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ORIGINAL ARTICLE

Comparative study between conventional and molecular tests to detect the incidence of brucellosis in cattle and buffaloes in Babylon and Karbala provinces

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ABSTRACT

Objective: The aim of this study, comparative efficacy of three methods for detection of brucellosis which included Rose Bengal test, Milk Ring Test and polymerase chain reaction as well as isolation of *Brucella* in cattle and buffaloes.

Methods: which have conducted from June to November 2017 on 141 of brucellosis suspected females, the samples were taken from blood, milk and vaginal swabs from farms in Babylon and Kerbala provinces.

Results: All samples examination showed no significant differences (P>0.05) with highest positive result when our study had used (RBT) between cattle and buffaloes, the animals had positive results from 74 cattle and 67 buffaloes to give 42 and 33 positive results respectively . While by using multiplex PCR technique the number of positive results were lower than that gave by (RBT) which recorded significant differences result with other tests , beside that the infected cases gave 31 and 19 isolates of *B. abortus* while there were no isolates and 5 isolates of *B. melitansis* for cattle and buffalo, respectively. The results showed there is no significant differences in positive results by using (MRT) between cattle and buffaloes and when compare it with other tests. as well as the results showed there is no significant differences in positive results by using culture between blood samples, milk samples and vaginal swabs and when compare it with PCR.

Conclusion: The results of RBT, MRT and culture in cattle and buffaloes suggested that these tests may be used for fast routine screening of herds but the confirmatory diagnosis should attempt by using molecular technique PCR test of brucellosis in individual animals. In addition that PCR the more reliable test important to detect the type of *Brucella* (*abortus or melitansis*) which cannot detected by using the other tests.

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INTRODUCTION

Genus *Brucella* is a bacterial causative agent of Brucellosis which considered a zoonotic disease, Brucellosis in domesticated animals causes a significant economic loss due to loss of fetuses, reduced milk yield, placentitis, infertility, and abortion in pregnant female and epididymitis and infertility in male while in human cause many signs of a flu-like infection, including undulating fever, headaches, back pains, sweats,

weakness, joint pain due to arthritis, in some patients, symptoms of acute brucellosis can endure over one year and eventually result in chronic persistence, complications of inflammation sacroilitis, peripheral, osteomyelitis, bursitis, and spondylitis but rarely results death[1]. The species that have the highest impact on domestic animals productivity are *Brucella abortus* infect cattle, *B. melitensis* infect small ruminants and B. suis infect swine as well as effect on human health[2,3] and preferentially, mixed living systems may cause cross species barriers infections[4,5]. *Brucella*e are difficult to diagnose and treat because its intra-cellular pathogens in cells of the immune system and for that, The isolation from the infected animals considered the definitive diagnosis required, *Brucella* spp. are have the specificity of slow growing intracellular bacteria and the chance of its culture from blood decreases with the disease progresses[6,7]. In humans, the direct contact though the intact skin or injuries by infectious material or fomites and ingestion of milk or milk products of domestic animals that carry the *Brucella* that were the general transmission ways[8]. While In animals, the direct contact through the mucous membranes and fomites as well as ingestion of infected materials are the ways of transmission in animale[9]. Clinical treatments are specially difficult and take a long time in addition to there is no vaccine against this disease for humans[10]. Therefore, protection of human against brucellosis, depends on the work on the infected animals carrying the disease by Control and prevention brucellosis is through testing and vaccines as well as treatment[11].

Diagnosis of brucellosis based on clinical signs is not sufficient to determine the size of the infection because there are cases of carrier of the disease and dose not show sufficient signs of diagnosis like male and non pregnant heifers, as abortion is the only chief clinical feature of this infection. Therefore, laboratory tests are necessary to confirm the diagnosis[6]. Diagnosis of brucellosis is divided into direct and indirect diagnosis. Isolation, *Staining* and molecular tests(like PCR) are a direct diagnosis because it deals with the germ directly, while the examination of the milk ring test and examination Rose Bengal test (RBT) (serological test) indirect diagnosis because it deals with antibodies[6].

diagnosis of *Brucella* spp. infection can be done using Serological tests via detection of antibodies in serum[12]. In addition, the organism can be detected by polymerase chain reaction (PCR) in blood, semen and abomasal fluid of aborted fetuses and, compare to culture method, PCR has more sensitivity and specificity[13,14]. The method that can detect small nucleotide differences of small quantity of samples is Multiplex PCR, therefor is considered appropriate in terms of cost, time and infection determination[15]. MRT is most widely used for screening and monitoring brucellosis in dairy cattle[16].

