Bulgarian Journal of Veterinary Medicine, 2020, **23**, No 3, 319–330
ISSN 1311-1477; DOI: 10.15547/bjvm.2217

Original article

DIAGNOSTIC PERFORMANCE OF RFLP-PCR AND SARCOSINE BASED INDIRECT ELISA VERSUS IMMUNOASSAYS IN *BRUCELLA* INFECTED AND VACCINATED SMALL RUMINANTS

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Summary

Soliman, S. M., H. S. Soliman, H. I. Mohamed, M. A. Salem & S. A. Ahmed, 2020. Diagnostic performance of RFLP-PCR and sarcosine based indirect ELISA versus immunoassays in *Brucella* infected and vaccinated small ruminants. *Bulg. J. Vet. Med.,* **23**, No 3, 319–330.

This study was carried out for evaluation of the diagnostic performance of different serological assays; buffered acidified plate antigen test (BAPAT), rose bengal plate test (RBPT), immunochromatographic assay (ICA), rivanol test (RivT), indirect ELISA using two types of coating antigens (smooth lipopolysaccharide; S-LPS and N-lauroylsarcosine-extracted antigens; SE) and complement fixation test (CFT). Relative sensitivity and specificity of various techniques were estimated. The traditional serological tests failed to distinguish the vaccinated from naturally infected animals. Using iELISA with extracted antigens (SE) as a coating antigen was a more accurate test to differentiate the naturally infected animals from vaccinated animals. Application of restriction fragment length polymorphism polymerase chain reaction (RFLP-PCR) on sera samples from seropositive animals, Rev-1 vaccinated sheep and *Brucella* field strain infected sheep and goats revealed that there were samples identified as *B. melitensis* biovar 3 field strain and other samples identified as *B. melitensis* Rev-1 vaccinal strain. The obtained results established that restriction fragment length polymorphism-polymerase chain reaction can differentiate between animals infected with *Brucella* field strains from animals vaccinated with the Rev-1 vaccine.

Key words: *B. melitensis* Rev-1 vaccine, ELISA, RFLP-PCR, sarcosine, serological assays

INTRODUCTION

Brucellosis is a highly contagious zoonosis that influences the general wellbeing and monetary policy execution of endemic and non-endemic countries (Wareth *et al.,* 2014). *Brucella melitensis* is the fundamental cause of ovine and caprine brucel-

losis. The clinical manifestations of brucellosis in sheep and goats are described by reproductive disorders (OIE, 2016).

Diagnosis of brucellosis is relying upon bacteriological examination and molecular methods (direct tests) as well as serological *in vitro* and allergic *in vivo* (indirect) tests (Donev *et al.,* 2010).

Vaccination is a method for diminishing the prevalence of the disease to a level where the eradication programme can be applied. The Rev-1 live *B.melitensis* vaccine is the most frequently used vaccine in control programmes against brucellosis in small ruminants in different countries. It induces a strong antibody response to the smooth lipopolysaccharide (S-LPS) and results in positive reactors in serological tests which leads to difficulties in distinguishing infected sheep and goats from vaccinated sheep by traditional serological tests, therefore hindering control programmes (Shome *et al.,* 2014). To encourage serological diagnosis, different techniques have been investigated, including tests that detect antibodies to proteins (Letesson *et al.,*1997), furthermore, to the smooth LPS-related native hapten (NH) polysaccharide (Diaz-Aparicio *et al.,* 1994).

An enzyme-linked immunosorbent assay (ELISA) utilising a soluble antigen extracted from *B. abortus* 544 by n-lauroylsarcosine (sarcosine extracts) was developed to diagnose brucellosis (Erdenebaatar *et al.,* 2003).

