Evaluation of ELISA Using Different Antigens in the Discrimination between Brucella Vaccinated and Infected Cattle

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Abstract: The main objective of the present study was the diagnosis and differentiation between Brucella infected and vaccinated cattle. 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 Immunoblot and iELISA using LPS or CPE as coating antigens.The highest values of the ability of serological tests to differentiate S19 vaccinated animals from those infected ones were detected in iELISA using CPE as coating antigen and lowest values were seen in iELISA using LPS as coating antigen.
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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)** also it is one of the major diseases that is responsible for reproductive failure in animal livestock**(Xavier *et al.,* 2009)**

Brucellosis eradication programs are based exclusively on serological screening of cattle as well as sheep herds to detect and remove infected animals and using attenuated vaccines such as *Brucellaabortus*S_{19} for cattle and *Brucellamelitensi*s Rev1 vaccine for sheep and goats.

The presence of smooth lipopolysaccharides (S-LPS) with an o-chain explains the appearance and persistence of antibodies in serum following administration of these vaccines is consider one of the most difficult tasks in the serological diagnosis of bovine brucellosis is the discrimination of infected from vaccinated animals **(Nielsen *et al.,* 1989).**

Indeed most standard serological tests, i.e. serum agglutination and complement fixation use whole smooth cell preparations as antigen for diagnosis of brucellosis **(Magee, 1980; De Klerk and Anderson, 1985).**

Since S-LPS is the major exposed antigen of the cell surface **(Debbarh*et al.,* 1995)** as the antibody response to the S-LPS that is usually measured. In addition, these techniques do not distinguish clearly infected from vaccinated animals, and also other gram negative bacteria may cross react with smooth brucella species such as *Yersinia enterocolitica 0:9.*

Consequently, the specificity of LPS-based assays is low Anti-LPS antibodies may persist for more than a year after acute brucellosis **(Almuneef and Memish, 2003).** It cannot differentiate vaccinated from infected animals by most of the LPS-based assays **(Samartino*et al.,* 1999)**. Therefore, the serological diagnosis of brucellosis is still a challenge in animal diseases. The development of LPS-free protein-based diagnostic may be the key to overcome all of these challenges

Thus it is reasonable to assume that the determination of the humeral response against Brucella proteins could help to circumvent those undesired reactivities although the internal antigen may be considered as an excellent antigen for its specificity as cytoplasmic proteins induce a higher antibody response than outer membrane proteins **(Letesson*et al.,* 1997)**. Additionally, the production of antibodies directed against proteins may be host specific, but this practical use seems to be limited since the antibodies cannot detected in early stages of infection **(Serikawa*et al.,* 1989)**. Therefore, the aim of this study was the extraction and purification of lipophysaccharides (S-LPS) and cytoplasmic protein extract (CPE) antigens and characterization of these antigens using sodium Dodcylesulphate polyacrylamide gel Electrophoresis (SDS – PAGE).

Apply immunoblot analysis on examined sera against extracted antigens to detect the immunogenic bands. Evaluation of indirect ELISA using lipopolysaccharides(LPS) or cytoplasmic protein extract(CPE) as coating antigens in diagnosis as well as estimation ability of applied tests in differentiation of brucella infected from vaccinated cows.

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 agewith 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 were collected from all animals for immunoblot and indirect ELISA. Blood samples were allowed to clot and the sera were separated by centrifugation and stored at at -20 ^oC in the deep freezer for serological tests.

2. 1. Antigen Preparation

The lipopolysaccharides(LPS) of *Brucellamelitensis* biovar3 (field stain)was extracted and purified from proteins and other contaminant by phenol-chloroform-petroleum ether method described by **Galanose*et al.***(1969) and modified by **Qureshi and Takayama (1982)**.

Extraction of cytosoluble antigen of *Brucellamelitensis* biovar3 (field strain) as described by **Chin and Turner (1990)** and Quantitation of the protein content by (**Lowry *et al.*, 1951**).

2.2. Polyacrylamide gel electrophoresis (SDS-PAGE):

It was performed according to the methods described by **Laemmli (1970)**

Samples were heated at 100c for 5 min. before they were loaded onto 10% (wt/vol) running gel with a 4%(wt/vol) stacking gel. Electrophoresis was carried out at a constant voltage until the dye had migrated to the bottom of the gel.

