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Amos PCR as a Rapid Screening Method for Differentiation of Infected and Vaccinated Cattle and Sheep with Brucellosis

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Abstract: This study was carried out on 55 animals (5 vaccinated cattle with S19 vaccine, 5 vaccinated cattle with RB51 vaccine, 5 vaccinated Sheep with Riv 1 vaccine, 20 cattle from suspected to be infected or had a history of brucellosis and 20 sheep from mobile flocks of known history of brucellosis). The results of serological tests which were applied on serum samples, revealed out of 5/5 vaccinated cattle with S19(100%), (100%) and (100.0%) were positive by RBPT, TAT and Riv.T respectively so vaccinated animals tend to yield persistent post-vaccinal immune responses. While out of 5/5 vaccinated cattle with RB51 were negative by the same serological tests as RB51 a rough strain of Brucella can not detected by these tests. But out of 20 cattle from infected farm with *Brucella* 5/20(25%), 5/20(25%) and 5/20(25%) were positive by RBPT, TAT and Riv.T respectively. Out of 5 vaccinated Sheep with Riv 1 vaccine 5/5(100%), 5/5(100%) and 5/5(100%) were positive by RBPT, TAT and Riv.T respectively while out of 20 sheep from infected mobile flocks 4/20 (20%), 4/20(20%) and 4/20(20%) were positive by RBPT, TAT and Riv.T respectively. Out of 5 milk samples were collected from seropositive naturally infected cattle and 4 milk samples from seropositive naturally infected sheep were positive PCR amplifies, 731 bp fragment indicated (*B. melitensis* biovar 3), which considered the most prevalent strain in Egypt. AMOS PCR results were recorded by electrophoretic mobilitis in agarose gels and photographic methods showed two products (498 and 364 bp) plus 178-bp were amplified from RB51 vaccinated cattle but only one product (498 bp) was amplified from the S19 vaccinated cattle. Amplified fragments obtained from vaccinated sheep with Rev 1 vaccine produce 2 bands, 282 bp and 238 bp, the product obtained from digestion of the amplified fragment. In contrast naturally infected sheep with *B.melitensis* field isolate (*B. melitensis* biovar3) produced only one band a 238-bp fragment. AMOS PCR was shown to be a valuable tool and a rapid screening test for differentiating the vaccinated animals with S19, RB51 or Riv 1 from infected one with *B. melitensis* biovar 3(field strains of *Brucella*) which means that Brucellosis eradication program could reliably use the *Brucella* AMOS PCR without any supplement to other diagnostic and epidemiological data to release sale animals from quarantine before the conventional identification methods are completed.

Key words: *Brucella* · Diagnosis · Amos Or Multiplex PCR Assay

INTRODUCTION

Brucellosis is more of a Mediterranean disease. In endemic areas, brucellosis causes high economic loss and has serious public health consequences. Worldwide, *B. melitensis* is the most prevalent species causing human brucellosis [1-3]. For this reason a test and slaughter policy is not realistic in the majority of places where *B. melitensis* is endemic due to lack of financial resources needed for compensation. Brucellosis caused by *B. melitensis*, is endemic in Egypt. It appears to be of particular risk in rural communities, especially in Upper Egypt [4]. Diagnosis of the causative agents is the cornerstone of any control program and is based on bacteriological and immunological findings. In the absence of culture facilities, the diagnosis of brucellosis

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traditionally relies on serological testing with a variety of agglutination tests which its positive results are confirmed by other serological and bacteriological tests [5, 6]. Many current serological tests have proved to be either too sensitive, giving false-positive results, or too specific, giving false-negative results [6-8]. In addition, the presence of antibodies dose not always mean an active case of brucellosis, since vaccinated animals tend to yield persistent post-vaccinal immune responses and other gram-negative bacteria such as *Yersinia enterocolotica* cross-react with *Brucella* spp. [9].