MATERIALS AND METHODS

Animals and samples

Samples were collected from 141 cattle and buffaloes case suspected of brucellosis after observation of some clinical signs like abortion, endometritis, metritis, infertility from different un-organized dairy farms in Babylon and kerbala provinces were used in this project. Samples (blood, milk and vaginal swabs) were collected between june 2017 to November 2017. All samples were collected in a sterile screw caps . the environmental contamination was avoided by the samples collected directly from infected mother into a sterile equipment. All samples were transfers recently in to the microbiological lab in kerbala university and classified in to many parts for bacteriological, immunological and molecular investigation (Figure 1).

Blood samples was taken from each patient for culture, Rose Bengal test and for extracting DNA. Bacterial cultures was attempt by using *Brucella* broth and *Brucella* agar as descripted by[16]. milk samples which collected from each animal included in the study were divided to three parts, one for culture for bacteriological isolation, the second for Milk ring test and the third for (PCR), the vaginal samples were collected by using sterile swabs which transferred to the laboratory and cultured in *Brucella* broth after that we use the broth for sub culture in *Brucella* agar and for PCR test to detect the positive samples . the study done by using three sections :

The first section was the culture for all samples on *Brucella* broth and sub culture to the *Brucella* agar and after that deal with the positive results, therefor the suspected Colonies which recognized by colony morphology were stained by gram stain, to detect the gram negative coccobacilli microscopically. After that biochemical tests were used (hydrogen peroxidase, Urease test, catalase and oxidase tests) as described in[16].

The second section through the isolation and blood samples for Rose Bengal test (RBT) and the milk for milk ring test as described in[16].

The third section by prepare 1 ml of freshly evacuated pellets directly to isolate DNA Extraction procedures and multiplex polymerase chain reaction. (multiplex PCR) used to confirm the *Brucella* spp.

Molecular techniques

Two Bacterial DNA Extraction Kits were directly used to extract bacterial cells from milk and blood FavorPrep Milk Bacterial DNA Extraction Kit (Favorgen Biotech Corp, Taiwan) and QIAamp DNA Tissue and Blood (Qiagen, Germany), respectively, according to the manufacture company.

For multiplex PCR , primers were used according to Mirnejad *et al* (2013)[17] based on the sequences of the gene mobile genetic element IS711 (Gen Bank accession no.M94960, have been a practical purpose target for molecular enactment of terrestrial *Brucella* species depend on the number and distribution of IS711 copies within the bacterial genomes, The *B. abortus* sequences were IS711 forward 5'-TGCCGATCACTTAAGGGCCTTCAT-3' and B1-F reverse 5'-AAATCGCGTCCTTGCTGGTCTGA-3'. The *B. mellitinsis* sequences were B2-F: 5'-GACGAACGGAATTTTTCCAATCCC-3' and IS711 reverse 5'-TGCCGATCACTTAAGGGCCTTCAT-3'.

These reactions were accomplished in volumes of 25µL with 1mM of MgCl2, 0.1mM of dNTPs, 1.5 U of Taq DNA polymerase (Invitrogen), BSA (10mg/ml), 10 pmol of each of the IS711 F and IS711R primers, 10 pmol of each BcF and BcR, and 3 µl of genomic DNA. The reaction was carried out pre-denaturation for 5-min at 94ºC followed by 30 cycles of denaturation at 94ºC for 45 sec, annealing at 66°C for 45 sec and extension at 72ºC for 60 sec, followed by final extension at 72ºC for 5 min.The products from the Multiplex PCRs were stained with ehtidium bromide (Invitrogen) solution and subjected to a Electrophoresis run on 1.5% agarose gel, in Tris-boric acide EDTA (TBE) buffer, together with the 100 bp molecular weight marker (Invitrogen). The results were obsorved in a transilluminator[18].