2.3. Immunoblots

The extracted antigens (LPS or CPE) were separated by SDS-PAGE and transferred to nitrocellulose sheets at 60V overnight with cooling following the method of **Towbin*et al.* (1979).**

2.4. ELISA

It was carried out according to the methods described by **Bassiri*et al.* (1993).**Coating**:**Disposable polystyrene microtiter plates with flat bottom were coated with either LPS diluted in carbonate buffer at 0.5 µg/ ml (100 µl /well) (**Kiltelberger *et al.*, 1998)** or CPE preparation at final concentration of 20 µL/ml each per well in100 µl of carbonate buffer (**Weynants*et al.*, 1996**) and incubated overnight at 4°C. The coated plates were incubated at 37 °c for 1 hour then over night at 4°c followed by three times of washing using washing buffer (200 µl/well). Blocking was performed by adding the blocking buffer with 200 µl/well of a solution of PBS-Tween (20 mM phosphate buffer(pH 7.4) containing 0.05% Tween20), 3% casein for one hour in incubator then washed different times. Two fold serial dilution of serum samples were added (l00 µl/well) and then incubated for one hour, washed different times using washing buffer. Alkaline phosphatase labeled anti-bovine IgGconjugate (Sigma Chemical Co) was added (l00 µl/well) at 37 °c for 1 hour then washed three times. Substrate indicator mixture (l00 µl/well) was added and the plates incubated at room temperature in dark place. Stopping of the reaction occurred using stopping buffer (50 µl/well) then reading at 492 nm wave lengths (versa max apparatus USA). Control positive and negative sera were included in each time at work. ELISA titers were expressed as the reciprocal of the highest serum dilution that gave an OD reading of at least two times the OD for negative serum

3.Results and Discussion

Extracted LPS preparation from *Brucellamelitensis*biovar 3 was obtained in almost pure form and results obtained in Table (1) and Figure (1) indicated that 4 bands in SDS-PAGE were detected after staining with silver nitrate method.

The molecular weight of these bands ranged between 6.79 to 11.32 KDa which is similar to that reported by **Bogard*et al.* (1987).** These bands represent the core region and clusters of polysaccharide side chains. Similar observation was recorded by **Grain-Bastuji*et al.* (1990).** It has been demonstrated that cytoplasmic proteins is the common internal antigens of smooth and rough strains of brucella **(Baldi*et al.*, 1999).** Therefore, it was assumed that the cytoplasmic proteins of genus brucella are specific for the genus and most of them are common to its entire species.

Table (1): Sodium dodecyl sulfate polyacrylamide gel electrophoresis pattern of lipopolysaccharides extracted from *Brucellamelitensis*biovars3.

Lanes bands	Marker		LPS	
	M.W(KDa)	Amount	M.W(KDa)	Amount
1	200	4.90	11.32	59.8
2	97.40	6.35	9.10	9.08
3	68	13.8	8.21	16
4	29	32.1	6.79	15.2
5	14.30	23.1	--	--
6	6.20	19.7	--	--

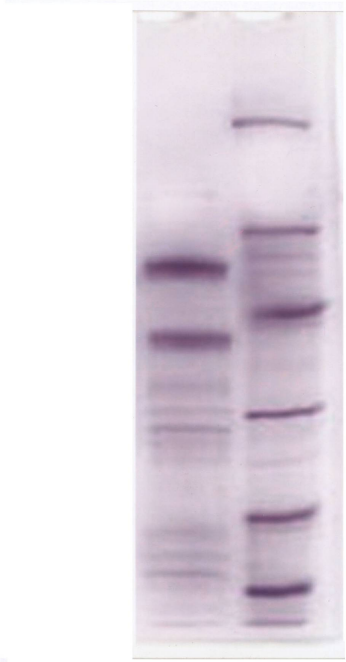
M.W : Molecular weight
K Da : Kilo Dalton

Lane (1) Lane (2)
Fig (1): Sodium dodecyl sulfate polyacrylamide gel electrophoresis pattern of lipopolysaccharides of *Brucellamelitensis* stained by silver stain
Lane (1) LPS
Lane (2) Marker

The SDS-PAGE of CPE extracted from *Brucellamelitensis*biovar 3 stained by (Coomassie blue) Table (2) Figure (2) revealed 8 protein bands ranging from 10.28 to 80.80 KDa. This result agrees with that recorded by **Debbarh*et al.* (1996)** who found CPE bands in the molecular weight mass ranging from 10 to 80 Kda.

