Serological and Molecular Studies on the Diagnosis of Bovine Brucellosis

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Abstract: The animals included in this study were180 naturally infected non vaccinated cows in governmental farm (group 1), 125 brucella free cows in which strain 19 vaccination had never been practiced (group 2) and 530 strain 19 vaccinated cows (group3). Sera from these animals were examined for brucellosis using RBPT, BAPAT, Riv.T, TAT, CFT. For cows suspected to be infected with brucellosis, the results revealed that the percentage of positive reactors for RBPT, BAPAT, Riv.T, TAT and CFT were 139(77.2%), 143(79.4%), 130(72.2%), 146(81.1%) and 131(72.8%) respectively. While for brucella free cows, the percentage of positive reactors were 2(1.6%), 4(3.2%), 1(0.8%), 5(4%) and 1(0.8%) respectively. cows vaccinated with s19 vaccine using RBPT, BAPAT, Riv.T, TAT, and CFT revealed that agglutinins were quite evidenced 2 weeks post vaccination. The number of animals positive for Brucella antibodies reached maximum at 4 weeks post vaccination. The incidence of isolation from supramamary and retropharyngeal L.n, liver , spleen and milk samples were 54%, 48%, 50% , 38%4and 30.3% respectively. the obtained results indicate that *brucella melitensis* biovar 3 still the prevalent type affecting cattle in Egypt. In the present study the results revealed that PCR assay able to differentiate S19 vaccinated animals from those infected ones.
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1. Introduction

Brucellosis is a zoonotic world wide infectious disease of animal that is caused by a number of host adopted species of gram negative intracellular bacteria of the genus brucella **(Ochali *et al.,* 2005)**, leading to tremendous economic losses as well as a potentially debilitating infection in man **(Hosein *et al.,* 2010)**.

Among the different species of genus brucella, *Brucella aboruts* is the common strain infecting cattle all over the world while *Brucella melitensis* is affecting mainly sheep, goats and also other species **(Alton, 1990)**.

Cattle Brucellosis is usually caused by *Brucella abortus* biovars and occasionally by *Brucella melitensis* **(OIE, 2008).** In Egypt, starting from 1998 *Brucella melitensis* was reported to be the common strain isolated from cattle as reported by **Shalaby *et al.* (2003); Sayour (2004) and Shehata (2004).**

The disease is mainly characterized by abortion, stillbirths or weak calves and lactating cows may show decrease in milk yield **(Matope *et al.,* 2010)**. In bulls, brucellosis may manifest as unilateral or bilateral orchitis and sterility, while in all age groups, hygromata involving one or more leg joints may be observed **(Muma *et al.,* 2007).**

Diagnosis of brucellosis is based on isolation of the organism from infected animals but this is a cumbersome and time consuming task, due to the fact that these fastidious organisms grow slowly on primary isolation **(Meyer, 1981)**. Moreover, it is not possible to isolate Brucella every time even from infected individual **(Ray, 1979)**, therefore, assessment of antibody response employing serological test play a major role in the routine diagnosis of brucellosis and supported where appropriate by bacteriological examination **(Alton *et al.,* 1988)**.

PCR is a quick and reliable diagnostic methods as the most sensitive of the developed technique is the amplification of nucleic acid by polymerase chain reaction (PCR) **(Saiki *et al.,* 1988; Kramer and Coen, 2001)**. The high sensitivity of this technique has the advantage that it may lead to the earlier detection of the disease (**Deacon and Lah, 1989; Gall and Nielsen, 2004)**.

PCR is a rapid tool for molecular biology, very sensitive that a single DNA molecule can amplified, and single – copy genes are extracted out of complex mixture of genomic sequences and visualized as a distinct band on agarose gel **(Persing, 1991 and Gupta *et al.,* 2006)**. The development of one day test for brucella on the easily performed, highly specific and extremely sensitive PCR, can detect brucella organisms directly in tissues and body fluids **(Fekete *et al.,* 1990; Klevzas *et al.,* 1995a; Bricker, 2002 and Yu and Nielsen, 2010)**. Therefore, The aim of this study was the evaluation of the most commonly employed serological test used for diagnosis of bovine brucellosis including Rose Bengal plate test(RBPT), buffered acidified plate test(BAPT) , rivanol test(Riv.T), tube agglutnation test(TAT) and complement fixation test(CFT). Monitoring antibody response of S_{19} vaccinated cows following vaccination up to 24 weeks post vaccination employing the above mentioned tests, isolation and identification of brucella strains affecting cattle, estimation of sensitivity, specificity and ability of applied tests in differentiation of brucella infected from vaccinated animals, evaluate PCR as rapid tool for diagnosis and differentiation of Brucella infected from S_{19} vaccinated cows were carried out

