Evaluation of PCR, ELISA, and Culture Methods for the Diagnosis of Animal Brucellosis .

Sahar A. Al-Bayatti* and Amina N. Al-Thwani**

*****Veterinary Laboratory and Research Department, General Veterinary Company, Ministry of Agriculture.
******Genetic Engineering and Biotechnology Institute for Post Graduate Studies, University of Baghdad.

ABSTRACT

The present study was undertaken to evaluate polymerase chain reaction (PCR) technique in the diagnosis of brucellosis in comparison with the enzyme linked immunosorbent assay (ELISA) and microbiological culture techniques in animal's blood and aborted fetuses.

Thirty animal blood samples were used 20 of them were rose bengal plate test (RBPT) positive and which were subdivided into two groups (11) vaccinated and (9) not-vaccinated, and 10 negative controls. All these samples were examined by PCR using primer pair to amplify a 223-bp region within a gene coding for 31-kDa *Brucella* antigen, ELISA and culture. Five aborted fetuses were also included in this study these were examined by PCR using the same mentioned pair of primers and culture only.

The results of the 20 RBPT +ve blood samples revealed that 13 and 7 were also positive by PCR and ELISA representing 65% and 35% sensitivity, none was positive by culture. There was no positive case among the control group by all the tests which included in this study so its specificity were 100%. The overall agreement between PCR and ELISA and PCR and culture were 73.33% and 56.66% respectively. From the five aborted fetuses which included in this study 4 were positive for *Brucella* infection by PCR and 3 of the 4 were also positive by culture, one was negative by both. The overall agreement between PCR and culture was high and reached 80%. On the basis of

biochemical characteristics results the two isolates which were from sheep fetuses were *Brucella melitensis* and one *Brucella abortus* isolates from aborted buffalo fetus.

Due to many advantages, like speed, safety, high sensitivity and specificity, PCR is recommended to use in the diagnosis of animal brucellosis but its results need more evaluation in the vaccinated animal.

INTRODUCTION

The term "brucellosis" is applied to a group of closely related infectious diseases, all caused by bacterial pathogens in the genus *Brucella*. Manifestation of the disease may be ranged from abortion in the infected female's to orchitis or epididymitis in the male or even death. Characteristically all *Brucella* species establish persistent infection in the mononuclear phagocyte system of the natural host species. Brucellosis causes major economic losses to the agriculture industry and the causative agent is classified as a category B pathogen by the Centers for Disease Control and Prevention (CDC ,2003). Most of the countries that are faced with the economic losses and public health issues caused by animal brucellosis have governmental programs for the eradication or control of the disease. Accurate diagnostic procedures are critical for the success of these programs (Nicoletti, 1982). Because of their potential to detect very small numbers of organisms, PCR –based assays have been applied recently to diagnose many infectious diseases. However there are only a few reports on the use of PCR for the diagnosis of animal brucellosis from blood samples and less from aborted fetuses. Moreover, the advantages of such techniques over the ELISA and culture have not yet been clearly established especially on vaccinated animals.

The aim of this study was to evaluate the new methods of PCR-based assay in the diagnosis of animal brucellosis in compare with ELISA and culture.

MATERIALS AND METHODS

Collection and treatments of samples

Thirty blood samples were collected, in fact that twenty were taken from animals which shows evidence of brucellosis (some are with history of abortion and other RBPT+ve) and ten apparently healthy controls.

Five ml blood sample volume were withdrawn from each animal, 2 ml of it was injected into prepared sterile trypticase soy broth with 2% sodium citrate and incubated for 48 hours at 37ºc, then it was sub cultured on duplicate agar plates one of them incubated aerobically and the other in an microaerophilic atmosphere (containing 5-10% carbon dioxide), *Brucella* spp. were identified using the standard methods advised by Alton *et al.* (1988). The rest of the blood samples were divided as follows: - one ml was used for DNA extraction, using the commercial purification system (Wizard Genomic DNA Purification Kit, Promega, Madison, WI) according to the manufacturer's instruction for DNA purification from blood. The rest of two ml injected into plane tubes and used for serum collection for serology tests. The serum then submitted to RBPT as described by Morgan , (1967) then used for ELISA, using the kit provided by the (NOVATEC-Germany) company and according to their instruction. Five aborted fetuses were also included in this study; two of them were aborted sheep fetuses the other two were aborted buffalo fetuses while one was aborted horse fetus, all were examined by direct culture on trypticase-soy-agar and using their stomach contents, which were withdrawed by a sterile syringe. The specimens were cultured on duplicate agar plates and incubated one

in air and the other in an atmosphere containing an Added 5-10% of carbon dioxide, as advised by Alton *et* al. (1975).