Statistical analysis

Statistical analysis can be evaluated by comparing the actual value among blood, milk and PCR against a critical value found in a Chi-Square distribution and one-way analysis of variance (ANOVA) was used to determine if there are any statistically significant differences between two or more routes for test of brucellosis by using SPSS program.

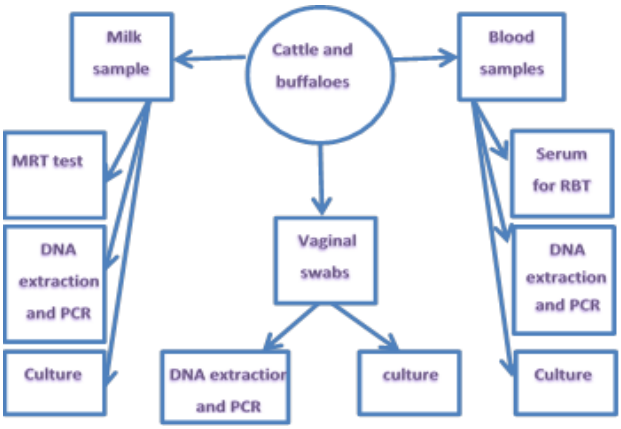

Figure 1: **schematic diagram showed the routes of *Brucella* manipulation**

RESULTS

Three techniques were used in this study which included serological methods (RBT, MRT) and molecular method (PCR) and bacteriological culture to detect the brucellosis in cattle and buffaloes had sings like abortion, endometritis, metritis, infertility and other sings and after the bacterial culture and the conformation by using biochemical tests and gram stain its appear under the microscope a Gram negative coccobacilli Figure 3. A total of 141 consist of 74 cattle and 67 buffaloes were designated for sample collection as milk samples , blood samples and vaginal swabs to investigate brucellosis. All samples examination showed no significant differences (P>0.05) with highest positive results when our study had used (RBT) between cattle and buffaloes (Figure 4) as 75 (53.2%)infection, the animals had positive results from 74 cattle and 67 buffaloes to give 42 and 33 positive results respectively (Table 1). On the other hand by using the multiplex PCR technique the number of animals which gave positive results 55 (39%) were lower than (RBT) which recorded significant differences results with other tests, and beside that the infected cases gave 31(100%) and 19 (79.2%) isolates of *B. abortus* while there were no isolates and 5 isolates (20.8%) of *B. melitansis* for cattle and buffalo, respectively (Table 2) (Figure 2). Table 3 showed there is no significant differences in positive results by using (MRT) between cattle and buffloes.as well as Table 4 showed there is no significant differences in positive results by using culture between blood samples, milk samples and vaginal swabs. moreover Table 5 showed the results of 74 blood samples of cattle gave 42 (56.7%) positive results with rose Bengal test and 14 (18.9%) positive results with isolation and 31 (41.8%) positive results with PCR assay while in buffaloes 67 blood samples gave 33 (49.2%) positive results with rose Bengal test and 15 (22.3%) positive results with isolation and 24 (35.8%) positive results with PCR assay while the Results showed the 74 milk samples of cattle gave 33 (44.6%) positive results with milk ring test and 17 (22.9%) positive results with isolation and 29 (39.2%) positive results with PCR assay while in buffaloes 67 milk samples gave 18 (26.8%) positive results with milk ring test and 16 (23.9%) positive results with isolation and 23 (34.3%) positive results with PCR assay (Table 6). Moreover, results showed the 74 vaginal swabs of cattle gave 17 (22.9%) positive results with isolation and 28 (37.8%) positive results with PCR assay while in buffaloes 67 vaginal swabs gave 14 (20.9%) positive results with isolation and 19 (28.3%) positive results with PCR assay (Table 7).