2. Material and Methods

Animals:

Naturally infected cows:

A total of 180 naturally infected non vaccinated cows in governmental farm where *Br. melitensis* is endemic. These cows had a history of abortion and reproductive troubles (group 1).

Brucella free cows:

A total of 125 animals from brucella free areas and strain 19 vaccination had never been practiced (group 2).

Strain 19 vaccinated cows:

A total of 530 cows, these were negative to serological tests at the time of vaccination. The animals were vaccinated between 3 to 8 months of age with a dose of 3-8x10^9 CFU. They were bled at 2 weeks post vaccination and every 2 weeks until 24 weeks post vaccination (group 3).

Samples:

Serum samples for serological examination:

Blood samples were allowed to clot and the sera were separated by centrifugation and stored at -20 ^oC in the deep freezer for serological tests.

Blood sample for polymerase chain reaction:

Five ml of blood were collected from the Jugular vein of cattle (animals having history of abortion or infertility problems) through a sterile dry needle into a sterile heparinzed vacationer tube. The samples were stored at-80 ^oC until used.

Milk sample for bacteriological examination:

About 20 ml of milk were collected from udder of reactors cattle into a sterile vacationer tube. Milk samples used for bacteriological examination were stored at 4ºC.

Collection of tissue specimens:

Different tissue specimens were collected from brucella seropositive slaughtered cows for bacteriological examination. Lymph nodes especially supramammary and retropharyngeal lymph nodes were taken from the carcasses including the surrounding fat and without cutting of the obtained lymph nodes. The collected lymph nodes and internal organs were packed in sterile disposable plastic bags and were transferred on ice packs to the brucella department laboratory (AHRI) as soon as possible. They were kept frozen at -20 ^oC until cultured.

Serological Examination: All sera were tested for antibodies against brucella by RBPT according to Morgan *et al.* (1969), BAPAT and Riv.T according to Anon (1984), TAT and CFT according to Alton *et al.,* 1988.

Bacteriological examination of organs for brucella:

Isolation, identification, detection of smooth colonies and biotyping of brucella organisms were carried out according (Alton *et al.,* 1988).

Polymerase Chain Reaction (PCR):

DNA extraction from blood samples

DNA was extracted from blood using Blood DNA Preparation Kit (Jena Bioscience Cat. No. PP-205S) . Extraction were performed by adding 300 µl of whole blood to a 1.5 ml microtube containing 900 µl RBC Lysis Solution ,invert 10 times and incubate for 3 min at room temperature with occasional inversion. Centrifuge for 30 sec at 15,000 g. and remove the supernatant with a pipet leaving behind the visible white cell pellet and about 10-20 µl of the residual liquid. Vortex the tube vigorously for 10 sec to resuspend the white cells in the residual liquid, then add 300µl Cell Lysis i Solution to the resuspended cells and pipet up and down to lyse the cells until no clumps are visible. For protein Precipitation add 100 µl Protein Precipitation Solution to the cell lysate. Vortex vigorously for 20 seconds to mix well and centrifuge at 15,000 g for 1 min. Transfer the supernatant into a clean1.5 ml microtube containing 300 µl Isopropanol >99%. Mix the sample by inverting gently for 1 min. and centrifuge at 15,000 g for 1 min. Discard the supernatant and drain tube briefly on clean absorbent paper. Add 300 µl Ethanol 80% and invert the tube several times to wash the DNA pellet. And centrifuge at 15,000 g for 1 min. Carefully discard the ethanol and dry at room temperature for about 10 to 15 min. Add 50-100 µl DNA Hydration Solution. and incubate the sample at 65°C for 30 min to accelerate rehydration. Store DNA at -20°C or -80°C till PCR performed.

