44.39 to 97.48) and specificity was 70% with 95% confident interval (45.72 to 88.11), whereas, the PCR assays were highly specific in acute and long evolution brucellosis cases 87.50 % with 95% confident interval (61.65 to 98.45). so it is very stable insertion sequence (IS) element with mobility have been demonstrated the Brucella genome called IS711 or IS6501, which are exclusively found in the genome of all qualified species of the genus Brucella. While the sensitivity was 57.14%, with 95% confident interval (28.86 to 82.34).the results of PCR assay were detected the IS711 gene (Fig 1).

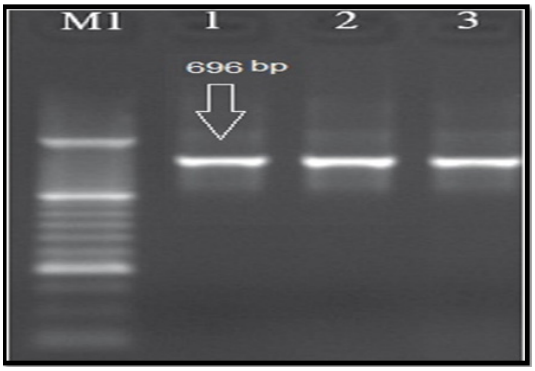

Fig. 1. Showed that amplification products of IS711 gene of 696 bp, extracted from B. ovis, Agarose gel electrophoresis of PCR products for IS711 sequence of *Brucella spp* , M1, 50 bp DNA , Ladder, 1-3 well, represented PCR product of *Brucella spp* have 696bp

Multiple studies have been demonstrated by using molecular assay to identify *Brucella ovis*(24), So, we can recommend that PCR provides the opportunity to identify ovine brucellosis rapidly in semen samples after artificial seminal insemination, As is known in many other regions of the world, there are currently no compulsory surveillance of the brucellosis in Iraqi flocks, all flocks have to undergo international or intra-community trade.

The study was performed visually on the testicular atrophy and epididymitis prior to the histological examination, it is seeninfections

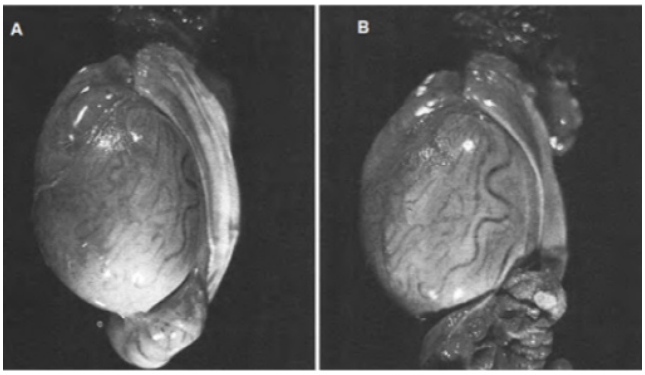

Fig. 2. Swelling and irregular conformation of the tail of the epididymis of a ram infected with *Brucella ovis* (A). Cross-section of the tail of the epididymis revealed a pocket of inspissated material resulting from extravasation of sperm (B)

of the accessory sex glands of males allows for dissemination of organisms through the semen. Some study revered that infections can occur in the accessory sex organs without testicular or epididymal lesions being present(25and 26). So it is common venereal transmission of *B. ovis* in sheep. In rams, epididymitis and orchitis are the most common presenting signs. Lesions are usually unilateral but may be bilateral. Our current study has not studied the quality and quantityparameters of the semen, It was just a comparison of two tests between molecular and serological test, in addition to enhanced the screening pathological changes by using histopathological study.*Brucella ovis* infections in rams predominately affect the epididymis with testicular lesions being uncommon. Palpable lesions in the epididymis of rams are frequently the result of infection with *B. ovis*, congestion and haemorrhages in the unilateral testis were noticed(Fig 2). Palpable enlargement of the epididymis, especially involving the tail portion epidydimis revealed congestion and focal degeneration,Epididymal lesions are characterized by hyperplasia and hydropic degeneration of tubular epithelium. Resulting extravasation of sperm leads to the formation of a spermatic granuloma,it is also noticed that scrotum was swollen, largely due to an inflammation of the tunica and fibrinopurulent exudate in the tunica vaginalis. There was infiltration of neutrophils and few mononuclear cells with focal necrosis.

In conclusion; our results showed that this molecular assays were slightly more specific than the ELISA, but somewhat less sensitive, The high specificity and acceptablesensitivity of the molecular assay support its probable interest for diagnosing ram brucellosis, , epididymitis and orchitis are the most common presenting signs of ram brucellosis.

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