Isolation and identification of *Brucella* offers a definitive diagnosis of brucellosis but it a time-consuming, require skilled technicians and require a minimum of 5 days. In addition, *Brucella* highly pathogenic for humans [10]. Vaccination with live, attenuated *B. abortus* strains has been effective in preventing *B. abortus* infections and abortions in cattle. Until recently, strain 19 (S19), a naturally occurring smooth and attenuated strain of *B. abortus*, lipopolysaccharide of S19 contains O side chain, which is responsible for an immunodominant antibody response after vaccination or infection with field strains. S19 vaccination causes the appearance of a transient serologic titer of antibody [11]. *B. abortus* vaccine strain RB51 is a stable, rough and attenuated mutant as described by Schurig *et al*. [12]. Since the lipopolysaccharide of *B. abortus* RB51 is devoid of O side chain, which do not interfere with the serology as mentioned by Stevens *et al*. [13], thus decreasing the incidence of false-positive reactors [14]. It was recognized that vaccination with S19 and Riv1 vaccine cause existence of positive reactors in serological tests among vaccinated (cattle and sheep) thus increasing the incidence of false-positive reactors [14] which lead to difficulties in distinguishing between infected and vaccinated animals by conventional serological tests [3, 15].

Polymerase chain reaction (PCR) is a recent advance for rapid and accurate diagnosis of brucellosis and overcome the limitations of conventional methodology. This robust test can differentiate in a single step all of the classical *Brucella* species, including vaccinal strains [3], with almost 100% sensitivity [16], useful when serological testing often fails [17].

AMOS PCR assay is a multiplex primer assay that uses a five-primer cocktail. One primer anneals to the IS711 element. As designed, *B. abortus* amplifies a 498-bp product, *B. melitensis* amplifies a 731-bp product, *B. ovis* amplifies 976-bp product and *B. suis* amplifies a 285-bp product. AMOS PCR assay was developed to differentiate between field strains, vaccine strain S19 and RB51. The primers amplify a 498-bp product present in *B. abortus* bv. 1, 2 and 4 plus two vaccine strains and they also amplify a 364-bp product from RB51. Identification of S19 is based on a PCR primer pair which amplifies a short sequence (178 bp) [18] of the *eri* gene (essential for erythritol catabolism), present in all *Brucella* strains except *B. abortus* S19 [19]. This PCR procedure has high potential as a rapid screening test for differentiating the two *Brucella* vaccine from the virulent field strains of Brucella. Ewalt and Bricker [20]. So the present study aimed at:

Detection and identification of *Brucella* spp. from infected cattle and sheep, differentiation between vaccinated animals with any type of vaccine (S19, RB51or Riv 1) from infected one using recent techniques AMOS PCR.

MATERIALS AND METHODS

This study was carried out on 55 animals (5 vaccinated cattle with S19 vaccine, 5 vaccinated cattle with RB51 vaccine, 5 vaccinated Sheep with Riv 1 vaccine, 20 cattle from suspected animals or had a history of brucellosis and 20 Sheep from mobile flocks of known history of brucellosis). Samples were collected between October -2011 till March 2012.

Sampling

Collection of Milk Samples: Milk samples were collected from seropositive dairy cattle and sheep for PCR assay.

Collection of Blood Samples: The two blood samples (with and without EDTA for PCR and serology) were collected from vaccinated cattle and sheep from Jugular vein after disinfection site of injection by ethyl alcohol 70%. About 8-10 ml of blood is collected from each animal in a plain vacutainer tube. The samples were divided into two blood samples (one with EDTA and other without for PCR and serology), coded and transferred to the laboratory with the minimal delay in an insulated ice box.

Serological Examination: Sera were prepared according to [21]. Seroprevalence of brucellosis was investigated using commonly used serological tests as: Rose Bengal Plate Test (RBPT) [21], Tube Agglutination Test (TAT) [21] and Rivanol Test(Riv.T) [21].

Molecular Examination (For Milk and Blood Samples)

Extraction of DNA from Milk and Blood Samples for PCR Assay: DNA was extracted from milk and blood using Gene JET™ Genomic DNA Purification Kit, Quick Protocol QP14. This method was applied in Biotechnology Department, Animal Health Research Institute (AHRI), Egypt.