DNA amplification:

Oligonucleotide primers:

Table (1): Sequences of oligonucleotide primers used for PCR

	Sequences	Amplified product
P1	5`TGGAGGTCAGAAATGAAC3`	282 bp
P2	5` GAGTGCGAAACGAGCGC3`	
Br. abortus	5'- GAC GAA CGG AAT TTT TCC AAT CCC	498 bp
IS711	5'- TGCCGA TCA CTT AAG GGC CTT CAT	

DNA amplification was done by 2 different PCR sets of primers. Oligonucleotide primers specific for *B. abortus* were used to amplify the insertion sequences IS711 **(Betsy and Shirley, 1994)**..

The PCR 25 µL of reaction mixture contained 10 mM tris HCl (pH 8.4), 50 mM KCl, 1 mM magnesium chloride, 200 µM each deoxyribonucleoside triphosphate (dATP, dGTP, dCTP, dTTP), 10 PM of each oligonucleotides primer , 1 µ of Taq polymerase (Fermentas), 2–4 µg of total DNA extracted from blood samples and 100 ng from the positive controls.

For P1 and P2 primers , PCR was performed as follows: 35 cycles of PCR with 1 cycle consisting of 20s at 95ºC for DNA denaturation, 1 min at 50ºC for primer annealing and 1 min at 72ºC for polymerase mediated primer extension. The last cycle included incubation of the sample at 72ºC for 7 min. Samples were considered positive when a single band of DNA at 282 bp

For IS711 and B. *ab*ortus primers, After an initial denaturation at 93 ºC for 5 min, the PCR profile was set as follows: template denaturation at 95 ºC for 1.25 min., primer annealing at 55.5 ºC for 2 min. and primer extension at 72 ºC for 2 min., for a total of 35 cycles, with a final extension at 72 ºC. Samples were considered positive when a single band of DNA at 498 bp**.** All PCR were performed in a DNA thermocycler (Perken Elmer model 9600).

Electrophoresis of PCR Products:

7 µl The PCR products were loaded on ethidium bromide stained 2% agarose gel. Analysis of species-specific pattern were done by comparing the molecular weight of DNA fragments with the reference DNA marker(100 bp DNA ladder Jena Bioscience Cat. No. M-214 and 50 bp DNA ladder Jena Bioscience Cat. No. M 202) , then photographed using a digital canon Camera.

3. Results And Discussion

Brucellosis is an infectious disease of animals that is caused by Gram negative intracellular bacteria of genus brucella. It is worldwide zoontic disease that is recognized as a major cause of heavy economic losses to live stock **(Adam, 2002).** The disease is characterized by abortion retained placenta, arthritis and epididmyitis

In apparent infection is however common and is an important source of transmission of the disease **(Acha and Szyfres, 1980)**. Because lack of clinical signs, laboratory diagnosis mainly by serological tests is essential.

Diagnosis of the disease is the cornerstone of any control program and is based on bacteriological and immunological findings . The use serological tests are recommended as a means of indirectly diagnosing the disease **(Farina, 1985).**

The most difficult tasks in the serological diagnosis of bovine brucellosis has been the discrimination of infected from vaccinated animals, since vaccinated animals tend to yield persist post vaccinal immune response and other gram negative bacteria such as *Yersinia enterocolitica* may cross react with smooth brucella spp.**(Baldi *et al.*, 1996).**

In the present study different serodiagnostic tests, including Rose Bengal plate test (RBPT), buffered plate antigen test (BAPAT), rivanol test (Riv.T), tube agglutination test (TAT) , complement fixation test (CFT) were used, Moreover trails for detection and isolating the organism from cattle tissues and milk were also done. PCR were employed for diagnosis of brucellosis among non-vaccinated cows suspected to be infected with brucellosis from governmental farm where *Br. melit*ensis is endemic as well as differentiation between naturally infected and S_{19} vaccinated cows.

In this study, it seemed that RBPT detected 139 (77.2 %) from 180 non vaccinated cow suspected to be infected with brucellosis (group1) and 2(1.6%) from 125 Brucella free cows (non reactors) (group 2),Table (2).

Table (2): Results of standard erological tests for detection of brucella infection in examined cows.