The genes coding for the *Brucella* major OMPs (*omp2a* and *omp2b*), their expression and the polymorphism of both porin genes have been studied by PCR–RFLP which allowed the identification of diversity of both *omp2a* and *omp2b* among *B. melitensis* strains (Bosseray, 1985). Pst I digests of a 282 bp fragment amplified from the *omp2* genes of *B. melitensis* Rev.1 should produce three bands, an intact 282-bp fragment from the amplified *omp2a* gene that lacks the Pst I site and two smaller fragments of 238 and 44 bp, the products obtained from digestion of the *omp2b*-amplified fragment (Saeedzadeh & Sharifiyazdi, 2013). In contrast, field isolates of all *B. melitensis* biotypes only show two fragment patterns, 238 and 44 bp produced by the digestion of *omp2b* and *omp2a* (Bardenstein *et al.,* 2002).

The present investigation was completed to evaluate the diagnostic performance of ELISA based on sarcosine versus immune assays in *Brucella* infected and vaccinated small ruminants and utilisation of the RFLP-PCR for differentiation between *Brucella* infected and vaccinated small ruminants.

MATERIALS AND METHODS

Study design

Serum samples were collected from four groups of sheep and goats. Two hundred and twenty-six blood samples were collected from unvaccinated sheep (Group 1) originating from different *Brucella* infected localities with recorded cases of reproductive disorders (late term of abortion & retained placenta) and previous history of *Brucella melitensis* recovery: El-Qaliubiya (n=25), El-Beheira (n=41), El-Sharkia (n=85), El-Gharbia (n=42) and Domietta (n=33). Two hundred and fourty-one blood samples were collected from unvaccinated goats (Group 2) located in *Brucella* infected localities with recorded cases of reproductive disorders (late term of abortion, retained placenta and orchitis) and previous history of *Brucella melitensis* recovery. These localities were El-Qaliubiya (n=47), El-Beheira (n=46), El-Sharkia (n=65), El-Gharbia (n=40) and Domietta (n=43).

Group 3 comprised healthy unvaccinated sheep and goats (50 each) from *Brucella* free areas where Rev-1 vaccination had never been practiced. Group 4 included 350 Rev-1 vaccinated sheep (3 to 8 months of age) treated with a dose of 1–2 $\times 10^9$ CFU. They were bled monthly until 6 post vaccination month.

Brucella melitensis Rev-1 vaccine originated from seed strain obtained from National Veterinary Services Laboratories "NVSL", 1800 Dayton Avenue, Ames, Iowa, 50010, USA.

Serological tests

BAPAT, RBPT, ICA, RivT, and CFT were carried out according to Nielsen *et al.* (2004); OIE (2016).

Antigens extraction and purification for ELISA

LPS extraction and purification: Extraction of *B. abortus* S99 lipopolysaccharide was done by the hot phenol-water method (Sharifat *et al.,* 2008). *Brucella abortus* S99 (biovar 1) was obtained from the Veterinary Sera and Vaccine Research Institute (VSVRI) Abassia, Cairo, Egypt.

Extraction of sarcosine was carried out according to Erdenebaatar *et al.* (2003). Quantitation of the protein content was performed as per Lowry *et al.* (1951). Polyacrylamide gel electrophoresis (SDS-PAGE) was done according to the methods described by Laemmli (1970). Indirect ELISA (iELISA) was performed as described by Erdenebaatar *et al.,* (2003); Garin-Bastuji *et al.* (1998).

Restriction fragment length polymorphism polymerase chain reaction (PCR) (Mullis & Faloona, 1987)

The oligonucleotide *Brucella*-specific primer was designed from *Brucella omp2* gene (Bardenstein *et al.,* 2002). The sequence of the primers was F- 5`TGG AGGTCAGAAATGAAC 3` and R - 5` GAGTGCGAAACGAGCGC 3`.

Pst I restriction enzyme was used according to the manufacturer's instruction (Biolabs, 1405). The amplified product was analysed by electrophoresis on 1.5% agarose gel, captured utilising a Polaroid camera.