DNA Amplification: Detection and Identification of PCR Product:

- PCR amplification for detection of Brucella DNA from milk samples of infected cattle and sheep as described by Leal-Klevezes *et al.* [22]. PCR products was analyzed by electrophoresis through 1.5 % agarose gel stained with Ethidium bromide solution (0.5mg/ml) and visualized under an ultraviolet transilluminator and photographed. Visible band of appropriate size of 498 bp for *B. abortus a*nd 731 bp for *B. melitensis* were considered positive.
- PCR amplification for differentiation between vaccinated cattle with RB51 and S19 according to Ewalt and Bricker [20], The amplified products were separated by electrophoresis in 1.5% wt/vol agarose gel were only one product (498 bp) was amplified from the DNAs of *B. abortus S*19; two products (498 and 364 bp) plus 178 bp were amplified from RB51 DNAs.
- PCR amplification for differentiation between vaccinated sheep with Riv 1 vaccine and infected sheep with *Brucella* field strain (*Br melitensis biovar* 3, was performed according to Mullis and Faloona [23].

The amplified products were separated by electrophoresis in 1.5% wt/vol agarose gel. *B. melitensis* Rev.1 vaccine strain digests revealed two visible bands 282 bp and another band 238-bp. In comparison, the PstI digestion of *B. melitensis* field strain revealed a single band 238-bp.

RESULTS AND DISCUSSION

Brucellosis particularly caused by B. *melitensis*, is endemic in Egypt, presumably affecting large numbers of animals as well as humans. It appears to be of particular risk in rural communities especially in Upper Egypt [4]. Rapid and accurate diagnosis is fundamental for control and eradication of brucellosis [24]. Culture provides the definitive diagnosis of brucellosis and it is considered the gold standard method for it [25]. Because of difficulty of performing culture in the field, its consuming for the time, its health hazard and lack sensitivity of the most culture procedures, the serological tests are the main tools used for detection of *Brucella* infection in animals [24]. Although it is well known that there is no single serological test gave high sensitivity and antigen combination showed 100% sensitivity and specificity simultaneously Munoz *et al*. [25]. The limitations of both isolation and serological detection procedures have resulted in increasing the use of PCR-based methods for detection and identification of *Brucella* species due to their accuracy, sensitivity, speed and ability to work with DNA as opposed to highly infectious live cultures [26].

From data illustrated in Table 2 for results of the different serological tests, the study revealed in 5/5 vaccinated cattle with S19(100%), (100%) and (100.0%) were positive by RBPT, TAT and Riv.T respectively. while the study revealed in 5/5 vaccinated cattle with RB51 (0.0%), (0.0%) and (0.0%) were negative by RBPT, TAT and Riv.T respectively, which explained as RB51 a rough strain of *Brucella* can not detected by this serological tests as it detected smooth strain of *brucella* since vaccinated animals tend to yield persistent post-vaccinal immune responses, which agree with Corbel, [10] and explained by Schurig *et al*. [12] and Stevens *et al*. [13], Woodard [11] who mentioned that similar to virulent *B. abortus* strains, the lipopolysaccharide of S19 also contains O side chain, which is responsible for an immunodominant antibody response after vaccination or infection with field strains. S19 vaccination usually causes the appearance of a transient serologic titer of antibody to *Brucella* O antigen and in some vaccinated cattle, these titers of antibody do persist.

Results in Table 2 revealed out of 20 cattle from infected farm with *brucella* 5/20(25%), 5/20(25%) and 5/20(25%) were positive by RBPT, TAT and Riv.T respectively.

While out of 5 vaccinated sheep with Riv 1 vaccine 5/5(100%), 5/5(100%) and 5/5(100%) were positive by RBPT, TAT and Riv.T respectively. while out of 20 sheep from infected mobile flocks 4/20 (20%), 4/20(20%) and 4/20(20%) were positive by RBPT, TAT and Riv.T respectively.

Nearly ratios of positive reactors by the same serological tests were reported by Samaha *et al.* [27] that detected antibodies against *Brucella* organisms in

Table 1: Sequences of the oligonucleotide primers used in PCR assay are in the following

