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COMPARATIVE CONVENTIONAL AND MOLECULAR TOOLS FOR DETECTION AND DIFFERENTIATION OF *BRUCELLA* FIELD AND VACCINAL STRAINS

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ABSTRACT

The current study was applied on 111 cows (78 *Brucella* suspected, 13 vaccinated with S19 and 20 with RB51) for differentiation of *Brucella* wild and vaccinal strains using conventional and molecular methods (PCR-RFLP, AMOS and real-time PCR). Serum samples were examined using rose Bengal (RBT), tube agglutination and rivanol tests. Milk samples were subjected to milk ring test (MRT), isolation and PCR assays. The diagnostic sensitivity and specificity of RBT were 100% and 75% respectively, while MRT were 60% and 71% respectively. The highest diagnostic sensitivity and specificity were recorded for PCR (100% and 82%) respectively. Serological tests of vaccinated cows with S19 and RB51 revealed different seropositive and seronegative results respectively for all applied tests. Five isolates of suspected cows were obtained and identified as *B.melitensis* biovar-3, two isolates of S19 vaccinated cows were obtained and identified as S19, and two isolates of RB51 vaccinated cows (one was identified as RB51 and the other as *B.melitensis* biovar-3). PCR-RFLP assay revealed two patterns (P1 with 238bp and P2 with bands 282, 238bp) were obtained for field *B.melitensis* and Rev.1 vaccine respectively. AMOS and real-time PCR revealed three different amplicons and three different dissociation peaks respectively specific for different *Brucella* species. Conclusively, PCR-RFLP can differentiate *B.meletensis* (wild and vaccinal) and appropriate for application in mixed farms, while AMOS-PCR assay is recommended to distinguish between S19 and RB51 vaccinal strains and *B.melitensis*. The use of more than one method provides a better reliable diagnostic approach for potent improvement of brucellosis control program.

Key words: *Conventional, Molecular, Brucella field, Vaccinal strains*

INTRODUCTION

Brucellosis is a highly contagious bacterial disease causing significant reproductive losses in animals. In Egypt, despite the implementation of the National Brucellosis Control Program, the disease is still endemic among ruminants and human due to the predominance of smallholdings that favor close contacts between humans and animals and presence of mixed populations of animals, and consumption of unpasteurized milk and dairy products (Hegazy *et al.*, 2011; Holt *et al.*, 2011).

Currently, the diagnosis of brucellosis is based on microbiological and serological laboratory tests. However many serological tests have proved to be either give false-positive, or give false-negative results (Ruiz-Mesa *et al.*, 2005). The gold standard for the diagnosis of brucellosis is isolation. However, handling live *Brucella* involves risk of laboratory infection and time consuming. In order to avoid these disadvantages, methods based on the polymerase chain reaction (PCR) are becoming very useful (Ibrahim *et al.*, 2002; Yu and Nielsen, 2010).

PCR-restriction fragment length polymorphism (RFLP) especially of outer membrane protein (OMP) genes of *Brucella* has been widely and successfully used for genotyping of several *Brucella* isolates (Cloeckaert *et al.*, 2001).

Brucella AMOS (Abortus, Melitensis, Ovis, Suis) PCR assay is a multiplex PCR designed to detect the IS711 insertion elements in the four *brucella* species (Ancora *et al.*, 2005).

Real-time PCR constitutes a further technological improvement for the molecular identification of the genus *Brucella* and the differentiation of its species (Probert *et al.*, 2004).

Vaccination of livestock is the cornerstone for the control and prevention of brucellosis (Cloeckaert *et al.*, 2002). Although the differentiation among vaccine strains and wild-type field isolates of

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Brucella is cumbersome, diagnostic laboratories are presented to distinguish between them due to the possibility of isolation of vaccine strains from milk or other biological samples (Miranda *et al.,* 2015).

Epidemiology of brucellosis is very multifarious due to the probability of involvement of different animal and different *Brucella* species (Kiril *et al.,* 2015). This reality highlights the importance of the diagnostic procedures for detection and typing of *Brucella,* as powerful epidemiological tools which are essential for a successful control program. Additionally, the control of human brucellosis remains largely dependent on the control of the disease in animals (Kiril *et al.,* 2015). This study aimed to identify and differentiate vaccinal strains and *Brucella* species field using conventional and molecular methods PCR-RFLP, AMOS and real-time PCR as a trial for potent improvement of control program of brucellosis.