Table (2): Sodium dodecyl sulfate polyacrylamide gel electrophoresis pattern of cytoplasmic proteinextracted from *Brucellamelitens*biovar 3.

Lanes bands	Marker		CPE	
	M.W(KDa)	Amount	M.W(KDa)	Amount
1	97.4	6.98	80.88	36.1
2	58.1	23.4	58.07	29.6
3	39.8	22.8	38.33	7.81
4	29	14.8	36.14	3.1
5	20.1	14.5	27.08	9.37
6	14.3	17.5	25.71	5.38
7	--	-	18.01	3.18
8	-	-	10.28	5.49

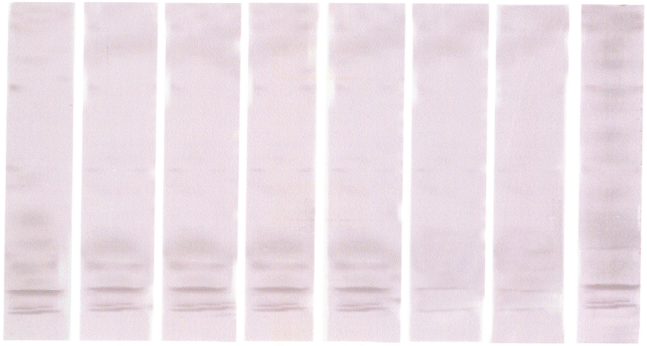


Lane (1) Lane (2)
Fig. (2): Sodium dodecyl sulfate polyacrylamide gel electrophoresis pattern of cytoplasmic protein of *Brucellamelitensis* stained by coomassie stain.
Lane (1) CPELane (2) Marker

Immunoblotting analysis using LPS, the antigen antibodies reaction of sera of brucella infected cows was typically similar to sera of S_{19} vaccinated cow As shown in Figure (3). In fact, the reactivity of brucella infected cows cannot be distinguished from that of cows vaccinated with S_{19} against LPS antigen.

These results are similar to that recorded by **Marin et al. (1999)** who suggested that the similarity in the S-LPS response hampered diagnostic effort to differentiate vaccinated from infected animals. Also **Baldi*et al.* (1996)** reported that S_{19} vaccinated animals developed anti LPS IgG level that overlapped with those developed by brucella infected cattle.

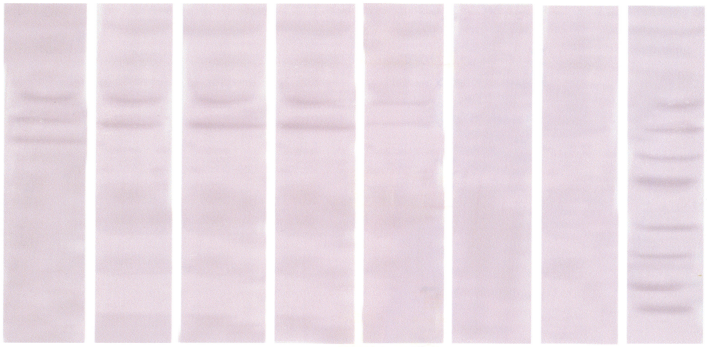
In this study immunoblot technique was used to analyze bovine antibody responses to CPE of *Brucellamelitensis*biovar 3 to determine whether there are antigen specific difference in serologic response of S_{19} vaccinated and naturally infected animals Figure (4)



Lane (1) Lane (2) Lane (3) Lane (4) Lane (5) Lane (6) Lane (7) Lane (8)

Fig. (3): Immunoblott analysis using LPS antigen.

Lane (1): S19 vaccinated cow sera (4 weeks post vaccination) against LPS antigen.
Lane (2): S_{19} vaccinated cow sera (6 weeks post vaccination) against LPS antigen.
Lane (3): S_{19} vaccinated cow sera (8 weeks post vaccination) against LPS antigen.
Lane (4): S_{19} vaccinated cow sera (10 weeks post vaccination) against LPS antigen.
Lane (5): S_{19} vaccinated cow sera (12 weeks post vaccination) against LPS antigen.
Lane (6): S_{19} vaccinated cow sera (16 weeks post vaccination) against LPS antigen..
Lane (7): S_{19} vaccinated cow sera (20 weeks post vaccination) against LPS antigen.
Lane (8): Brucella infected cow sera against LPS antigen.