Primer	Sequence (5′-3′)
The sequences of the oligonucleotide primers for detection of *brucella* from milk as described by Leal-Klevezes *et al*.[22].	
B. abortus-specific primer	5' GAC-GAA-CGG-AAT-TTT-TCC-AAT-CCC
B.melitensis-specific primer.	5' AAA-TCG-CGT-CCT-TGC-TGG-TCT-GA
IS711-specific primer	5' TGC-CGA-TCA-CTT-AAG-GGC-CTT-CAT
The sequences of the oligonucleotide primers for differentiation between RB51 and S19 as described by Ewalt and Halling [20]	
RB51/2308	5' CCC-CGG-AAG-ATA-TGC-TTC-GAT-CC
eri primer 1,	5' GCG-CCG-CGA-AGA-ACT-TAT-CAA
eri primer 2,	5' CGC-CAT-GTT-AGC-GGC-GGT-GA
The sequences of the oligonucleotide primers for differentiation between Riv 1 vaccine and field strain (Br melitensis biovar 3 as described by Mullis and Faloona [23]	
Forward P1	5`TGGAGGTCAGAAATGAAC
Reverse P2	5`GAGTGCGAAACGAGCGC
Digestion of the Amplified Products: Fast Digest PstI, restriction enzymes was used as described by Fermentas 5/ ….C T G C A G..3/ 3/ ….G A C G T C..5/ www Fermentas.com	

Table 2: Serological profile of samples collected from vaccinated and infected cattle and sheep

		RBPT		SAT			Rivanol T.	
Species	No. of examined animals	+	%	+	±	%	+	%
Vaccinated cattle with S19	5	5	100	4	0	80	5	100
Vaccinated cattle with RB51	5	--	--	--	--	--	--	--
Cattle from infected farm	20	5	25	4	1	25	5	25
Vaccinated Sheep with Riv 1	5	5	100	3	1	80	5	100
Sheep from infected	20	4	20	4	0	20	4	20
Total	55							

[RBPT: Rose Bengal Plate Test, TAT: Tube Agglutination Test Riv.T: Rivanol Test].

107 cows (5.44%), 98 cows (4.98%),. 93 cows (4.73%) and 88 cows (4.48%) when they examined by BAPAT, RBPT, TAT and Riv.T, respectively and very lower ratios of positive reactors by the same tests were reported by Abdel-Hamid *et al.* [28] and disagree with that of Al-Azeem *et al.* [29] who mentioned the study revealed presence of the antibodies against *Brucella* organisms in 32 cows (100%), 30 cows (93.8%), 29 cows (90.6%) and 27 cows (84.3%) from the examined 32 cows by BAPAT, RBPT, TAT and Riv.T respectively.

It was noticed that serological tests can not differentiated between vaccinated and infected animals by as vaccinated cattle and sheep with S19 and Riv 1 vaccine gave positive results as in Table 2. In addition, the presence of antibodies dose not always mean an active case of brucellosis, since vaccinated animals tend to yield persistent post-vaccinal immune responses and other gram-negative bacteria such as *Yersinia enterocolotica* may cross-react with *Brucella* spp. [30]. Also these results confirmed by the findings of Pefanis *et al.* [31] who mentioned that standard serological tests can not differentiated between vaccinated and infected animals.

All the *Brucella* organisms detected and identified by PCR were identified as *B. melitensis* which agree with that of Bricker and Halling [18], Bricker and Halling [19], Khoudair, [32], who reported that *B. abortus* (biovars 1, 2 and 4) amplifies a 498-bp product, *B. melitensis* (all biovars) amplifies a 731-bp product, *B. ovis* amplifies 976-bp product and *B. suis* (biovar 1) amplifies a 285-bp product. Also agree with Molina-Flores, [4] and Refai, [24] who reported that brucellosis particularly caused by *B. melitensis*, is endemic in Egypt, presumably affecting large numbers of animals as well as humans.

Isolation of *B. melitensis* indicates that it is still the prevalent species in cattle and buffaloes may be attributed to presence of mixed populations of sheep, goats, cattle and buffaloes in the villages, [33], in addition to that most sheep or goat flocks in Egypt are mobile and subsequently the infected flocks can contaminate pastures and spread the disease to the other animals in another herds or areas [26]. In addition to that the major route of human infection with brucellosis is consumption of raw milk or its products [34].

The higher sensitivity of PCR assay than the culture method may be attributed to the high sensitivity of PCR to detect fewer number of *Brucella* organisms present in the milk sample than that can be detected by culture [35, 22]. Also, PCR detects DNA which present in both living and dead *Brucella* organisms while culture detects

only the living organisms and due to massive contamination of the milk samples with other organisms [35]. The same finding was found by, Abdel-Hamid *et al.* [28] and Samaha *et al*. [27].

