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 in the ram 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 tissues were castrated from ram for histopathological diagnosis of Ovine brucellosis. Sensitivity and specificity of ELISA and PCR were 80%, 70%; and 87.5%, 57.14%; respectively. 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 with a pocket 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), Brucella ovis (sheep), Brucella abortus (cattle) and (pigs), generally, the main features of brucellosis are reproductive failure, such as in the female cause abortion stillbirth or birth of unthrifty newborn, and in the male cause epididymitis, orchitis and frequent sterility. This facultative intracellular bacteria for that considered persistent infection and the infected animals shed the organism by mammary 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 human heath, many countries have program to control and eradicate brucellosis from the domestic animals and its considered the key to protect the human.

Control and prevention programs have two principal methods: start with accurate diagnosis and vaccination of animals and slaughter of infected animals, usually on the basis of a reaction to a molecular and serological tests.

Brucellosis has been annihilated from cattle in different climatic regions of the world and is nearly eradicated in others. However, it is still diffuse and is an economically substantial agricultural disease in many countries. There are still many cases of human brucellosis reported each year in regions where the disease has not been eradicated in livestock farming animals.

The epidemiological, clinical and pathological picture of B. melitensis is has similar
to *B. abortus* infection incattle. Only sheep are affected with *B. ovis* naturally and the ewe is less susceptible than the ram, there is bacteremia initially, with mild systemic reaction usually, and the bacterium can be isolated from different edible parts of the slaughtered animal. In Ram, the clinical signs of the disease results from infection, localization and inflammatory processes in the epididymis. Inflammation in this part results in extravasation and spermstasis with a series of immunological stimulation which causing aspermatocele and therefore leads to reduced fertility. Not all rams that infected by brucellosis have sensible lesions in the epididymis: infection can also manifest in the seminal vesicles and the wall of the ampulla. The ejaculate of the infected ram is the way to shed the organism.

However, many researchers have demonstrated the persistence of *Brucella* spp. DNA in asymptomatic patients after the conclusion of therapy for long periods of time. The diagnosis by using molecular methods is more sensitive and faster than traditional methods, for brucellosis detection.

Various PCR assays were used to detection of *Brucella* DNA in clinical samples and considered rapid (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. Identification at the level of genus provided by the use of different DNA targets (which is substantial to start antibiotics medication) or at the level of organisms species (which is important for epidemiological and epizootological analysis). PCR a highly accurate screening test of clinical samples and improves analytical sensitivity. Multiplex PCR technique enables genotyping the bacterial pathogen and strain description.

Histologically, In the acute stage, there is inflammatory oedema in the dartos and scrotal fascia, exudates in the tunica vaginalis and early granulation tissue forming. 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 extended to the testicular tissues. In progressive stages they undergo granulomatous inflammation associated with caseous necrosis. As the epididymis form the scrotal enlargement it is also atrophied, *B. ovis* can commonly be isolated from any of the organs of reproduction system, particularly the tail of epididymis, and rarely seen in the visceral lymph nodes and internal organs, the *B. abortus* is characterized by edematous thick placenta, with fins, elevated yellow-white plaques in the intercotyledonary areas and varying degrees ovine focal cotyledonary necrosis, In Iraq, brucellosis is still one of the endemic diseases and infects domestic animal species causing economic losses and most their species can infect human. In the present study, our results have addressed these points and determined the feasibility of ELISA, 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 at the same area and the same time but the study was allocated on the rams and conducted on the incidence abortion in pregnant sheep and goat in some flocks of Karbala province with some of mortality rate 3% which suspect infection by brucellosis and the study dealt with 30 rams have clinical signs and seropositive brucellosis by using enzyme linked immunosorbent assay (*Brucella ovis* Ab Test). The study was beginning from November 2016 to January 2017 before the state-sponsored brucellosis vaccination in governmentally campaign with omitted any animal have biased seropositivity. Fresh semen samples were directly collected from rams by testicular fine-needle aspiration with avoiding any contamination (such as urine, soil), the semen was collected inside the sterile container and then directly transmitted in to the ice box to veterinary microbiological laboratory University of Karbala for molecular investigation within one hour.

**Animals**

Thirty Rams were clinically investigated by naked eye and checked macroscopically and microscopically in order to knowledge the testicular atrophy and epididymitis, three unilateral testicular of three infected rams were collected in a manner castration, 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 sera have been collected by sterile syringe from jugular vein of animal and these sera were collected in tubes without anticoagulant 12. A commercially available *Brucella ovis* Ab Test kit (Chekit, IDEXX Laboratories, LasRozas) was used according to the manufacturer’s instruction, the optical density was read and analyzed in relation to positive and negative controls to calculate a serum of ram/positive ratio and cut-off values were interpreted as: >50% positive; <10% to <50% suspicious; and <10% negative, our data was reported that sera from suspicious ELISA is intermittent positive.

**Sample handling**

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

**Molecular study**

The current study was used PCR with a oligonucleotide primers pair targeting the IS711 sequence (ISP1: 5'-GGTTGTTAAAGGAGACACGC-3' and ISP2: 5'-GACGATACGTTTCAACTTG -3') designed from the nucleotide sequence of the *Brucella ovis* (14, 15). The reaction was performed using approximately 10 pmol of each primer at 25 µM, 1.25U of Taq Polymerase (Invitrogen), 25 µL 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; followed by 35 cycles of (95°C for 35 sec), annealing (62°C for 45 sec), and extension (72°C for 45 sec); and a final primer extension at 72°C for 6 minutes.

**Histopathological study**

Testicular tissue with gross visible lesions was kept in ten percent neutral buffered formalin and operation for histopathological screening using standard protocol (16). The paraffin embedded tissues were cut into sections of five micrometer thickness and 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 prevalence, specificity and sensitivity with a 95% confidence level for both test according to the formula equation:

Prevalence of Disease = True positive/ 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 performed prior 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 signs when 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 of Brucella ovis Ab Test, they are considered as a rapid, sensitive and specific assay for detecting antibodies against Brucella ovis in serum and plasma of rams, it can also be used for other ovine 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. Several studies of this test have been reported this assay as indirect ELISA and used ABTS (2,2’-azino-bis-[3-ethylbenzthiazoline-6-sulphonic acid]) as chromogen, these results different from 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.

<table>
<thead>
<tr>
<th>ELISA test Diseases NO.</th>
<th>None diseases NO.</th>
<th>Total Number</th>
</tr>
</thead>
<tbody>
<tr>
<td>PCR assay Positive NO.</td>
<td>True positive</td>
<td>False positive</td>
</tr>
<tr>
<td></td>
<td>8</td>
<td>6</td>
</tr>
<tr>
<td>PCR assay Negative NO.</td>
<td>False negative</td>
<td>True negative</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>14</td>
</tr>
<tr>
<td>Total</td>
<td>10</td>
<td>20</td>
</tr>
</tbody>
</table>

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).

Multiple studies have been demonstrated by using molecular assay to identify Brucella ovis, 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 seen infections
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 (25 and 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 quantitvparameters 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 epididymis 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 serticatic 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.

**REFERENCES**