Using the same (Wizard Genomic DNA Purification Kit) DNA was extracted from the stomach contents of the aborted fetuses, then the DNA which was extracted from the blood and from the stomach contents of the aborted fetuses submitted to PCR amplification presses.

PCR assay

DNA amplification system provided by Promega-company, USA and genus specific B4/B5 primer pair designed by Baily to amplify a 223-bp region within agene coding for 31-kDa membrane protein specific to the genus *Brucella* (Baily *et al.* 1992).

Primers name	**Sequences**
B4	5'-TGGCTCGGTTGCCAATATCAA-3'
B5	5'-CGCGCTTGCCTTTCAGGTCTG-3'

The reaction mixture contained 5µl of 10X PCR buffer, 3.5µl of 25 mM MgCl2 , 1.5µl of the dNTP mix (10mM each), 5µl of B4 primer of 10 pmol/µl 6µl of B5 primer of 8 pmol/µl, 0.5µl of 5υ/µl Taq DNA polymerase and 10 µl of sample DNA in a volume of 50µl.

The reaction was performed in a thermal cycler (Techgene, Cambridge Ltd. UK). The cycling condition were an initial denaturation at 95ºc for 5 min, template denaturation at 94ºc for 1 min, annealing at 55ºc for 1 min, and primer extension at 72ºc for 1 min, for a total of 40 cycles, with a final extension at 72ºc for 7 min.

Ten µl of the amplified products were mixed with 3µl of loading buffer and analyzed by electrophoresis in 2% agarose gel stained with 0.5µg/ml ethidium bromide, at 100v for 25 min, in 0.5x TBE buffer, then visualized under UV light using ultraviolet transelumenater(USA). DNA ladder (100-1000 bp) was used.

A sample was considered positive for *Brucella* spp. when a specific fragment of 223 bp was detected in the gel (Baily *et al.*,1992).

Statistical analysis

The percentages of positive, specificity, sensitivity and the overall agreement between PCR and ELISA and PCR and culture were calculated (Showman , 1986).

RESULTS AND DISCUSION

1- Animal's blood samples:

The results of ELISA, PCR and culture detection in animal's blood are shown in Table (1). RBPT results were positive in all the animals included in this table, the ELISA results were positive in only 7 of the infected animals groups which represent 35% sensitivity, but it was negative in the entire 10 control group, representing 100% specificity. The infected animals group was subdivided into two groups **a**-vaccinated, in which the percentage of positive results by ELISA was 54.5% higher than that of the second **b**-non vaccinated group which was 11.1%. Table (2).

PCR was positive in 13 out of 20 infected animals, Table (1), and thus represent 65% sensitivity while it was negative in the control group representing a100% specificity. Figure (1) shows the results of some animal's blood PCR amplification process. The percentage of PCR positive in the vaccinated group which was 81.8% higher than that of the non-vaccinated group which was 44.4% (the same effect previously noticed in ELISA), Table (2).

The results of PCR which compared to that of ELISA showed that the PCR results were identical to that of ELISA only in the control (RBPT-ve) group, while it differ in the infected (RBPT+ve) as shown in Table (3), which revealed that from the 13 positive cases in PCR only 7 were also positive by ELISA with a 46.15% identical rate and from the 7 PCR negative 6 were also negative by ELISA with a 85.71% identical rate.

There were no positive cases by culture among both the infected and the control animal groups, Tables (1 & 2).

Table 1. ELISA, PCR and culture results of animals infected with brucellosis.