Occurrence of false-negative bacteriological results due to massive contamination of the milk samples or from inhibition of some *Brucella* spp. in the selective medium that are major factor that limit the use of conventional bacteriological methods. In these circumstances *Brucella* DNA can still be detected by PCR assay [36]. Here, it must be mentioned that survival of *Brucella* organisms in milk is altered by the change in pH and storage temperature [37] and that *Brucella* content of milk depends on stage of the lactation [36, 38].

The result were recorded by photographic methods showed two products (498 and 364 bp) plus 178-bp were amplified from RB51 DNAs (Lane1,3 and 5). But only one product (498 bp) was amplified from the DNAs of *B. abortus* S19 (Lane 2,4 and 6). The products are clearly differentiated by their electrophoretic mobilitis in agarose gels.

Our results similar to that of Ewalt and Bricker [20] who stated that AMOS PCR assay was developed to differentiate field strains from vaccine strain (S19) and RB51 and the primers amplify a 498-bp product present in *B. abortus* bv. 1, 2 and 4 plus two vaccine strains and they also amplify a 364-bp product from *B. abortus* RB51.. Identification of S19 is based on a PCR primer pair which amplifies a short sequence (178 bp) [18] of the *eri* gene (essential for erythritol catabolism), present in all *Brucella* strains except *B. abortus* S19 [19]. Thus, the identification of S19 is based on the absence of amplification of this target. This PCR procedure has high potential as a rapid screening test for differentiating the two *Brucella* vaccine from the field strains of *Brucella*. [20].

Also explained by Woodard [11] who mentioned that vaccination with live, strain 19 (S19), a naturally occurring smooth and attenuated strain of *B. abortus*, the lipopolysaccharide of S19 also contains O side chain, which is responsible for an immunodominant antibody response after vaccination.

This robust test can differentiate in a single step all of the classical *Brucella* species, including those found in marine mammals and the S19, RB51 and Rev 1 vaccine strains [3].

Our results agree with Munoz *et al.* [25]. who mentioned that there is no single serological test gave high sensitivity and antigen combination showed 100% sensitivity and specificity simultaneously and Foster *et al.* [26] who said that the limitations of both isolation and serological detection procedures have resulted in increasing the use of PCR-based methods for detection and identification of *Brucella* species due to their accuracy, sensitivity, speed and ability to work with DNA as opposed to highly infectious live cultures.

Agarose gel electrophoresis of Pst1 digest of amplified fragments omp 2 gene from *Brucella* strains. The Figure 4 shows the 282 pb DNA lacking the Pst1 site and the large, Pst1 digested DNA fragments.

The PCR test was performed with vaccinated sheep with Rev 1 vaccine and naturally infected one with *B. melitensis* biovar 3 field strain, a single band with the expected size of 282 bp (Lane1-4) was obtained in Fig. (3).

Digestion of the amplified fragments from the obtained strains with PstI restriction endonuclease gave different bands on agarose gels (Fig. 4). *B. melitensis* standard Rev.1 vaccine strain digests revealed two visible bands 282 bp. Due to the lack of PstI site and another band 238-bp in size (Lane 1,3 and 5). In comparison, the PstI digestion of *B. melitensis* field strain digests revealed a single band, 238-bp in size (lanes 2, 4, 6 and 7). Our results agree with that of Mullis and Faloona [23] and Nashwa, *et al*. [39]. In contrast naturally infected sheep with *B. melitensis* field isolate (*B. melitensis* biovar3) produced only one band a 238-bp fragment this results came in close agreement also to that described by Bardenstein *et al.* [40] and Banai *et al.* [41] who demonstrated that the Rev 1 vaccine led to the adverse effects of strain persistence in the vaccinated animals and was occasionally spread horizontally, In two cases it was shown that the vaccine strain caused human infection demonstrating the zoonotic hazards of its virulence.

From the above results by PCR indicated its the highly sensitivity and specificity of PCR than that other methods which agree with that of Hamdy and Amin [35] who mentioned that the most specific diagnostic test involves isolation of the causative organism, but this suffers from the drawback of requiring along incubation period and low sensitivity especially in the chronic stage of the disease and Brucella organism is pathogenic to human.