Examined animals	No.	RBPT		BAPT		RIVT		TAT		CFT	
		No.	%	No.	%	No.	%	No.	%	No.	%
Suspected cows	**180**	**139**	**77.2**	**143**	**79.4**	**130**	**72.2**	**146**	**81.1**	**131**	**72.8**
Brucella free cows	**125**	**2**	**1.6**	**4**	**3.2**	**1**	**0.8**	**5**	**4**	**1**	**0.8**

The test gave the lower incidence of positive reactors among suspected and brucella free cows compared with BAPT and TAT. This finding may be attributed to inhibition of non specific agglutinins by acidic pH of antigen as reported by **Rose and Roepk (1957); Oomen and Waghela (1974)** who considered the results of the test as specific and recommended the use of such test as field one to distinguish between specific and non specific agglutinins.

Nicoleltti (1967) recorded that the RBPT is more accurate indicator for brucella infection than TAT. **Morgan *et al.* (1969)** suggested that RBPT similar to CFT and highly sensitive than TAT beside its ease of application. **Corbel (1972)** showed that RBPT activity is associated only with the IgG class immunoglobulin especially IgG1 which is enhanced in acidic solution.

It appeared in this study that the BAPAT detected 143 (79.4%) positive reactors among suspected cows and 4(3.2%) among non reactors (Brucella free cows) Table (2). The high sensitivity of this test mainly is due to the fact that it detects both IgG and IgM molecules **(Nelson, 1989)**. Even IgG1, which is not agglutinating material at neutral pH, is active at low pH of BAPAT **(MacMillan, 1990).**

Our results agree with **El-Gibaly *et al.* (1990)** who concluded that BAPAT is sensitive test for diagnosis of *Br. melitensis* infected cows**. Refai (1989)** reported that it was decided to use BAPAT as presumptive test due to its high sensitivity, and added that positive samples should be then tested by other serological confirmatory test.

Regarding to RivT where its results were presented in Table (2) which were 130 (72.2%) positive reactors among suspected cows and 1(0.8%) among Brucella free cows. Our results are in agreement to those reported by **Hamdy (1992) and Anwar (1999).**

The lower positive incidence than RBRT and BAPAT may be due to the precipitating activities of Rivanol solution of the IgM So the test only detect IgG_1 and IgG_2 immunoglobulin as recorded by **Margan (1967) and Pietz and Gowart (1980)**.

The specificity of RivT was reported to be high in diagnosis of brucellosis in the examined farm animals which agreed with the results reported by different authors **(Nicoletti, 1992 and El-Enbawy *et al.*, 1995).**

Application of TAT on serum samples of suspected and non reactors (Brucella free cows) were recorded in Table (2) which is 146 (81.1%) and 5 (4%), respectively. It is appeared that the TAT among all tests used in this study gave the highest rate of positive animals compared with other traditional serological tests. This may be explained by that test has a high sensitivity with respect of IgM rather than IgG as reported by **Alton (1977).** On the other hand, **MacMillan (1990)** concluded that TAT failed to show significant titres in recent and chronic brucella infection. while **Corbel (1972**) reported that TAT gives false positive reaction as a results of cross reaction between the antigen of brucella and other organisms or due to the presence of non specific agglutinins in bovine sera.

From the obtained results it is noticed that the presence of some samples collected from non reactors (Brucella free cows) reacted positively with RBPT, BAPT and TAT. This may be attributed to the presence of some bacteria as *Escherichia coli*, *Salmonella Dublin*, *Yesinia enterocolitica* 0:9 and *Pasteurella tularensis* in the body fluids and secretions which react positively with the tests used in diagnosis of brucellosis causing faults or error in the interpretation of the results.

Employing of CFT in this study revealed 72.8% positive reactors among suspected cows and 0.8% among non reactors (Brucella free cows). The test as shown from collective data of different serological tests Table (2) gave negative results in many serum samples that were identified reactors in other tests such reaction may be regarded as false positive reactions. **El-Gibaly *et al.* (1975)** concluded that TAT must be confirmed by CFT to prove that all animals are brucella free. The test has been recommended as a confirmatory test by several authors **(Salem *et al.*, 1987; Hosein, 1996 and Ghanem, 1998).** In addition **Morgan *et al.* (1978)** stated that in old standing chronic infection CFT is often positive while the other agglutination tests are negative. Another advantage of this test as reported by **Jones *et al.*(1963)** that in recently infected herds, cattle developed complement fixation titers before agglutination tests.