Sample No.	ELISA* titer	PCR	Culture	animal's Kind	vaccine state
1	23.7	Neg.	Neg.	Goat	Vaccinated
2	16.8	Pos.	Neg.	Goat	Vaccinated
3	2.8	Pos.	Neg.	Goat	Vaccinated
4	1.1	Pos.	Neg.	Goat	Vaccinated
5	0.9	Pos.	Neg.	Goat	Vaccinated
6	1.1	Pos.	Neg.	Goat	Vaccinated
7	1.2	Neg.	Neg.	Sheep	not- Vaccinated
8	0.9	Neg.	Neg.	Cattle	not- Vaccinated
9	1	Pos.	Neg.	Buffalo	not- Vaccinated
10	15.7	Pos.	Neg.	Buffalo	not- Vaccinated
11	1.1	Pos.	Neg.	Buffalo	not- Vaccinated
12	1.8	Neg.	Neg.	Goat	not- Vaccinated
13	2.2	Neg.	Neg.	Cattle	not- Vaccinated
14	12.5	Pos.	Neg.	Goat	Vaccinated
15	37.4	Pos.	Neg.	Sheep	Vaccinated
16	2.3	Pos.	Neg.	Cattle	not- Vaccinated
17	2.7	Neg.	Neg.	Cattle	not- Vaccinated
18	23.8	Pos.	Neg.	Sheep	Vaccinated
19	18.6	Pos.	Neg.	Sheep	Vaccinated
20	8.2	Neg.	Neg.	Sheep	Vaccinated

*A titer < 9 is considered negative (Neg.) while > 11 positive (Pos.) 9-11 grey zone.

Table 2. Characteristics of infected animals and control groups and the percentages of the tests results.

Groups	**No. of Samples**	**ELISA**			**PCR**			**Culture**		
		Pos.	**Neg.**	**%**	Pos.	**Neg.**	**%**	**Pos.**	**Neg.**	**%**

RBPT (+ve) infected animals	**vaccinated**	11	6	5	54.5	9	2	81.8	0	11	0
	Not-vac.	9	1	8	11.1	4	5	44.4	0	9	0
	Sumtotal	20	7	13	35	13	7	65	0	20	0
control RBPT(-ve)		10	0	10	0	0	10	0	0	10	0

When the total results of both the sensitivity and specificity of each tests was analyzed statistically it was found that PCR was the only efficient test in this group of animal samples.

The figures (3) and (4) illustrate PCR evaluation results in animal samples.

Table 3. Comparison of animals PCR results with ELISA and Culture.

characteristic state of PCR		**No. of Samples**	**ELISA**			**Culture**		
			Pos.	**Neg.**	**Identically%**	**Pos.**	**Neg.**	**Identically%**
PCR+ve in infected groups	**vaccinated**	9	5	4	55.5	0	9	0
	Not-vac.	4	1	3	25	0	4	0
sum total		13	6	7	46.15	0	13	0
PCR-ve in infected groups	**vaccinated**	2	1	1	50	0	2	100
	Not-vac.	5	0	5	100	0	5	100
sum total		7	1	6	85.71	0	7	100

control RBPT -ve	10	0	10	100	0	10	100

From Table (3) the overall agreement (overall agreement: is the proportional similarity of the results of both tests) between the PCR and each of ELISA and Culture were calculated as following:

$$PCR \text{ and ELISA} = \frac{6+6+10}{30} \times 100 = 73.33\%$$

$$\text{PCR and culture} = \frac{7+10}{30} \times 100 = 56.66\%$$

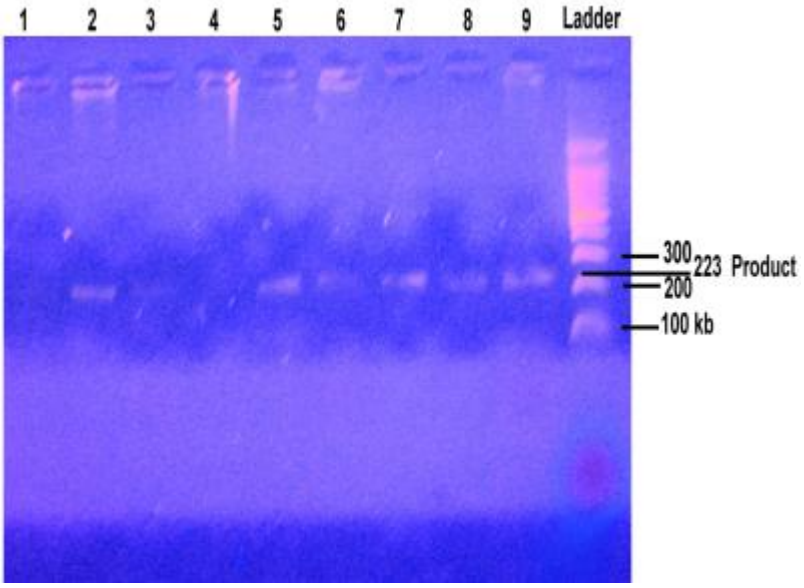

Fig. 1. Results of PCR detection. The amplified fragments were separated by electrophoresis on a 2% agarose gel, stained with ethidium bromide at 100 volts for 25 min. photographed under UV light. First lane is marker, 100-bp ladder.

2- Aborted fetuses.

The results of PCR detection and microbiological culture are shown in Table (4). Three samples were positive by both PCR and culture; one was positive by PCR and negative by culture, while one negative by both PCR and culture, the percentage of overall agreement between them reached 80% as

shown in figure (4).From the three aborted fetuses which were positive by culture 2 were aborted sheep fetuses and one was buffalo's aborted fetus.

Brucella organisms first recognized in smears obtained from fetal membranes stained with modified Ziehl-Neelsen stain, which stained red against a blue background, figure (2), while *Brucella* culture recognized then on the basis of colonial morphology and slide agglutination test, subsequent examination by routine bacteriological methods confirmed the genus *Brucella*, so the obtained isolates were Gram-negative, coccobacilli, arranged singly, in pairs, short chain and with small groups, negative for haemolysis on blood agar, and it does not grow nor lactose- fermenting on MacConkey agar, negative for nitrate reduction and indol production, while they were positive for oxidase and catalase tests. The colony morphology and the bacteriological and biochemical characteristics of the isolates revealed that the isolates which obtained from the aborted sheep fetuses differ from that which obtained from aborted buffalo fetus in that they grew well without CO_2 requirement and they were negative for H_2S production, while the isolate obtained from the buffalo fetus required the CO_2 to grow and was able to produce H_2S, beside the other differences like the host preference and size. On the basis of biochemical results, the isolates from the sheep fetuses were diagnosed as *Brucella melitensis* while the isolate from the buffalo fetus was *Brucella abortus*. Only one case was positive by PCR and negative by culture and was from aborted horse fetus.

Table 4. The results of PCR and culture in aborted animal's fetuses.

Animal's Kind	No. of Samples	PCR		Culture		Identically %
		Pos.	Neg.	Pos.	Neg.	
Sheep fetuses	2	2	-	2	-	100
Buffalo	2	1	1	1	1	100

fetuses						
Horse fetus	1	1	-	-	1	0

80% is the overall agreement between the PCR and Culture.