MATERIALS and METHODS

S**amples:** Blood and milk samples were collected aseptically from 111 cows (78 suspected or had history of brucellosis, 13 vaccinated with strain19 (S19) and 20 vaccinated with strain RB51 (RB51) Table (1).

The serological tests were applied on 111 serum samples using rose bengal test (RBT) (Alton *et al.,* 1988), standard tube agglutination test (SAT) (MacMillan, 1990) and rivanol test (RT) (Alton *et al.,* 1988) was standardized and performed as described by Carpenter (1997). The 111 milk samples were subjected to immunological tests using milk ring test (MRT) as described by (Alton *et al.,* 1988) and were plated onto *Brucella* agar medium (Oxoid) supplemented with antibiotics at 37 °C with 5% CO2 for 5 to 7 days for isolation. Identification of the isolates was conducted biochemically by routine methods (Alton *et al.,* 1988).

Vaccinal strain: Rev.1 was used in the study as a reference strain

Statistical analysis: Sensitivity and specificity were calculated according to Thrusfield (1986).

Molecular assays: DNA was extracted from bacterial strains by boiling procedure according to Reischl *et al.* (1994). DNA was extracted from Milk samples with slight modification of Walsh *et al.* (1991) using100µl Chelex 100® (Fluka, USA).

PCR- RFLP assay: This method carried out according to Samadi *et al.* (2010) by targeting *Brucella* OMP2 gene and the amplified products were digested using *Pst*I endonuclease enzyme. PCR assay was performed in total volume of 25ul reaction mix contain 5ul of template DNA, 20 pmol of each primer Table (2) and 1X of PCR mix (Fermentas). The analysis of PCR products was carried out using 1.5% ethidium bromide stained agarose gel. *Pst*I restriction enzyme was used according to the manufacturer's instruction (Thermo Scientific). Digestion products were electrophoresed using 3% agarose gel.

AMOS PCR assay: The assay was performed according to Bricker and Halling (1994) using a single reverse primer, targeting the *Brucella* insertion element IS*711,* and three different forward primers Table (3). The assay was performed using total volume of 50ul reaction mix contain 5ul of template DNA, 50 pmol of each primer and 1X of PCR mix.

SYBR Green real time PCR: In an extension of the AMOS-PCR, a multiplex SYBR Green real time PCR was performed. The assay was carried out using Rotor-Gene Q Series (QIAGEN). The assay was performed using total volume of 30ul reaction SYBR Green Master Mix (Fermentas) contain 5ul of template DNA, 10pmol of each primer (same primer of AMOS assay). After amplification, analysis of melting temperature (Tm) was carried out by continuous recording of fluorescence at gradual increase of temperature (0.2°C/s) over the range 50–95°C. Thermal profiles of all PCR assays were listed in Table (3).

RESULTS

Results of 78 serum samples of suspected cows, 23 (29.5%), 20 (25.64%) and 16 (20.5%) were positive for RBT, SAT and RT respectively. While in milk samples 24 (30.77%), 5 (6.41) and 18 (23.08%) were positive for MRT, isolation and PCR respectively Table (4). All five isolates were identified bacteriologically as *B.melitensis* biovar-3 and by PCR as *B.melitensis*. The diagnostic sensitivity and specificity of RBT, MRT and PCR compared to bacteriological culture were recorded in Tables (5 and 6) where PCR recorded highest sensitivity (100%) and specificity (82%).

The diagnostic sensitivity and specificity were determined as 100% and 86%, respectively for OMP2-PCR assay when the results were compared with the bacterial culture results (5 samples tested positive and 63 samples tested negative with both methods), while in milk samples were 55% and 98% respectively when the results were compared with BSCP-PCR assay results (as 10 samples tested positive and 59 samples tested negative with both methods). On the other hand, BSCP-PCR assay demonstrates the same sensitivity and specificity in both cases (bacterial culture or milk samples).