Strain 19 is the most commonly used in vaccination program against bovine brucellosis in Egypt and all over the world. The main advantage of S_{19} is it gives a considerable humeral and cellular protection against brucellosis even when we use it at a reduced dose. Yet its main disadvantages are the production of smooth antibodies which interfere with the diagnosis of disease using conventional serological tests **(Alton *et al.*, 1984 and Crawford *et al.*, 1991).** In the present work the profile of antibody producing in 530 cows following vaccination with S_{19} using conventional serological tests (RBPT, BAPT, Riv.T, TAT and CFT) are shown in Table(3). The antibodies gave evidenced 2 weeks post vaccination reached their peak 4 weeks post vaccination.

The results showed in Table (3) revealed that 4 weeks post vaccination the percent of vaccinated cows showed positive reaction by using RBPT, BAPT, Riv.T, TAT and CFT (97.7%, 99.4%, 89.8 %, 98.5% and 84.9%) respectively.

These results agreed with that of **Jones *et al.*(1980)** who reported that in animals that have been vaccinated with smooth strain vaccines give false positive reaction by using traditional serological test.

As **Crasta *et al.* (2008)** reported that conventional serological methods principally measure antibody to S-LPS either as presented on the intact bacterium or immobilize on a plastic matrix. The antibody response of animals to S-LPS from smooth vaccines or field strains decrease by time but antibody titers persist longer in naturally infected animals, so these conventional serological tests have limited ability to discriminate vaccinated from naturally infected animals.

In this concern **Nielsen *et al.* (1989)** reported that conventional serological methods such as agglutination or complement fixation test measure antibodies to smooth LPS since animals vaccinated with S_{19} and animals naturally infected with field strains develop similar anti-LPS responses, so it is difficult to establish their status by means of conventional test.

Typing of brucella organism isolates from lymph nodes (Supramammry, and retropharyngeal lymph nodes), spleen and liver from slaughtered cattle which proved to be serologically positive are presented in Table (4). Results in this Table show that only 27 isolates out of 50 slaughtered reactor cows were recovered from examined samples, all typed as *Brucella melitensis* biovar 3.

Results showed in Table (4) revealed that the rate of isolation from examined lymph nodes (Supramammry, and retropharyngeal lymph nodes), spleen and liver were 54%, 48%, 50% and 38%.

Table (3) Results of standard serological tests to evaluate Brucella S_{19} vaccinated cows sera.

Examined animals	No of examination	Time of examination	RBPT		BAPT		RIVT		TAT		CFT	
			No.	%	No.	%	No.	%	No.	%	No.	%
Vaccinated cows	530	0	0	0	0	0	0	0	0	0	0	0
		2	330	62.3	361	68.1	320	60.4	415	78.3	319	60.2
		4	518	97.7	527	99.4	476	89.8	522	98.5	450	84.9
		6	520	98.1	521	98.3	505	95.3	519	98	453	85.5
		8	420	79.2	489	92.3	323	60.9	431	81.3	312	58.9
		10	376	70.9	355	67.0	219	41.3	295	55.7	151	28.5
		12	149	28.1	151	28.5	89	16.8	248	46.8	39	7.4
		16	46	8.7	53	10	12	2.3	160	30.2	2	0.4
		20	7	1.3	9	1.7	0	0	42	7.9	0	0
		24	4	0.8	0	0	0	0	22	4.2	0	0

Table (4): Results of isolation and identification of Brucella organism from lymph nodes organs and milk of examined cows.

suspected examined Animal	No.	Organ								Milk		Type of isolate
		Supranamary L.n		Retropharyngeal L.n		Spleen		Liver				
		No.	%	No.	%	No.	%	No.	%	No.	%	
Slaughtered	50	27	54	24	48	25	50	19	38	ND	0	Br.mel. biovar 3
live	13	ND	0	ND	0	ND	0	ND	0	4	30.8	Br.mel. biovar 3

ND = not done; Br. mel. = *Brucella melitensis.*

These findings come in accordance with **Esmail *et al.* (2002)** who isolated *Brucella melilensis* from supramanmry lymph nodes (3 out of 16) cases naturally infected cows. On the other hand, a higher rate of isolation of Brucella organism from supramammary lymph node was reported by **Laing *et al.* (1988).**

Out of 33 milk samples collected from suspected live animals 10 brucella isoletes were recovered and all the isolated identified on the base of biochemical and serological reaction as *Brucella melitensis* biovar 3 as shown in Table (4).