Lane (1) Lane (2) Lane (3) Lane (4) Lane (5) Lane (6) Lane (7) Lane (8)

Fig. (4): Immunoblott analysis using CPE antigen.

Lane (1): Brucella S_{19} vaccinated cow sera against CPE (4 weeks post vaccination).
Lane (2): Brucella S_{19} vaccinated cow sera against CPE (6weeks post vaccination)
Lane (3): Brucella S_{19} vaccinated cow sera against CPE (8 weeks post vaccination)
Lane (4): Brucella S_{19} vaccinated cow sera against CPE (10 weeks post vaccination)
Lane (5): Brucella S_{19} vaccinated cow sera against CPE (12 weeks post vaccination)
Lane (6): Brucella S_{19} vaccinated cow sera against CPE (16 weeks post vaccination)
Lane (7): Brucella S_{19} vaccinated cow sera against CPE (24 weeks post vaccination)
Lane (8): Brucella infected cow sera against CPE.

By using peroxidase rabbit anti bovineIgG conjugate, sera from naturally infected cows showed strong antibody reaction against 80.88, 58.07, 38.33, 36.14, 27.08, 25.71, 18.01 and 10.28 KDa bands. However sera from S_{19} vaccinated animals 4 weeks post vaccination showed week immunoglobulin reactivity against 80.88, 58.07 and 38.33 KDa while, no reaction was observed against 18.01 KDa bands. Sera from vaccinated cows 6,8,10 and 12 weeks post vaccination developed weak antibody reaction against 80.88 and 58.07 KDa only.

So, our results confirm the finding reported by **Debbrah et al. (1995)** who found that vaccinated animals not developed anti 18 KDa protein response. Also **Hemmen*et al.* (1995)** could not find by competitive ELISA, any anti – 18KDa protein response in S_{19} vaccinated cattle.

Enzyme likedimmunsorbent assay using purified antigens and/or monoclonal antibodies have developed in order to eliminate the problem of low specificity (**Oncel, 2005).** This technique has been evaluated for many years for their diagnostic performance to detect serum antibodies to brucella species.

Indirect ELISA have several advantages being that the antibodies to be detected react with the antigen without performing secondary function as agglutination precipitation or activation of complement **(Nielsen and Kwok, 1995).**

A variety of *Brucellamelitensis* surface antigens contribute to the diagnosis of infection in cattle. Although the internal antigen may be considered as an excellent antigen for its specificity, its practical use seems to be limited since the antibodies cannot detected in early stages of infection **(SeriKawa*et al.*, 1989).**

It has long been recognized that the LPS is the major antigen of the surface of smooth brucella (**Zygmunt*et al.*, 1994).** Naturally infected animals have continuous exposure to antigen because of the persistence of pathogenic strains and produce LPS antibody response **(Nielsen *et al.*, 1989).**

In this study the results of antibody reactivity of sera of suspected cows and non-reactors (brucella free cows) are shown in Table (3).

The positive cases are 153(85%) and 7(5.6%), respectively. These results coincided with that of **Nielsen and Gall (1994)** who reported that LPS who commonly used as an antigen in most indirect ELISA formats.

Table (3): Antibody reactivity of sera of examined cows using LPS as coating in indirect ELISA .

Animal examined		Antibodes titer														Total reactor	
		20		40		80		160		320		640		1280			
		No.	%	No.	%	No.	%	No.	%	No.	%	No.	%	No.	%	No.	%
Su	180	2	1.1	14	7.8	14	7.8	30	16.7	32	17.8	40	22.2	21	11.7	153	85
Sr	125	4	3.2	2	1.6	1	0.8	0	0	0	0	0	0	0	0	7	5.6

Non specific cross reacting antibodies in uninfected animals against brucellalipopolysaccharidies (LPS) have been shown due to several pathogens including *Escherichia coli,Yersinia enterocolitica* and *Pseudomonas salanacearum* **(Nielsen and Duncan, 1982).**

Cytoplasmic proteins are the predominant components of the soluble Brucella species fraction from both smooth and rough strains. Thus they are relatively easy to obtain and it has been reported that both humoral and cellular immune responses against them occur **(Goldboum*et al.*, 1992).**

Results showed in Table (4) revealed that131 (72.8%) of suspected cows developed IgG against CPE and all non reactors (brucella free cows) showed negative reaction against CPE.