Results of serology of vaccinated cows, revealed that out of the thirteen vaccinated cows with S19 10 (76.92%),8 (61.54%) and 5 (38.46%) were positive using RBT, SAT and RT respectively. The positive results of the thirteen milk samples were 8 (61.54%), 2 (15.38%) and 4 (30.77%) for MRT, isolation and PCR respectively. The two isolates were identified as S19 by bacteriological identification (has the same properties of *B. abortus* biovar 1strain, but does not require CO2 for growth, does not grow in the presence of benzyl penicillin (3 µg/ml), thionin blue (2 µg/ml), and ierythritol (1 mg/ml) and by PCR (gave one specific amplicon of 498bp). While, the twenty vaccinated cows with RB51 revealed no positive serological results for RBT, SAT and RT. The positive results in milk samples were 1 (5%), 2 (10%) and 3 (15%) for MRT, isolation and PCR respectively. The two isolated strains, one of them was identified bacteriologically as *B.melitensis* biovar-3 and by PCR as *B.melitensis*, while the other isolate was identified as RB51 by bacteriological identification (rough morphology and growth in the presence of rifampicin (250 µg per ml of media) and by PCR (gave two amplicons of 364 and 498bp) Table (7).

In 78 milk samples of (suspected infected cows) studied by BCSP gene based PCR 18 samples could give positive amplification of 223bp specific for genus *Brucella* Fig. (1). An amplicon of 731bp specific for *B. melitensis* was obtained for all the five field isolates and positive milk samples indicating high presence of *B. melitensis* Fig. (2). However, only10 samples gave positive amplification of 282bp by using OMP2 gene based PCR Fig. (3). Concerning toPst1 enzymatic digestion of amplified fragment the results revealed two patterns (P1 and P2), P1 with two bands the large 238bp and the small 44bp (not shown) and P2 with three bands 282, 238 and 44bp Fig. (4). P1 was found in all *B. melitensis*, S19, RB51 isolates and all milk samples tested positive while P2 was found only in *B. melitensis* Rev-1 vaccine.

Results of AMOS-PCR revealed specific amplifications of different amplicons, 498bp specific for S19, two amplicons of 498 and 364bp specific for RB51 and an amplicon of 731bp specific for *B. melitensis* field and vaccinal strains Fig. (5). Regarding to the multiplex SYBR Green real time PCR using the same primer of AMOS the results revealed a fluorescent signal for isolates and Rev-1 vaccine while no fluorescent signals were present in negative control Fig. (6). The analysis of melting curves of the amplified *Brucella* isolates, yielded three (closely but distinct) dissociation peaks of melt temperatures (Tm) of 80°C and 80.5°Cfor RB51, S19 and 81.2°C for Rev-1vaccine and *B.melitensis* field isolate respectively Fig. (7).

Table 1: Samples and tests used in the study

Animal status	Samples	Test					
		RBT	SAT	RT	MRT	Isolation	PCR
Suspected cows (n=78)	Serum	78	78	78	--	--	--
	Milk	--	--	--	78	78	78
Vaccinated (S19) (n=13)	Serum	13	13	13	--	--	--
	Milk	--	--	--	13	13	13
Vaccinated (RB51) (n=20)	Serum	20	20	20	--	--	--
	Milk	--	--	--	20	20	20

n: number of animal

Table 2: Oligonuclutide primers used in the study

Target	Sequence (5' - 3')	Amplicon size	Purpose
All *brucella* OMP2	TGGAGGTCAGAAATGAAC GAG TGC GAA ACG AGC GC **Samadi*et al.* (2010)**	282bp	Identification and PCR-RFLP
Brucella cell surface protein (BCSP)	TGGCTCGGTTGCCAATATCAA CGCGCTTGCCTTTCAGGTCTG **Mukherjee *et al.* (2007)**	223bp	Identification
B.abortus S19	**F:**GAC GAA CGG AAT TTT TCC AAT CCC	498bp	AMOS assay and multiplex SYBR Green real time PCR
B. melitensis	**F:**AAA TCG CGT CCT TGC TGG TCT GA	731bp	
*B. abortus*RB51	**F:**CCC CGG AAG ATA TGC TTC GAT CC **R:**TGC CGA TCA CTT AAG GGC CTT CAT **Bricker and Halling (1994); Kumar *et al.* (2014)**	364bp	