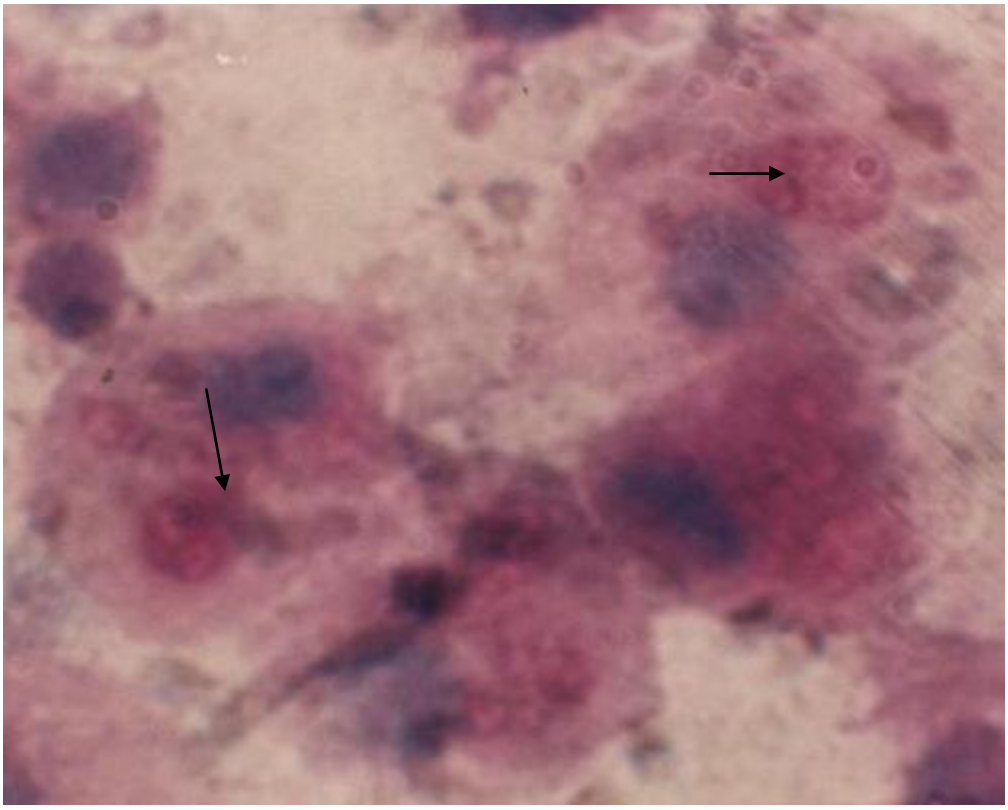

Fig. 2. Fetal membranes stained with Ziehl-Neelsen stain (100X). Notice the round red clumps within the tissue cells.

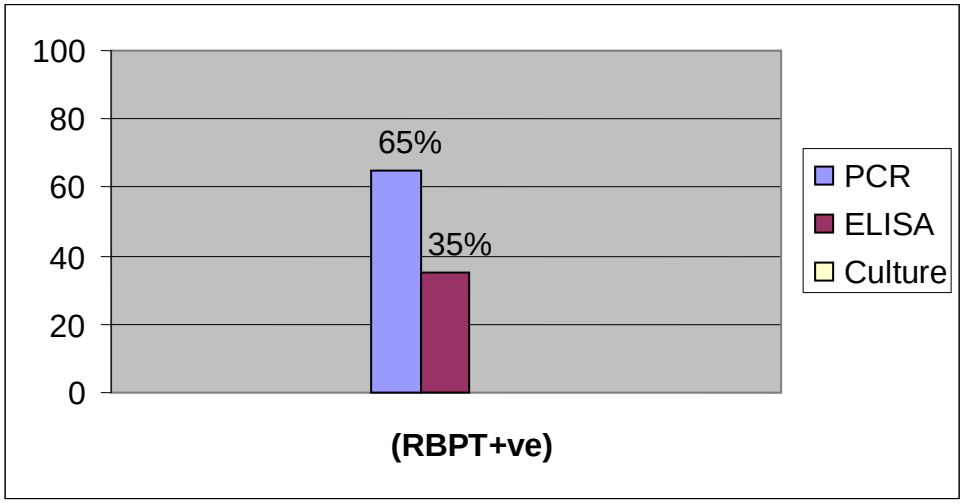

Fig. 3. Chart illustrate the percentages of positive according to PCR, ELISA and culture.

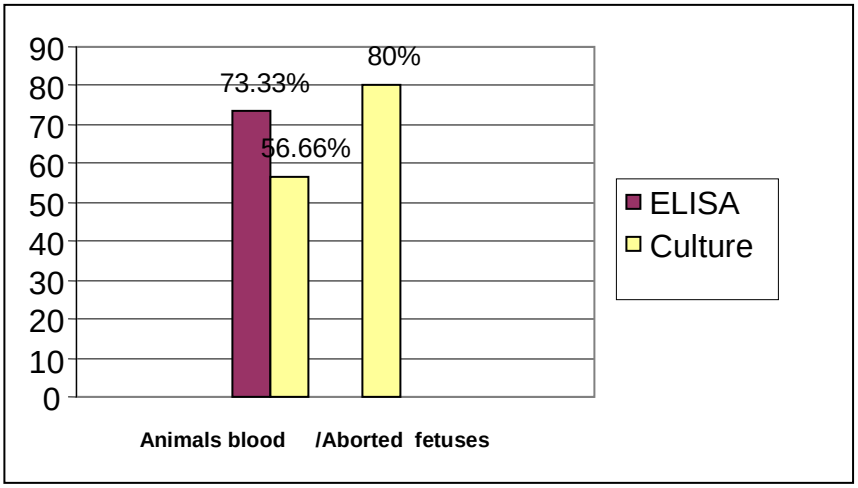

Fig. 4. Percentages of overall agreements between the PCR and each of ELISA and culture.