Relative sensitivity and specificity

CFT is considered the test of choice as it has a standardised result that can be interpreted in unified international units and correlates well with the isolation results and positive immune response against *Brucella* infection. Moreover, the CFT is considered by the OIE the official prescribed test for testing animals for international trade (OIE, 2016). CFT was employed in this study as a gold standard test, (Yohannes *et al.,* 2012). Relative sensitivity and relative specificity were calculated according to Parikh *et al.*(2008) from the following equations: Sensitivity= 100×True positive / (True positive + false negative); specificity= 100×True negative / (True negative + false positive); where true positive or negative reactions were those affirmed as being positive or negative by at least two tests. False positive or negative reactions are those affirmed as being positive or negative by another test or not tested.

RESULTS

Table 1 illustrates the immunoassay results in a total number of 226 sheep and 241 goats suspected to be infected with *Brucella* and originating from *Brucella* infected localities with previous history of *B. melitensis* recovery. The buffered acidified plate antigen test detected 186 (82.3%) out of 226 examined sheep

Table 1. Results of conventional serological tests for detection of *Brucella* infectivity in examined sheep and goats

Examined Animals	BAPAT		RBPT		ICA		RivT		CFT	
	No.	%	No.	%	No.	%	No.	%	No.	%
Suspected sheep (n=226)	186	82.30	183	80.97	181	80.09	173	76.55	165	73.01
Suspected goats (n=241)	200	82.99	197	81.74	194	80.50	185	76.76	180	74.69
Healthy unvaccinated sheep (n=50)	1	2	1	2	1	2	0	0	0	0
Healthy unvaccinated goats (n=50)	4	8	2	4	2	4	1	2	1	2

BAPAT: Buffered Acidified Plate Antigen Test, RBPT: Rose Bengal Plate Test, ICA: Immunochromatographic Assay, RivT: Rivanol Test, CFT: Complement Fixation Test.

Table 2. Results of conventional serological tests for monitoring of *Brucella* antibodies in vaccinated sheep

Animals	Time P.V. (months)	BAPAT		RBPT		ICA		RivT		CFT	
		No	%	No	%	No	%	No	%	No	%
Vaccinated sheep (n=350)	0	0	0	0	0	0	0	0	0	0	0
	1	342	97.71	322	92	330	94.29	293	83.71	285	81.43
	2	332	94.86	303	86.57	283	80.86	201	57.43	191	54.57
	3	283	80.86	205	58.57	210	60	52	14.86	44	12.57
	4	103	29.43	98	28	99	28.29	0	0	0	0
	5	63	18	56	16	58	16.57	0	0	0	0
	6	44	12.57	32	9.14	36	10.29	0	0	0	0

P.V: post vaccination

(group 1), 200 (82.99%) out of 241 examined goats (group 2). The RBPT detected 183 (80.97%) out of 226 examined sheep (group 1), 197 (81.74%) out of 241 examined goats (group 2). The corresponding picture for the ICA was 80.09% of examined sheep (group 1), and 80.5% out of examined goats (group 2). Riv. T detected 173 (76.55%) out of 226 examined sheep (group 1) and 185 (76.76%) out of 241 examined goats (group 2). The detected infectivity by the gold standard (CFT) is 165 sheep (73.01%) in group 1 and 180 (74.69%) in goats' group 2.

The results of serum samples (n=350) obtained from vaccinated sheep and examined by some immunoassays (BAPA, RBPT, and ICA) used in the diagnosis of brucellosis for monitoring of *Brucella* antibodies against vaccinal strain (Table 2) recorded the existence of vaccinal antibodies until the 6th month post vaccination. In contrast, confirmatory tests (CFT & Riv T) recorded the existence of vaccinal antibodies till the 3rd post vaccination month and negative results thereafter (4th, 5th, and 6th post vaccination month).