Table 3: Thermal profile of PCR assays performed in the study

Target	Technique	Cycling condition				No. of cycle
		Step	**Temp.**	**Time**		
OMP2 gene	**PCR-RFLP**	**Initial denaturation**	**94˚C**	**4 min**		**One cycle**
		Denaturation	**94˚C**	**1min**		**35 cycles**
		Anealing	**50˚C**	**1min**		
		Extension	**72˚C**	**1min**		
		Final extension	**72˚C**	**10 min**		**One cycle**
Brucella*specific insertion element IS*711	**AMOS-PCR and real time PCR**	**Initial denaturation**	**95˚C**	**4 min**	**10 min**	**One cycle**
		Denaturation	**95˚C**	**1.15 min**	**30 s**	**35 for AMOS and 45 for real time PCR**
		Anealing	**55˚C**	**1.15 min**	**45 s**	
		Extension	**72˚C**	**1.30 min**	**60 s**	
		Final extension	**72˚C**	**10 min**		**One cycle**

Table 4: The prevalence of brucellosis in suspected infected cows

Type of samples (n =78)	Test Positive					
	Serology			MRT	Isolation	PCR*
	RBT	SAT	RT			
Serum	**23 (29.5%)**	**20 (25.64%)**	**16 (20.5%)**	**--**	**--**	**--**
Milk	**--**	**--**	**--**	**24 (30.77%)**	**5 (6.41)**	**18 (23.08%)**

*BSCP-PCR assay

Table 5: Correlation between bacteriological culture with RBT, MRT and PCR results of milk samples of suspected infected cows

Test	Result	RBT		MRT		PCR*	
		+	-	+	-	+	-
Bacteriological culture	+ (n=5)	5	0	3	2	5	0
	-(n=73)	18	55	21	52	13	60
Total	78	23	55	24	54	18	60

*: BSCP-PCR assay n: Number of samples

Table 6: Sensitivity and Specificity of RBT, MRT, and PCR in comparison with bacteriological culture results

Test	Compared with	Sensitivity %	Specificity %
Bacteriological culture	RBT	100	75
	MRT	60	71
	PCR*	100	82

*: BSCP-PCR assay

Table 7: Prevalence of brucellosis in vaccinated cows

Test Positive		Type of vaccination			
		Vaccinated with S19 (n=13)		Vaccinated with RB51 (n=20)	
		+ Serum	+ Milk	+Serum	+Milk
Serology	RBT	10(76.92%)	--	0	--
	SAT	8 (61.54%)	--	0	--
	RT	5 (38.46%)	--	0	--
MRT		--	8 (61.54%)	--	1 (5%)
Isolation		--	2 (15.38%)	--	2 (10%)
PCR*		--	4 (30.77%)	--	3 (15%)

*: BSCP-PCR assay, n: number of animals

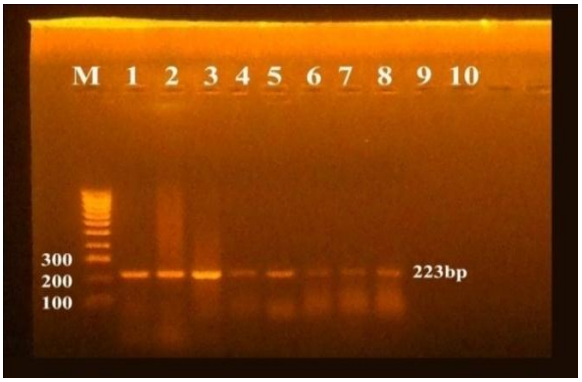

Fig. (1): Ethidium bromide stained 1.5% agarose gel electrophoresis of bcsp PCR assay of milk samples. **Lane M:** 100 bp DNA ladder, **Lane 1:** Positive control, **Lanes 2-8:** Milk samples. **Lane 9:** Negative control.

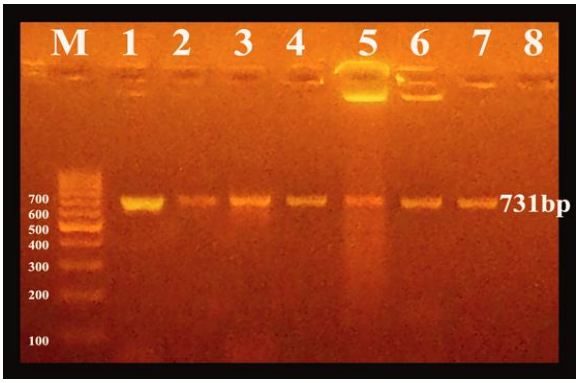

Fig. (2): Ethidium bromide stained 1.5% agarose gel electrophoresis of PCR-amplified IS711 element specific for *B. melitensis*. The figure shows a specific amplicon of 731-bp DNA. **Lane M:** 100 bp DNA ladder, **Lane 1:** Positive control, **Lanes 2,3:** Field isolates, **Lanes 4-7:** Milk samples, **Lane 8:** Negative control.