DISCUSSION

Brucellosis in animals is a serious economic disease. Losses due to abortions, stillbirths, irregular breeding, and loss of milk production are economic consequences, so accurate diagnosis is required to achieve success in disease control. There are probably more procedures to diagnose brucellosis in animals than any other disease. Yet, problem remains such as diagnosing incubative or latent infections and differentiating residual vicinal titers. As none of the available serological test conferred unequivocal specificity, new means of diagnosis depends on genetic tools were adopted. So in this study an evaluation of PCR-based assay capability in diagnosing animal brucellosis was undertaken.

Twenty animal blood samples which were positive by RBPT selected for subsequent tests by ELISA, microbiological culture and PCR. They were subdivided into two groups. 11 vaccinated in which ELISA was positive in 6 of them in the percentage 54.5% and the other 9 not vaccinated group in which ELISA was positive in only one of them in the percentage 11.1%. Seven was the

total number of positive by ELISA. Thus, its sensitivity was 35% and as none of the control group was positive; its specificity reached 100%. ELISA failed to detect 13 RBPT positive samples, 6 of them were also negative by PCR and culture, thus they are truly negative while 7 were positive by PCR which mean that they may be in the early stage of infection when IgG is under the level of detection by ELISA or it maybe false PCR results due to the high sensitivity of the test which lead it to detect the gene of the vaccine strain (there is no study on the vaccination of *Brucella* and its effects on the PCR results to compare the results with). Anyhow no single test appears to be free from demerits, this is the reason why many workers carried out comparative studies to determine the efficacy of different tests (Nielsen, 2002), and this is what was concluded by Gall and Nielsen, (2004) after reviewing various serological tests, (although they found that ELISA was more sensitive), besides Guarino *et al.*, (2000) during their study on buffalo noticed that there were 5 samples negative by ELISA but positive by PCR. Also Gupta *et al.* ,(2006) in their study on goat found that PCR was able to detect 12 sample which was negative by ELISA. However, many others revealed that ELISA is of higher sensitivity among other serological tests (Kerby *et al.*,1997 ; OIE, 2004 ; Kanani, 2007 ; Tanmay,2007).

PCR was able to detect 13 out of 20 RBPT positive animal cases, 6 of them were positive also by ELISA, while one of the ELISA positive was negative by PCR, which may be considered as false negative PCR result according to Navarro *et al.* ,(2004) who mentioned that if there are clinical and serological finding to support the presence of brucellosis in patients with negative blood culture and negative PCR should considered as false negative PCR and blood culture results, and this is were in the chronic granulomatous stage of infection when the DNA is under the threshold of detection (10-100fg) by the specific primers B4/B5.

PCR-based assay was more sensitive, 65% in comparison with ELISA and culture, while its specificity reached 100%. Other researchers also conclude that PCR was more sensitive in comparison with ELISA and other conventional serological tests (Guarino *et al.* ,2000 ; Gupta *et al.* ,2006 ; Amin *et al.,* 2001 ; Kanani , 2007 ; Tanmay**,** 2007).

Of all infected and control blood samples, none was positive by culture, this may be due to that most of them were vaccinated. Anyhow Guarino *et al.* (2000) also could not detect *Brucella* or any other organisms in buffalos blood of 44 samples which included in their study while they were able to detect 13 of them positive by PCR.

Some researchers tried to overcome some of the PCR and the ELISA techniques limitations in diagnosis of brucellosis by the adoption of a combination between them (Morata *et al.* ,2003).