These results were in agreement with those obtained by **Montasser (1995); Hosein *et al.* (2002) and Al-Ani *et al.* (2004).**

Therefore the above mentioned results indicated the importance of using several procedure to overcome of the problem of escaping of some infected animals in diagnosis of brucellosis as emphasized by **Necoletti and Muraschi (1966).** Therefore, it is importance to use more than one diagnostic test for the diagnosis of brucellosis.

Regarding for PCR assay, the optimal reaction condition for amplifying a template DNA was optimized in relation to different factors such as: Primer structure, magnesium ion concentration, annealing temperature and DNA polymerase enzyme.

The effect of these factors was qualitatively evaluated by determination of the PCR amplification products fractionated on agarose gel and visualized under U.V. light after staining with ethidium bromide.

The obtained data, indicated that the optimal concentration of magnesium ion in the reaction was 2 Mm, Taq polymerase enzyme concentration was 1U, primer concentration was 20 Pm and optimal annealing temperature was 55ºC for specific–PCR and 37 for RAPD – PCR where the strongest amplification was obtained.

These results agreed with **Fekete *et al.* (1990)** who mentioned that the optimal PCR condition for amplifying a template DNA vary from one primer to another and necessary to be determined empirically.

The results of the PCR tests using the P1 and P2 primers specific for *Brucella* melitensis which were performed on *Brucella melitensis* field strain as positive control and the blood samples of infected animals, are shown in (Fig.1). The *Brucella melitensis* field strain as positive control and 18 out of 20 blood samples of infected animals were brucella positive as indicated by the size of the PCR product in agarose gel (approximately 282 bp).

The results of the PCR tests using IS711 and *Br. ab*ortus primers which were performed on *Brucella abortus* vaccine strain as positive control and the blood samples of vaccinated animals, are shown in (Fig.2). The *Brucella abortus* vaccine strain as positive control and 14 out of 15 blood samples of vaccinated animals were positive as indicated by the size of the PCR product in agarose gel (approximately 498 bp) .

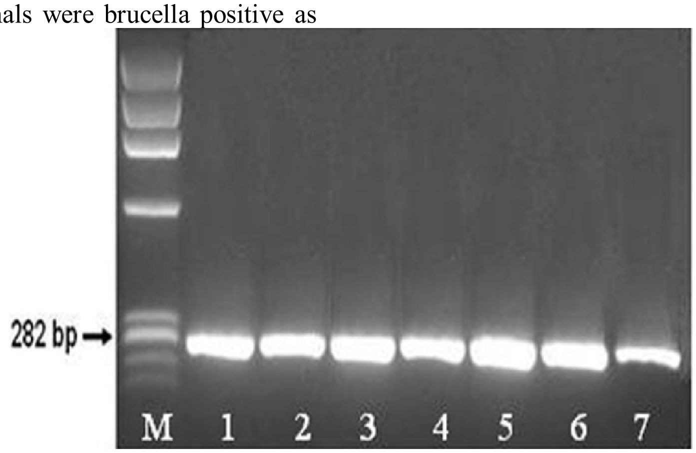

Fig. (1) : Agarose gel electrophoresis of PCR-amplified *omp 2* gene fragments from *Brucella melitensis* strains. The figure shows a single band 282-bp DNA fragment. M: ØX 174 RF DNA *HaeIII* digest marker (Biolabs). Lane , 1 *Br. melitensis biovar 3* field strain. Lanes:2 – 7, represintive blood samples of infected animals.