Fig. (3): Ethidium bromide stained 1.5% agarose gel electrophoresis of *OMP2* PCR assay. **Lane M:** 100 bp DNA ladder, **Lane 1:** Positive control, **Lanes 2-5:** Field isolates, **Lanes 6-13:** Milk samples. **Lane 14:** Negative control.

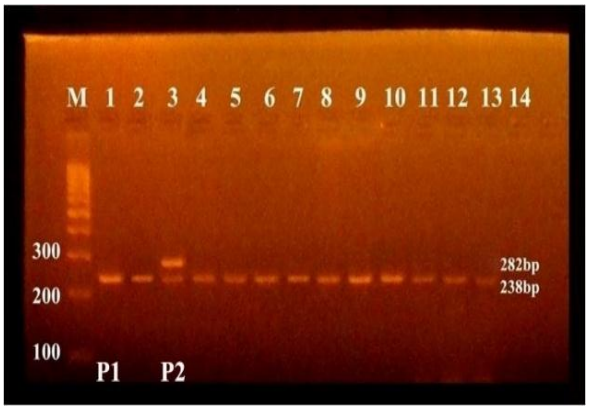

Fig. (4): Ethidium bromide 3% agarose gel electrophoresis of PCR-RFLP Pst1 digests demonstrates two patterns. Pattern 1 with two bands 238bp and 44bp (not shown). pattern 2 with three bands 282, 238 and 44bp. **Lane M:** 100 bp DNA ladder, **Lane 1:** S19, **Lane 2:** RB51, **Lane 3:** Rev-1 vaccine, **Lanes 4, 5:** *B. meletinsis* field isolates **Lane 6-13:** Milk samples.

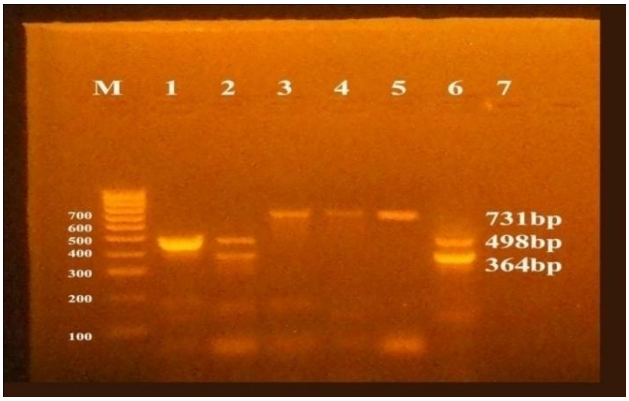

Fig. (5): Ethidium bromide stained 1.5% agarose gel electrophoresis of AMOS PCR assay **Lane M:** 100 bp DNA ladder, **Lane 1:** S19 isolate show one specific band 498bp, **Lane 2:** RB51 isolate show two specific bands 498 and 364bp, **Lane 3:** Rev1 vaccine show specific band 731bp, **Lane 4,5:***B.melitensis* field isolates, **Lane 6:** Milk sample show RB51 pattern.

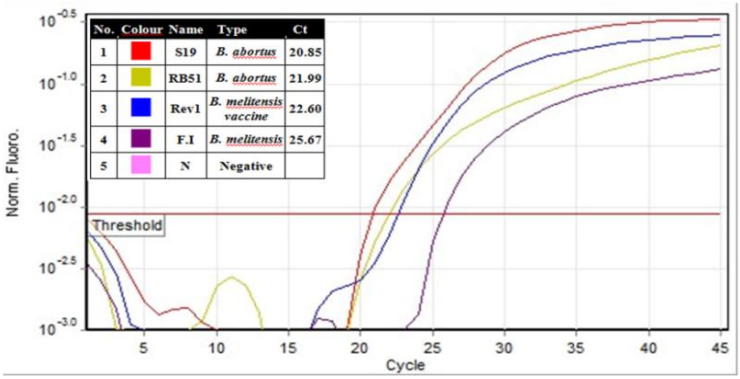

Fig. (6): Amplification curve showing positive amplification of the isolates (S19, RB51 and *B. melitensis*) and the Rev1 vaccine while negative control shows no amplification.