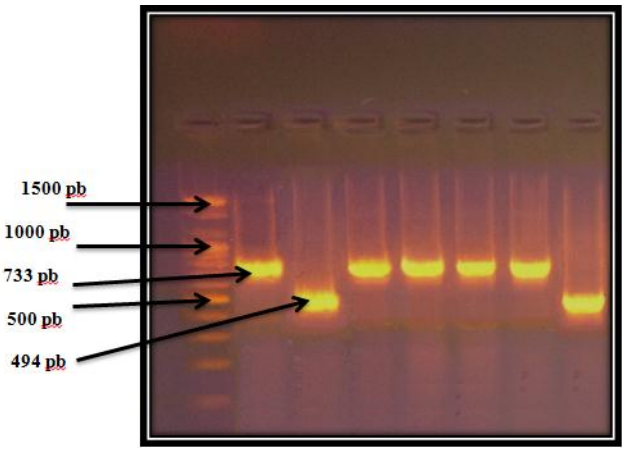

Figure 2: **represents a typical result after agarose gel electrophoresis of PCR products, the first lane represents the M: ladder DNA marker,while the lanes 2 and 7 represent : 494 bp for *B. abortus*, and the lanes 1,3,4,5 and 6 represent 733 bp for *B. melitensis*.**

Table 1: **Shows Numbers of infected animals with *Brucella* according to the highest test recorded (RBT) in the study**

Cases	Examined	Positive	Negative	Percentage
Cattle	74	42	32	(56.7%)
Buffaloes	67	33	34	(49.2%)
Total	141	75	66	(53.2%)
Statistical analysis		X^2= 0.244, P>0.05 no significant		

Table 2: **Shows Numbers of infected animals with *Brucella* according to the multiplex PCR test and the number of animals which infected by *B. abortus* and *B. melitansis***

Cases	Examined cases	Positive	Negative	Brucella abortus	Brucella melitansis	Statistical analysis	
Cattle	74	31	43	31 100%	0 0%	X^2=2.93 No significant p>0.05	X^2=0.036 Significant P<0.05
Buffaloes	67	24	43	19 79.2%	5 20.8%		
Total	141	55	46	50 90.9%	5 9.1%		

Table 3: **Shows Numbers of infected animals with *Brucella* according to the (MRT) test in the study**

Cases	Examined	Positive	Negative	Percentage
Cattle	74	33	41	(44.59%)
Buffaloes	67	22	45	(32.83%)
Total	141	55	86	(39%)
Statistical analysis	X^2= 0.902, P>0.05, No significant differences			

Table 4: **Shows Numbers of infected animals with *Brucella* according to the culture results in the study**

Cases	Examined	Positive Negative			Statistical analysis
		Blood samples culture	Milk samples culture	Vaginal swabs culture	
Cattle	74	14 (18.9%)	17 (23%)	17 (23%)	F test= 0.065 P>0.05 No significant differences
Buffaloes	67	15 (22.4%)	16 (23.9%)	14 (20.9%)	
Total	141	29 (20.6%)	33 (23.4%)	31 (22%)	

Table 5: **Shows the comparison between tests to determine brucellosis from blood samples**

Cases	Examined	Positive results			Statistical analysis
		RBPT	Culture	PCR	
Cattle	74	42 (56.7%)	14 (18.9%)	31 (41.8%)	F test= 12.1 P<0.05 Significant differences
Buffaloes	67	33 (49.2%)	15 (22.3%)	24 (35.8%)	
Total	141	75 (53.2%)	29 (20.5%)	55 (39%)	

Table 6: **Shows the comparison between tests to determine brucellosis from milk samples**

Cases	Examined	Positive results			Statistical analysis
		MRT	Culture	PCR	
Cattle	74	33 (44.6%)	17 (22.9%)	29 (39.2%)	F test= 1.3 P>0.05 No Significant differences
Buffaloes	67	18 (26.8%)	16 (23.9%)	23 (34.3%)	
Total	141	51 (36.2%)	33 (23.4%)	52 (36.8%)	