Table 3 reveals the immune response reactivity of sera for examined sheep and goats using *Brucella* S-LPS as coating antigen in indirect ELISA. It gave the highest positive reactors at first post vaccination month – 344 (98.29%). This response declined to attain the lowest rate at the 6th post vaccination month (44; 12.57%). Furthermore, suspected sheep

Table 3. Antibody reactivity of sera for examined sheep and goats using smooth lipopolysaccharide (S-LPS) and sarcosine extract (SE) as coating antigen in indirect ELISA

Animals examined	Time of examination (months)	Positive for ELISA LPS		Positive for ELISA SE	
		No.	%	No.	%
Vaccinated sheep (n=350)	0	0	0	0	0
	1	344	98.29	10	2.86
	2	333	95.14	5	1.43
	3	284	81.14	0	0
	4	103	29.43	0	0
	5	64	18.29	0	0
	6	44	12.57	0	0
Suspected sheep (n=226)		195	86.28	165	73.01
Suspected goats (n=241)		203	84.23	183	75.93
Healthy unvaccinated sheep (n=50)		0	0	0	0
Healthy unvaccinated goats (n=50)		1	2	0	0

Table 4. Relative sensitivity and specificity of different serological tests compared with CFT for diagnosis of brucellosis in sheep and goats

Serological test	Sheep		Goats	
	Sensitivity	Specificity	Sensitivity	Specificity
BAPA	98.79	78.38	99.45	78.18
RBPT	98.79	81.08	99.45	82.72
ICA	96.97	80.18	97.24	81.81
RivT	98.79	90.99	96.67	90.00
ELISA(LPS)	99.39	72.02	99.44	78.18
ELISA(SE)	98.18	97.297	99.45	97.27

Table 5. Detection and identification of *Brucella* from sera of vaccinated and infected animals by using RFLP-PCR

Examined animals	Number of *Brucella* detected	*Brucella* species
Vaccinated (n=5)	4	*B. melitensis* Rev-1 vaccine
Infected (n=5)	4	*B. melitensis* biovar 3 field strain

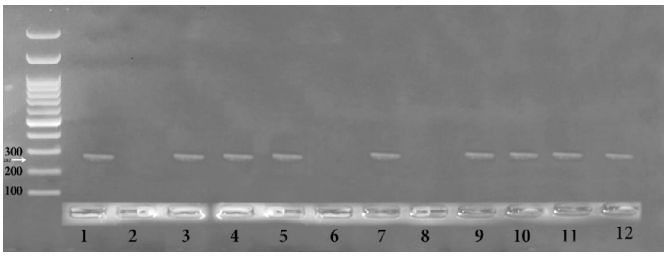

Fig. 1. Agarose gel electrophoresis of PCR-amplified *omp* 2 gene fragments from *Brucella* strains. The figure shows a single band 282-bp DNA fragment Digest. Lane 1; control positive *B. melitensis* Rev -1; lane 2; control negative; lanes 3, 4, 5 & 7, samples from vaccinated sheep with *B. melitensis* Rev-1vaccine; lanes 9, 10, 11 & 12, samples from *B. melitensis* biovar 3 infected animals.

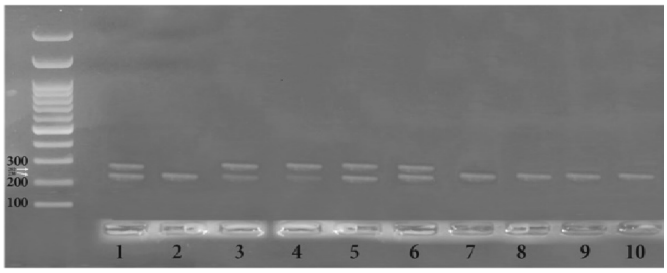

Fig. 2. Lane 1: control positive *B. melitensis* Rev-1; lane 2; control positive *B. melitensis* biovar 3 field strain; lanes 3, 4, 5 & 6: *B. melitensis* Rev-1 strain from vaccinated sheep; lanes 7, 8, 9 & 10; *B. melitensis* biovar 3 from sheep and goats infected with a field strain.

and goats in *Brucella* infected localities reacted strongly and gave higher positive reactors than other serological tests (195 and 203 respectively). In contrast, iELISA using sarcosine extracts gave only 10 (2.86%) at first post vaccination month, 5 (1.43%) reactors in second post vaccination month post-vaccination and none by the 3rd month. In suspected sheep and goats from *Brucella* infected localities, ELISA gave 165 (73.01%) and 183 reactors (75.93%) respectively.