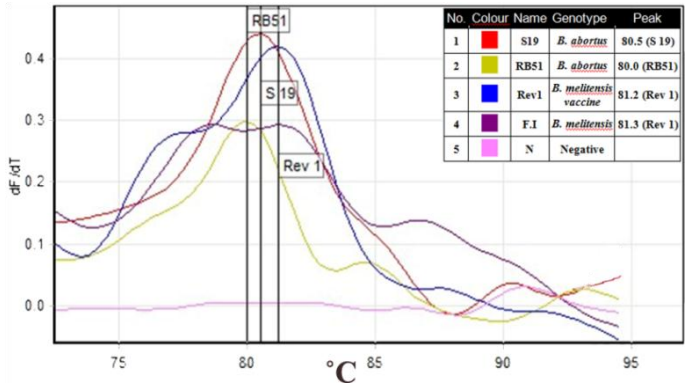

Fig. (7): Dissociation curve analysis of amplified isolates (S19, RB51 and *B. melitensis*) and Rev1 vaccine. The melting points were demonstrated at the legend at left side.

DISCUSSION

Brucellosis is one of the world's major zoonoses that still has veterinary, public health and economic concern. The retrospective data of serological tests presented in Table (4) revealed high incidence of brucellosis in Egypt, these results coincided to some extend to what reported before by samaha *et al.* (2008); Kaoud *et al.* (2010).

The discrepancy between results of the used serological tests proved that these tests served as important diagnostic aid but is not fully reliable (Akhtar *et al*., 2010; Eman *et al.*, 2014). The highest positivity was observed with RBT as this test can give false positive reaction. Despite these limitations, RBT may be used as a screening test to ascertain *Brucella* infection (Kaltungo *et al.*, 2014).

The lowest positivity was observed with RT. This finding was referred to that RT detects principally IgG1 and rivanol test have low sensitivity but high specificity (Acosta-González *et al.*, 2006). It is noticeable from Table (4) that the highest percentage was obtained with MRT, which may be raised from false positives as a result of many causes, including mastitis or hormonal disorder (Bercovich and Moerman, 1979). However, it is cost effective, easy to perform and can cover a large population in a short time (Cadmus *et al.*, 2008).

Culture and isolation of the organism has remained the only unequivocal proof (Poester *et al.*, 2010). Current study revealed only five (6.41%) isolates out of 78 milk samples of the suspected cows, this was probably returned to that *Brucella* was present in very low numbers (O'Leary *et al.*, 2006; Eman *et al.*, 2014). Also, Refai (2003) declared that the sensitivity of the bacteriological culture methods depends on the numbers and viability of *Brucella* in the sample. Thus, culture methods are not always successful as they are time consuming and the handling of microorganism is hazardous. All five isolates were identified phenotypically as *B.melitensis* biovar-3 this finding comes in agreement with previous reports that described *B.melitensis* as the most prevalent in Egypt (Samaha *et al.*, 2008; Eman and Ibrahim, 2014; Menshawy *et al.*, 2014). Unlike isolation, PCR based molecular tests do not require the presence of vital bacteria, can detect bacterial DNA even in the samples with small number of *Brucellae*, provide quick and objective results and are safe and relatively easy to perform (Poester *et al.*, 2010). The running results revealed higher positivity of PCR assay than culturing method (18 versus to 5), similar finding was achieved previously by Ilhan *et al.* (2008) who explained that PCR assay can also detect the dead organisms.

The data illustrated in tables (5 and 6), showed the diagnostic sensitivity of RBT was extreme (100%) and low specificity (75%), while MRT gave 60% sensitivity and 71% specificity. These results corresponded to Saegerman *et al.* (2004) who mentioned that these tests showed different sensitivities and specificities depending on numerous variables, such as dose and route infection, the presence cross-reactive bacteria and the kinetics of the induced immune response. The highest diagnostic sensitivity and specificity were recorded for PCR (100% and 82%), respectively this finding agreed with Hamdy and Amin (2002); Gupta *et al.* (2006); Al-Mariri and Haj-Mahmoud (2010). Moreover, Ibrahim *et al.* (2002); Alcina *et al.* (2012) added that because of the high specificity and strict sensitivity, PCR is the only test which was able to detect the occurrence of *Brucella* organisms in milk samples.

The use of more than one marker-based PCR (BCSP and OMP2) performed in the current study provides a better reliable molecular diagnostic approach for screening of *Brucella* in field samples, the same attitude was reported by (Mukherjee *et al.,* 2007; Ya and Nielsen 2010).