Table 7: **Shows the comparison between tests to determine brucellosis from vaginal swabs samples**

Cases	Examined	Positive results		Statistical analysis
		Culture	PCR	
Cattle	74	17 (22.9%)	28 (37.8%)	X^2=0.71 No Significant P>0.05
Buffaloes	67	14 (20.9%)	19 (28.3%)	
Total	141	31 (22%)	47 (33.3%)	

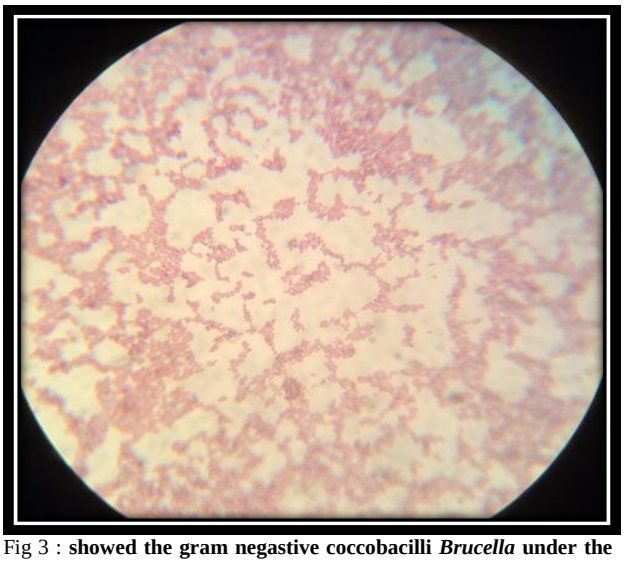

Fig 3 : **showed the gram negative coccobacilli *Brucella* under the microscope**

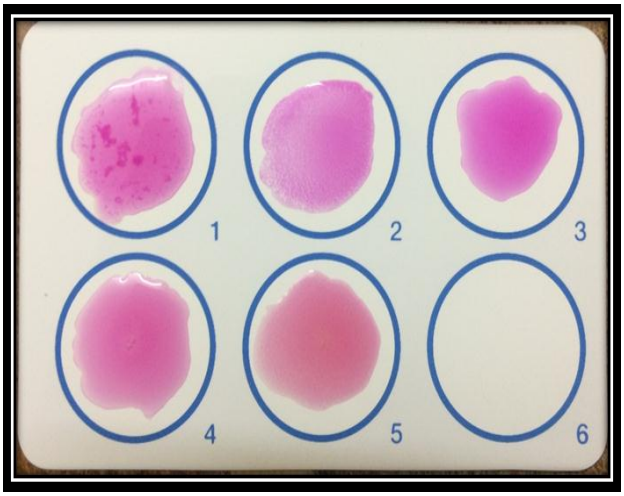

Fig 4: **Showed the positive results of infected animals by using Rose Bengal test. Circle 3 represent control positive, Circle 5 represent control negative while circles 1,2,4, represent the positive cases**

Discussion

Brucella is one of the major etiologies associated with zoonotic disease that cause acute infection signs in Human and animal[19], Diagnosis of brucellosis is the base for control and eradication the disease[16]. This study looking for the more accurate methods to detect *Brucella* infection in cattle and buffalo and by using those tests the data recorded appear variable difference between cattle and buffaloes which infected by brucellosis but the prevalence in cattle little more than

buffaloes which appear non significant. while Abbas and Aldeewan(2009)[20] reported a higher prevalence of brucellosis in cattle than that in buffalo. The higher prevalence in cattle may be attributed to the specie specificity, but in our study there is no differences may be due to mix living system and there is no accuracy in vaccination system especially in buffalo. The infected cattle samples recorded only the *B. abortus* species by using multiplex PCR test while the infected buffaloes samples recorded both *B. abortus* and B. melitansis, mixed living systems may cause cross species barriers infections[4].