Considering CFT as the gold standard, sensitivities were estimated and rearranged in descending order as followed (Table 4): ELISA (S-LPS), BAPA, RBPT, Riv.T, ELISA (SE) and ICA in sheep. The order in goats was ELISA (SE), BAPA, RBPT, ELISA (S-LPS), ICA and Riv. T. Specificities in sheep were arranged in

descending order as ELISA (SE), Riv. T, RBPT, ICA, BAPA and ELISA (S-LPS) while in goats the order was ELISA (SE), Riv. T, RBPT, ICA, ELISA (S-LPS) and BAPA.

Out of five serum samples collected from vaccinated sheep and examined by RFLP-PCR, 4 were detected as *B. melitensis* Rev-1 vaccine (Table 5). The same was figured out when it comes to the five examined serum samples originated from infected small ruminants as the RFLP-PCR detects only 4 as *B. melitensis* biovar 3 field strain.

The agarose gel electrophoresis of PCR-amplified omp 2 gene fragments from *Brucella* strains is illustrated on Fig. 1 and 2. Fig. 1 shows eight DNA amplifications out of 10 serum samples by RFLP-PCR at a single band 282-bp DNA fragment Digest, while Fig. 2 showed that the Pst I restriction endonuclease was capable to digest the amplified fragments of studied *Brucella* strains to give different bands that manifested on the agarose gel.

DISCUSSION

The intention of the immunoserological tests used in brucellosis control programmes is to identify infected animals that may spread the disease. Considering the CFT as the gold standard test, serological tests sensitivity and specificity were calculated (Yohannes *et al.,* 2012).

Table 1 shows higher specificity of CFT than BAPAT and RBPT suggesting that the BAPAT and RBPT positive samples should be confirmed by this test. Al Dahouk *et al.* (2003) considered that CFT should be utilised just as a confirmatory test and noticed that in functional terms sensitivity and specificity could vary broadly.

Relative sensitivity and relative specificity of BAPAT were estimated as 98.79% and 78.38% respectively in sheep and 99.45% and 78.18% respectively in goats (Table 4). This result is in line with that of Gall & Nielsen (2004) who reported that BAPAT was more sensitive and accurate than other conventional tests for recognition of *Brucella* infection. Relative sensitivity and relative specificity of RBPT were estimated at 98.79% and 81.08% respectively in sheep and 99.45% and 82.72% respectively in goats (Table 4). These outcomes concur with Aggad (2003) who found that RBPT is more efficient and sensitive in screening and detection of *Brucella* infection. This indicates that RBPT remains the most reliable serological test for large-scale surveillance/ eradication purpose (Garin-Bastuji *et al.,* 2006).

This result agrees with Hosein *et al.* (2017) who affirmed that the buffered *Brucella* antigen tests, BAPAT and RBPT have a higher sensitivity but also have a fewer reliable specificity resulting in a diminished number of false negatives and a critical number of false positives.

Interestingly, it was seen that few samples gathered from healthy unvaccinated sheep and goats reacted with BAPAT and RBPT. This might be because of the impact of a few Gram-negative bacteria as *Escherichia coli*, *Salmonella dublin*, *Yersinia enterocolitica* 0:9 and *Pasteurella tularemia* which may raise a response with the tests utilised in the diagnosis of brucellosis causing faults in the interpretation of the results.

Immunochromatographic assay was less sensitive than BAPAT and RBPT. Relative sensitivity and relative specificity were estimated at 96.97%, and 80.18 % respectively in sheep and 97.24% and 81.81% respectively in goats (Table 4).