Aborted fetuses due to brucellosis showed nonspecific lesions. In view of the considerable problems related to direct diagnosis of brucellosis in aborted fetuses, one of the main objectives of the present study was to evaluate the PCR using the same specific primers used in all the study as a tool for the detection of *Brucella* spp. in aborted fetuses. Taking the microbiological culture as a standard for comparison in this group, three samples were positive by PCR and culture, one was positive by PCR and negative by culture and one was negative by both PCR and culture, considering the one that was negative by culture is not a guarantee of the absence of *Brucella* infection. This makes PCR more sensitive than culture while the specificity was 100% and identical to culture. The results of the present study confirmed the results of Scarcelli *et al.*, (2004) who analyzed samples obtained from 67 aborted bovine fetuses by means of bacteriological methods and PCR and also found that the samples that were positive by PCR (34/67) more than that of culture (26/67), so he concluded that

PCR was more sensitive than culture. Other researchers have observed the same divergences between PCR and culture results (Fekete *et al.*,1992; Cetinkaya *et al.*,1999 ; Marques *et al.* ,2001) while Leyla *et al.* ,(2003) who detected *Brucella* by PCR and culture from fetal stomach contents found 38 of 39 culture positive were also positive by PCR, which was negative in all of the culture negative samples. So PCR sensitivity and specificity were determined as 97.4 and 100% respectively.

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تقييم كفاءة اختبارات PCR ، الاليزا والزرع الجرثومي في تشخيص داء البروسيلات في الحيوانات .

سحر احمد البياتي* **آمنه نعمه الثويني****

* قسم المختبرات والبحوث البيطريه / الشركه العامه للبيطره/ وزارة الزراعه

** معهد الهندسة الوراثية والتقنيات الاحيائيه للدراسات العليا/ جامعة بغداد/ وزارة التعليم العالي والبحث العلمي

الخلاصة

استهدفت الدراسة الحالية تقييم الفحص المعتمد على تقنية PCR (polymerase chain reaction) بالمقارنة مع كل من فحصي الاليزا (enzyme linked immunosorbant assay) والزرع الجرثومي في تشخيص داء البروسيلا في دم الحيوانات واجنتها المجهضه.

تم أستخدام 30 عينة دم لحيوانات منها 20 موجبه لفحص الروزبنكال [Rose (RBPT+ve) Bengal Plate Test] والتي بدورها تم تقسيمها الى مجموعتين الأولى ملقحه بعدد(11) والثانيه غيرملقحه بعدد(9) و 10 سيطرة سالبه للفحص المذكور.كل هذه العينات تم فحصها باللأليزا وال PCR باستخدام زوج البادئات المخصص بمضاعفة جين بوزن 223 زوج قاعدي مشفرلمستضد البروسيلا ذو وزن 31 كيلودالتون كما تم أستخدام 5اجنة مجهضة اختبرت بالـ PCR وباستخدام نفس البادئات المشار اليها والزرع الجرثومي فقط.

تم تأكيد الأصابة بالبروسيلا في عينات دم الحيوانات العشرين والتي كانت موجبة لفحص الروزبنكال في 13 حسب اختبار PCR و7 حسب أختبار الأليزا وهي بهذا تمثل نسب حساسية بلغت 65% و 35%على التوالي. لم يتم الحصول على اي عزل جرثومي من هذه العينات. كما ان الأختبارات الثلاثة كانت سالبة في جميع افراد مجموعة السيطرة بهذا تكون الخصوصية لكل منهم 100%. نسبة التوافق الكلية بين PCR والأليزا و PCR والزرع الجرثومي بلغت 73.33% و 56.66% على التوالي.

خمسة اجنة مجهضة تم شمولها في هذه الدراسة ، اربعة منها اظهرت نتيجة موجبة لفحص PCR وثلاثة من هذه الأربعة كانت ايضا موجبة للزرع الجرثومي. واحدة كانت سالبة لكلا الاختبارين. نسبة التوافق الكلية بين PCR والزرع الجرثومي كانت الأعلى وقد بلغت 80% . على اساس نتائج

الأختبارات البايوكيميائية، العزلتين اللتين تم الحصول عليهما من اجنة الأغنام المجهضة كانتا من نوع *Brucella melitensis* بينما العزلة التي تم الحصول عليها من جنين الجاموس كانت من نوع *Brucella abortus* .

بلنظر للفوائد العديده لل PCR والمتمثله في السرعه والامان والحساسيه والخصوصيه العاليتين نوصي باستخدامه في التشخيص. الان قدرته على تمييز الحيوانات المصابه من الملقحه غير دقيقه وتحتاج مزيد من الدراسه.