It was recognized that vaccination with S19 and Riv1 vaccine cause existence of positive reactors in serological tests among vaccinated population (cattle and sheep) which lead to difficulties in distinguishing between infected and vaccinated animals by conventional serological tests [15, 3], because of these difficulties the development of new diagnosis tests for the direct detection of brucella species [42].

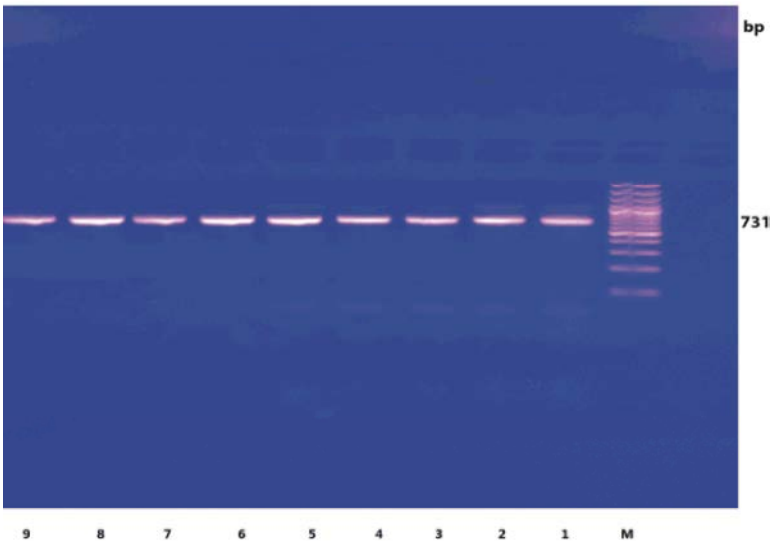

Fig. 1: In Photo PCR products on an agarose gel 1.5% stained by Ethidium bromide following electrophoresis. Lane M: molecular weight Marker Lane 1-5: DNA were extracted from milk samples of naturally infected cattle, Lane 6-9: DNA were extracted from milk samples of naturally infected sheep. PCR amplifies, 731 bp fragment indicated (*B. Melitensis* biovar 3) (Lane1-9)

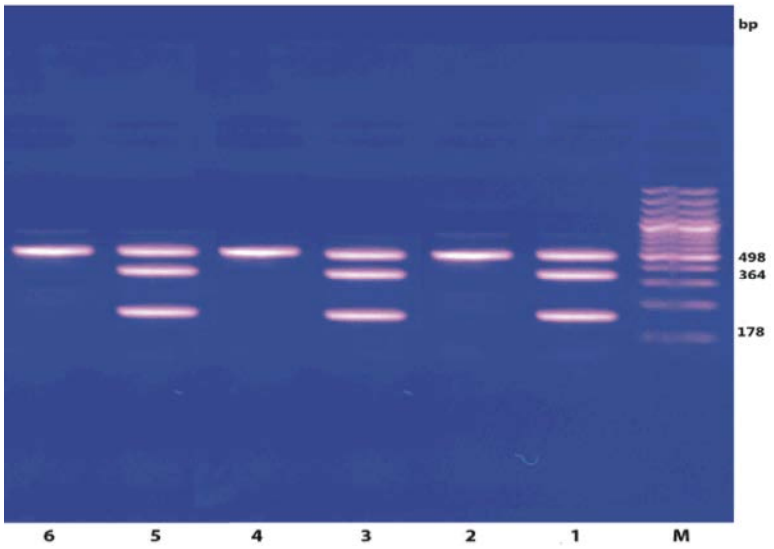

Fig. 2: Lane M: Marker Lane 1,3 and 5: vaccinated cattle with RB51 vaccine Lane 2,4 and 6: vaccinated cattle with S19 vaccine. DNA was amplified by AMOS PCR assay and visualized under UV light

In this study, PCR proved to be a valuable tool for differentiating vaccinated animal from infected one in less than 24 hours which overcome the limitations of both isolation and serological detection procedures have resulted in increasing the use of PCR-based methods for differentiation between vaccinated and infected animals due to their accuracy, sensitivity, speed and ability to work with DNA as opposed to highly infectious live cultures which in coordination with Munoz *et al*. [25] and Foster *et al.* [26] who mentioned that there is no single serological test gave 100% sensitivity and specificity like PCR and it can be used in clinical samples directly. The conventional methods of identification require a minimum of 5 days to identify an isolate to *Brucella* species and biovar level. This can delay the movement of animals between different owners and have a negative impact.