These results were in agreement with Nielsen *et al.* (2004) who revealed that ICA can detect both IgG and IgM antibodies to *Brucella*.

Rivanol test showed 98.79% relative sensitivity and 90.99% relative specificity in sheep and 96.67% and 90% respectively in goats (Table 4). This test is based on disposing of some non-specific responses by precipitation of high molecular weight serum glycoprotein from serum solutions; which is essentially IgM, leaving generally IgG in the serum (Poiester *et al.,* 2010).

Diagnosis and differentiation of infected sheep and goats from those vaccinated against brucellosis are sophisticated and need special techniques since vaccination leaves animals with persistent post-vaccinal immune response (Baldi *et al.,*1996). It is noteworthy that no single test can identify all infected animals at all stages of the infection and therefore a combination of serological tests (BAPAT, RBPT, RIV.T) should be included to diminish the number of both false negative and false positive reactors (Cordes & Carter, 1979).

BAPAT gave the maximum number of positive reactors of vaccinated sheep (342; 97.71%) at the first post vaccination month that declined gradually till 44 (12.57%) by the 6th month, while RBPT showed that 322 (92%) of vaccinated sheep reacted during the first month post vaccination, to attain 32 (9.14%) at post vaccination month 6. These outcomes are in line with data of Adone & Pasquali (2013) who mentioned that serological tests capable of detecting the S-LPS were the most delicate for small ruminant brucellosis, but if the animals have been before hand immunised or presented to Gram-negative bacteria with LPS O-chains like those of brucellae, they may yield false positive outcomes.

The results of ICA (Table 2) showed that 330 (94.29%) of vaccinated sheep reacted in the first post vaccination month, and only 36 (10.29%) by the 6th month in concordance with Nielsen & Yu (2010) who reported that the test detected non-specific IgM and specific IgG antibodies and that a high sensitivity was assured for all stages of the disease.

Rivanol test showed that 83.71% of vaccinated sheep reacted in the first month; 57.43% in the second month and 14.86% in the third month. The confirmation with rivanol test is also recommended due to its high specificity and reliability in detecting the infected animals (Poiester *et al.,* 2010).

Regarding CFT titres of the complement-fixing antibodies in the blood serum of sheep post-vaccination, they were present in 81.43% of vaccinated sheep in the first-month and disappeared completely by the 4th month. The limitation of the CFT is that it requires laboratory facilities and well-skilled laboratory staff. Although accurate, this test does not allow differentiation between antibodies due to infection from vaccinal antibodies (Poiester *et al.,* 2010). In spite of these issues, the CFT is a generally utilised test, and has been viewed as the most particular serological test for determination of brucellosis; it is a prescribed test for global trade (OIE, 2016).

Our results agree with those of Blasco & Molina-Flores (2011); OIE (2016) that when using serological tests, it is important to take vaccination status into consideration given that there are no currently serological tests that could differentiate vaccinated sheep and goats from the natural infected animals.

The study clearly showed the existence of vaccinal antibody in the vaccinated sheep beyond long period post-vaccination as also affirmed by Abboud (2015).

Relative sensitivity and relative specificity of ELISA using S-LPS were estimated as 99.39% and 72.07% respectively in sheep and 99.44% and 78.18% respectively in goats (Table 4). This agrees with Crowther (2001) who reported the superior sensitivity of ELISA as a primary binding immunoassay that detect the existence of all antibodies regardlessof their isotype or biological activity. The test was not able to differentiate between sheep vaccinated with Rev-1 vaccine from sheep and goats infected with brucellae in agreement Blasco (1997) who reported that *B. melitensis* Rev-1 had a long-lasting antibody response. Furthermore, Adone & Pasquali (2013) detailed that serological tests fit for detecting the S-LPS were the most sensitive for identifying small ruminant brucellosis, yet they may yield false positive outcomes, if the animals have been already vaccinated or presented to Gram-negative bacteria with LPS O-chains like those of brucellae.