The highest positive were recorded by using Rose Bengal test, which showed no significant difference in the incidence of brucellosis between cattle and buffaloes, followed by PCR and MRT and the lower record made by isolation. Stěrba (1987)[21] showed that Rose bengal tests are easy to perform in a short time and it does not require expensive equipment and skilled personnel, with the long shelf lives reagents. In other words, An assays are desirable to diagnose *Brucella* infection in cattle and buffalo, especially when low levels of Antibodies are exist in the blood. The reason for consider the Rose bengal test is gold standard due to fast, sensitive, simple diagnostic techniques for the detection of *Brucella* infection causing abortion in cattle and buffalo, while in the case of using a PCR it needs more experts especially in PCR amplification, or may be the choice of oligonucleotide primer is critical for accurate diagnosis in the clinic[22]. the polymerase chain reaction (PCR) has been adapted with high sensitivity and specificity to detection of DNA[23].

the serological tests like RBT may have cross–reactions with other gram negative bacteria including E. coli, Salmonella[24] or interfere of the vaccine with natural infection, meaning that it is unable distinguish between them[25].

The *B. abortus* vaccine (S19) may interfere with natural infection diagnosis by using RBT which give false – positive or the lipopolysaccharide (LPS) O-chain of other gram negative may cause false positive result with that test while early incubation of the disease may give false- negative by using RBT[26].

As well as, Corbel, M. J (1985)[27] mentioned some of RBT can give false positive result, due to other microorganisms that share epitopes with *Brucella* species during immune response of an animal to infection.

The low specificity of RBT give a larger number of animals testing false positive than in the confirmatory tests[28].

The difference in results between PCR and MRT which appear that the PCR record variable differences in cattle and buffaloes with MRT but it was non significant is possible due to the false – negative which given by MRT when there are small quantities of IgM and Ig A antibodies in milk samples, or decrease of the fat clustering factors[29], and the other reason that milk protein may hamper isolation many of *Brucella* antibodies[30], while, the sensitivity of PCR is high because could detect *Brucella* genome from 30fg of total DNA[31]. On other hand the vaccination, mechanical agitation and mastitis and other factors may be influence the MRT result and give false – positive[32].

Some samples gave with isolation negative results but while PCR gave positive results cause its ability to detect the bacterial DNA regardless living or dead organism while culture detects only living organisms[33]. As well as, PCR could detection of *Brucella* particles and this is evidence the presence of *Brucella* organisms in the samples of suspected cases[34]. The researchers Blasco, J. M (1992)[35] noted that mix infection or contamination can interfere or affect on the results of the research by culture and thus give false negative results or the bacteria lose their ability for culture while the DNA can still be diagnosed by using PCR. A small number of *Brucella* germs are sufficient for diagnosis by PCR as opposed to culture. Since the small number of *Brucella* germs is sufficient for transmit and induce infection, for that the PCR has a high sensitivity to bacterial detection through DNA- extraction protocol and the amount of field sample processed by the assay[36].

The researchers noted that the rate of isolation of the pathogen is high at the beginning of the disease but decrease after relapse and the lowest rate of isolation in chronic cases[36,37].

When the PCR test has the ability to determine the incidence of brucellosis higher than the culture and need just hours compared to the days needed for bacterial culture and detection of the organism and with the availability of the PCR test for laboratories easily, therefor , as suggested by many researches such as, the PCR test for detection of brucellosis is the golden test and can be used to confirm the diagnosis during the epidemiological survey and determine the number and the type of infection conjunction with other serological tests and culture to give the best image and closer to the truth in determining the size of the risk of infection[22].

Although the serological tests have sensitivity higher than the culture in brucellosis infection, the specificities are low due to false positive result[38].

CONCLUSION

The results of RBT, MRT and culture in cattle and buffaloes suggested that these tests may be used for fast routine screening of herds but the confirmatory diagnosis should attempt by using molecular technique PCR test of brucellosis in individual animals. Further more confirmation through PCR is needed for accurate test method to detect Brucellosis in the herd. In addition that PCR the more reliable test important to detect the type of *Brucella* (*abortus or melitansis*) which cannot detected by using the other tests.

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