However, the two genus specific PCR assays clarified that BCSP-PCR assay is more sensitive than OMP2-PCR assay in detecting *Brucella* in milk samples (100% in BCSP versus to 55% in OMP2-PCR assay). Such difference was not seen in both PCR assays when carried out on bacterial isolates, indicating that the presence of host DNA could affect sensitivity of primers, as observed previously by Navarro *et al.* (2002); Mukherjee *et al.* (2007) during their studies on bovine blood samples. Similarly, Baddour and Alkhalifa (2008); Ya and Nielsen (2010) reported that the sensitivity vary substantially for the different primers and primer targeting BCSP can provide the greatest sensitivity. Moreover, this difference in both PCR assays may be due to sequence of BCSP gene is better conserved than the OMP2 sequence in the genus *Brucella* so that, variation in the *omp2* sequence has been used as a basis for typing strains (Bardenstein *et al.,* 2002; Mukherjee *et al.*, 2007).

Vaccination of livestock is the cornerstone for the control and prevention of brucellosis but unfortunately, *Brucella* vaccine could be excreted in cow's milk (Leal-Hernandez *et al.,* 2005). Through the current study, serum samples of vaccinated cows with S19 showed seropositivity for all serological tests (table, 5) this finding agree with earlier report of OIE (2009) that S19 causes persistent titers which could not be distinguished from titers of natural infection because it is a smooth but attenuated strain (Siadat *et al.,* 2012). While serum samples of vaccinated cows with RB51 gave seronegative results for all applied serological tests. This finding was backed to that vaccination with RB51 vaccine did not result in the production of antibodies against the O-side chain of lipopolysaccharide (LPS), as measured by serological tests (Poester *et al.,* 2006).

In the present study, The MRT of vaccinated cows with S19 and RB51 gave 61.54%and 5% positive results, respectively. It is wrong to decide that milk has been found infected with *Brucella* based on positive MRT results. Indeed, only the presence of anti-*Brucella* antibodies is detected in milk by the MRT and such antibodies may be induced by vaccination and/or infection with wild type *Brucella* spp. (Godfroid *et al.,* 2013).

The bacteriological culture of milk samples of vaccinated cows with S19 revealed (2/13) isolates and were identified as S19 (OIE, 2009). Bricker and Halling (1995) reported that, though S19 can be distinguished from the wild type by culture methods, these methods are not rapid. While, the bacteriological culture of milk samples of vaccinated cows with RB51gave (2/20) isolates, one of them was identified as RB51 (OIE, 2009) and the other isolate was *B. melitensis* biovar-3 (Alton *et al.,* 1988). The results of PCR showed (4/13) and (3/20) for vaccinated cows with S19 and RB51 respectively this results agreed with the study of Arellano-Reynoso *et al.* (2013). These results declared that these *Brucella* vaccines could be excreted in cows' milk (Leal-Hernandez *et al.,* 2005; OIE, 2009). It was worth that *B.melitesis* biovar-3 (wild strain) was isolated from one seronegative RB51 vaccinated cow. This finding was shown before by Samaha *et al.* (2008). The fact that the negative serology could be due to insufficient number of bacteria to stimulate immunological response capable of generating antibodies feasible for detection by serological tests, although adequate enough to be shed (Arellano-Reynoso *et al.,* 2013).

It is significant to declare again that contact between small ruminants and cattle are almost always as the source of *B. melitensis* infections in cattle (Alvarez *et al.,* 2011). As reported by previous study in Egypt where cattle and buffalo kept in a household with sheep and goats had 6.32 times of testing seropositive for *Brucella* spp., compared to seronegative (Holt *et al.,* 2011). It is obvious from the results of PCR-RFLP presented in this study Fig. (3) that PCR-RFLP is more suitable for differentiation of ovine and caprine *Brucella* (vaccinal and field strain) as it could differentiate between *B. melitensis* field isolates (showed P1) and *B. melitensis* REV-1 vaccine (showed P2). This result is supported by previous result of Bardenstein *et al.* (2002); Samadi *et al.* (2010). So it is appropriate for application, especially in mixed farms when raising sheep and/or goats along with cattle or buffalo. By contrast, AMOS-PCR assay was unable to differentiate different *B. melitensis* (vaccinal and field strain) this finding was sustained previously by

OIE (2013). However, AMOS-PCR can differentiate between different types of S19 and RB51 vaccinal strains and *B.melitensis* Fig. (5) This result was also reported by previous study of Darla and Betsy (2000).