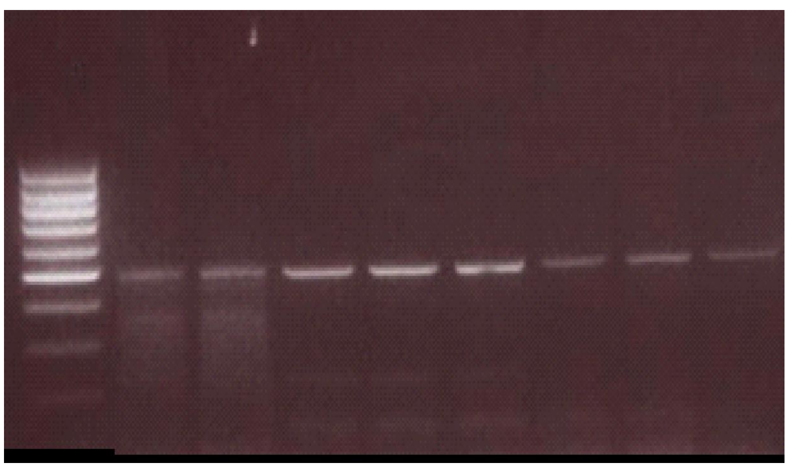

Fig. (2): Agarose gel electrophoresis of PCR-amplified *product* from *Brucella abortus* vaccine strains and blood samples of vaccinated animals. The figure shows a single band 498-bp DNA fragment. M: 100 bp DNA ladder (Promega). Lane 1 ,*Brucella abortus* vaccine strains. Lanes:2 – 8, represintive blood samples of vaccinated animals.

The PCR is a highly sensitive method which makes it possible to detect nucleic acid amplification products. The results can be obtained rapidly so that they can be used not only to support bacteriological investigation but also to make them more reliable (**Fekete *et al.*, 1990; Leal-Klevezas *et al.*, 1995b and Gallien *et al.*, 1998).** Because of the serological cross reactivity of brucella with other Gram negative bacteria such as *Yersinia enterocolitica* serotype 0:9, false positive results must be excluded for this reason, the primer P1, P2 and *Brucella aboruts* and IS711 were used in the PCR because they do not show such cross activity (**Betsy and Shirley, 1994).**These primers were also tested on DNA extracted from *Enterobacter* *aerogene*, *Legionella Pneumophilia* and *E. coli*, no amplification was observed denoting the specifity of PCR amplification of brucella sequences **(Bardenstein *et al.*, 2002).**

In the Present study,a number of variables were tested to determine the most suitable condition for DNA amplification as magnesium ion concentration, annealing temperature and DNA polymerase enzyme. Optimal amplification of a target sequence occurred when the primer annealing temperature was 55°C magnesium concentration was 2.5 mM and DNA polymerase enzyme was 1 U. The extracted DNA from blood samples was washed with TE X 100 buffer where chelex 100 was found useful for concentrating and removing polyvalent cantions from DNA **(Romero *et al.*, 1995).**

In the present study, 18 out of 20 of blood samples of infected cows and 14 out of 15 of blood samples of vaccinated cows had a positive PCR Tables(5,6). The sensitivity therefore being 90% for infected and 93.3% for vaccinated cows. The results revealed that PCR assay also able to differentiate S_{19} vaccinated animals from those infected ones **(Leal-Klevezas *et al.*, 1995a; Doosti and Dehkordi, 2011).**

Table (5): Detection and identification of brucella from blood of infected cow by using PCR.

No of blood sample examined	No. of brucella organism detected	Brucella species
20	18	*Brucella melitensis*

Table (6): Detection and identification of brucella from blood of vaccinated cow by using PCR.

No of blood sample examined	No. of brucella organism detected	Brucella species
15	14	*Brucella abortus* Vaccine strain

The presence of polymerase inhibitors could account for a PCR-negative result in the remaining samples. Mainly substances has been suggested to be amplification inhibitors, including hemoglobin, heparin, phenol, EDTA and SDS **(Jackson *et al.*, 1992 ; and Yu and Nielsen, 2010).** Other factors that may account for the false-negative PCR result are a number of brucella organisms below the detection limit, the degradation of target DNA in the sample and insufficient DNA extraction.(**Radwan and Ibrahim, 2000).**

In conclusion, PCR is considered to be the more reliable and accurate technique in comparison with serogical tests and tissue cultures. The major advantage is the speed with which the assay can be performed where, results could be obtained within less than 24 hours, and able to differentiate S_{19} vaccinated animals from those infected ones.

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