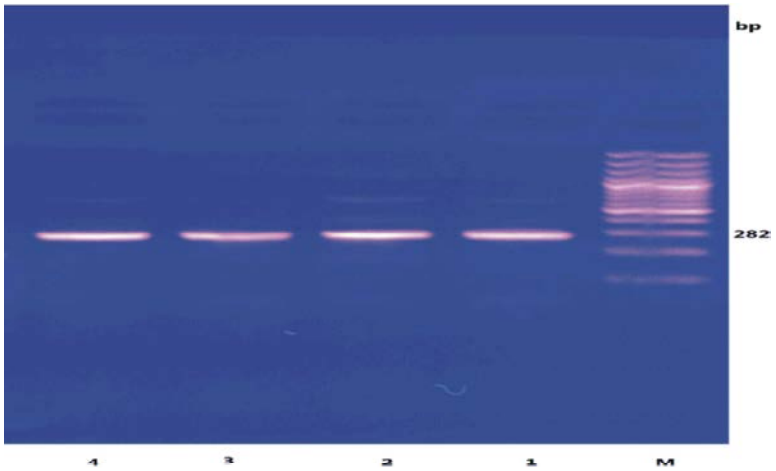

Fig. 3: Agarose gel electrophoresis of PCR-amplified omp 2 gene fragments from *Brucella* strains.The figure shows a single band 282-bp DNA fragment. Lane M: digest Marker. Lane 1 and,2 vaccinated sheep with *B. melitensis* Rev 1. Lanes:3 and 4 *B. melitensis* biovar 3 field strain. only one product (282 bp) was amplified

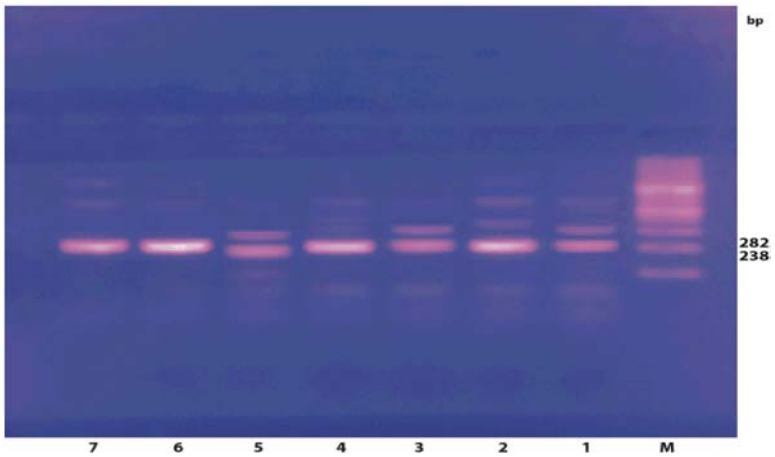

Fig. 4: Lane M: Marker, Lane 1,3 and 5 *B. melitensis* biovar 3 field strain from infected sheep. Lanes:2,4,6 and 7 *B. melitensis* Rev.1 strain from vaccinated sheep

Brucellosis eradication program personnel could reliably use AMOS PCR without supplement other diagnostic and epidemiological data (such as herd history and serological test results) to release sale animals from quarantine before the conventional identification methods are completed which agree with that of Nashwa *et al.* [39].

CONCLUSIONS

AMOS PCR proved to be a valuable tool for differentiating the vaccinated animal with S19, RB51 or Riv 1 from infected animals with *B. melitensi*s biovar 3 (field strains of *Brucella*). Also overcome slaughtering of vaccinated animals. It's a considered a rapid tool for detection and identification of *Brucella* spp. from infected animal in comparison with serology and culturing. The true there is no single serological test can differentiate between vaccinated and the infected animals. Finally brucellosis eradication program could reliably use the *Brucella* AMOS PCR without any supplement to other diagnostic and epidemiological data.

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