In this study, the practical usefulness of the ELISA with sarcosine extracts was tested as a method to differentiate *Brucella*-infected animals from vaccinated animals. Relative sensitivity and relative specificity of ELISA using sarcosine extracts were estimated as 98.18% and 97.297% respectively in sheep, and 99.45% and 97.27% respectively in goats (Table 4). The sarcosine extracts sturdily reacted with sera from suspected animals, but not with sera from *Brucella* free animals. Sera from sheep vaccinated with strain Rev-1 which were positive by conventional serological tests, were negative with sarcosine extracts (Table 3). These results established that an ELISA with sarcosine extracts is a useful tool for differentiating vaccinated from naturally infected animals. This result may be attributed in fact to the low concentration of LPS in the sarcosine extracts and matched with other results (Erdenebaatar *et al.,* 2003; Soliman *et al.,* 2014) which noticed that fortyfold-diluted sera from vaccinated animals did not exceed an OD492 of 0.5, and 1/50- to 1/800-diluted sera from infected animals had OD492 values higher than 0.5 when tested using ELISA with sarcosine extracts. Therefore sera from small ruminants vaccinated with strain Rev-1, which were positive by conventional serological tests, were negative with sarcosine extracts. The results obtained from this study, suggested that the ELISA with sarcosine extracts could be helpful for the identification of *Brucella*-infected sheep and goats (Erdenebaatar *et al.,* 2003) but further studies are necessary on its usage in the differentiation between *Brucella* infected and vaccinated small ruminants.

A molecular biotyping approach has been proposed on the foundation of restriction endonuclease polymorphism in the genes encoding *omp2* gene. The PCR test was performed with *B. melitensis* standard strain Rev-1 and *B. melitensis* biovar 3 field strain. The *omp2* gene exists as a locus of two almost homologous repeated copies (*omp2a* and *omp2b*) that somewhat differ among *Brucella* spp. (Ficht *et al.,* 1988). According to this information, we utilised unequivocal primers that intensify a 282-bp fragment (Fig. 1), flanking upstream sequences of the 5 termini of the two genes (*omp2a* and *omp2b*) and mounting downstream of the Pst I sites (Ficht *et al.,* 1990).

Pst I restriction endonuclease was capable to digest the amplified fragments of studied *Brucella* strains to give different

bands that manifested on the agarose gel (Fig. 2). Our results revealed that 4 out of 5 serum samples examined by RFLP-PCR were identified as *B. melitensis* Rev-1 vaccine abd 4 out of 5 –as *B. melitensis* biovar 3 field strain (Table 5).

Results obtained from this research, demonstrated that DNA fragments obtained from *B. melitensis* standard Rev-1 vaccine strain from seropositive animals (vaccinated) distinguished as *B. melitensis* Rev-1 strain produced four bands, an intact 282-bp fragment from the amplified *omp2a* gene that lacks the Pst I site, and another smaller four bands 238-bp fragments. Moreover, *B. melitensis* field infection (*B. melitensis* biovar 3) produced four bands 238-bp fragments from both *omp2a* and *omp2b*. These outcomes came in close consent to previous results (Ficht *et al.,* 1990; Bardenstein *et al.,* 2002; López-Goni *et al.,* 2008).

CONCLUSIONS

The indirect ELISA with sarcosine extracts was helpful to a certain extent in differentiating vaccinated from naturally infected sheep and goats but needs further investigations. Sarcosine based indirect ELISA was a sensible tool for accurate detection of *Brucella* infection in sheep and goats, which may be usefully utilised in the diagnosis of brucellosis and offers the advantage of simplicity and celerity. Our results confirmed that restriction fragment length polymorphism-polymerase chain reaction can produce a distinction in a very sensible single step between sheep and goats infected with *Brucella* field strains from sheep vaccinated with the Rev-1 vaccine.

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Paper received 03.10.2018; accepted for publication 05.11.2018

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