Comparing to conventional PCR the use of real-time PCR has the major advantage of obtaining results in a shorter time and does not require electrophoresis analysis (Zahidi *et al.*, 2015). This is the first trials to carry out multiplex SYBR Green real time PCR using the same primers of AMOS-PCR technique in differentiating *Brucella* species. The results revealed three (closely quiet distinct) dissociation peaks of melt temperatures therefore, this assay need more evaluation before introducing to routine identification and differentiation of *Brucella* species. However, the major advantage of the real-time PCR performed in this study over probe-based genotyping is that it is much cheaper, although some inability to detect very slight changes in melt temperatures. The same opinion was recorded by Gopaul *et al.* (2014) during their assessment of high resolution melting assay as a tool for rapid identification of *Brucella* species.

CONCLUSION

PCR-RFLP assay is appropriate to differentiate ovine and caprine *Brucella* (vaccinal and field strains) especially in mixed farms. However, AMOS-PCR assay is recommended to distinguish between S19 and RB51 vaccinal strains and *B. melitensis.* The use of more than one method (conventional and molecular) provides a better reliable diagnostic approach for potent improvement of control program of brucellosis.

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طرق مقارنة تقليدية وجزيئية للكشف والتمييز بين العترة الحقلية والعترة اللقاحية لميكروب البروسيلا

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تم تطبيق الدراسة الحالية على عدد ١١١ بقرة (٧٨ مشتبه بإصابتهم بميكروب البروسيلا ، ١٣ محصنين بلقاح S19 و ٢٠ محصنين بلقاح RB51 للتمييز بين العترات الحقلية واللقاحية لميكروب البروسيلا بإستخدام الأساليب التقليدية والجزيئية مثل (تقنية الPCR-RFLP، الأموس وإختبار إنزيم البلمرة المتسلسل الكمى). تم فحص عينات السيرم بإستخدام إختبار الروز بنجال وإختبار التلزن الأنبوبى وإختبار الريفانول وتم تعريض عينات اللبن لإختبار اللبن الحلقى والعزل وإختبارات إنزيم البلمرة المتسلسل. كانت نسبة الحساسية التشخيصية والتخصصية للروز بنجال ١٠٠% و٧١% علي التوالى وكانت بالنسبة لإختبار اللبن الحلقى ٦٠% و ٧١% على التوالى فى حين سجل إختبار إنزيم البلمرة المتسلسل أعلى نسب للحساسية والتخصصية (١٠٠% و٨٢%) على التوالى. كشفت الإختبارات السيرولوجية للأبقار المحصنة بلقاحى الS19 و RB51 وجود نتائج إيجابية وسلبية على التوالى لجميع الإختبارات المستخدمة. تم عزل ٥ معزولات *B.melitensis* من الأبقار المشتبه فى إصابتهم وعدد ٢ معزولة S19 من الأبقار المحصنة بلقاح S19 وأيضا عدد ٢ معزولة من الأبقار المحصنة بلقاح RB51 تم تصنيف إحداهما *B.melitensis* والأخرى RB51 كشف إختبار PCR-RFLP عن وجود نمطين مختلفين الأول معه باند بطول ٢٣٨ والثانى معه ٢ باند بطول ٢٣٨ و٢٨٢ زوج قاعدى تم الحصول عليهم مع العترة الحقلية لل*B.melitensis* وعترة اللقاح الRev-1 على التوالى. فى حين كشف إختبار الأموس وإختبار إنزيم البلمرة المتسلسل الكمى ثلاث أطوال وثلاث قمم تفكك حرارى مختلفة على التوالى مميزة لإنواع البروسيلا المختلفة. من هذا نستطيع أن نستخلص الأتى: إختبار الPCR-RFLP مناسب للتفريق بين عترات ال*B.melitensis* الحقلية واللقاحية وهو مناسب للتطبيق فى المزارع المختلطة. بينما نوصى بإستخدام إختبار الأموس للتميز بين سلالات الS19 ، RB51و *B.melitensis*. إستخدام أكثر من وسيلة تشخيصية يتيح لنا نهج تشخيصى أفضل موثوق به لتحسين فعال فى برنامج مكافحة البروسيلا.