Table (4): Antibody reactivity of sera of examined cows using CPE as coating antigen in indirect ELISA.

mal exa min	No.	Antipodes titer														Total reactor	
		20		40		80		160		320		640		1280			
		No.	%	No.	%	No.	%	No.	%	No.	%	No.	%	No.	%	No.	%
ted	180	0	0	3	1.7	12	6.7	24	13.3	38	21.1	32	17.8	22	12.2	131	72.8
af	125	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0

These results agree with **Debbrah*et al.* (1995)** who added that the antibody response against cytosoluble proteins however was always less intense and more heterogenous than antibody response against LPS but it is more specific.

In addition, IgG response against LPS preceded that against cytosoluble proteins in all infected animals which confirmed that smooth LPS antigen is the major immunodominant antigen in smooth brucella infection **(Zygmunt*et al.*, 1988).**

Results of antibody reactivity of sera of vaccinated cows using LPS as coating antigen in indirect ELISA was shown in Table (5) revealed that 100% of vaccinated cows 4 weeks post vaccination showed IgG anti LPS antibodies. This may attributed to the presence of large amount of smooth LPS on vaccinalBrucella strains induce a high level of anti-LPS antibody response **(Jimenez de Bague*set al.*, 1992).**

The usefullness of antigen other than LPS for differential diagnosis of bovine brucellosis has been poorly investigated.

Table (5): Antibody reactivity of sera of vaccinated cows using LPS as coating antigen in indirect ELISA.

No of examined animals	Time of examination (weeks)	Titers														Total reactor	
		20		40		80		160		640		1280		2560			
		No.	%	No.	%	No.	%	No.	%	No.	%	No.	%	No.	%	No.	%
530	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	2	164	30.9	181	34.2	68	12.8	0	0	0	0	0	0	0	0	413	77.9
	4	140	26.4	163	30.8	126	23.8	100	18.9	1	0.2	0	0	0	0	530	100.0
	6	125	23.6	131	24.7	143	27.0	86	16.2	44	8.3	0	0	0	0	529	99.8
	8	114	21.5	189	35.7	28	5.3	39	7.4	42	7.9	0	0	0	0	412	77.7
	10	99	18.7	123	23.2	118	22.3	0	0	0	0	0	0	0	0	340	64.2
	12	110	20.7	45	8.5	57	10.8	0	0	0	0	0	0	0	0	212	40
	16	93	17.5	36	6.8	18	3.4	0	0	0	0	0	0	0	0	147	27.7
	20	29	5.5	19	3.6	0	0	0	0	0	0	0	0	0	0	48	9.1
	24	11	2.1	1	0.2	0	0	0	0	0	0	0	0	0	0	12	2.3

The result of antibody reactivity of sera of vaccinated cows using CPE as coating antigen in indirect ELISA was shown in Table (6) and revealed that only 4.2%, 3.2% and 0.9% of vaccinated animals were positive with low titer 4, 6 and 8 weeks post vaccination, respectively.

Table (6): Antibody reactivity of sera of vaccinated cows using CPE as coating antigen in indirect ELISA.

No. of examined animals	Time of examination (weeks)	Titers														Total reactor	
		20		40		80		160		640		1280		2560			
		No.	%	No.	%	No.	%	No.	%	No.	%	No.	%	No.	%	No.	%
530	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	2	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	4	20	3.8	2	0.4	0	0	0	0	0	0	0	0	0	0	22	4.2
	6	14	2.6	3	0.6	0	0	0	0	0	0	0	0	0	0	17	3.2
	8	5	0.9	0	0	0	0	0	0	0	0	0	0	0	0	5	0.9
	10	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	12	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	16	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	20	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	24	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0

The marked difference anti-CPE levels between vaccinated and infected cattle is probably explained by differences in the duration of the exposure to brucella antigen.

In the case of infection with virulent strains the exposure to brucella antigen persists for longer periods, producing a stronger antibody response. This fact indicates the potential usefulness of protein antigens for diagnosing active brucellosis **(Baldi*et al.*, 1996).**

It is obvious that an iELISA test for determining antibodies directed to cytoplasmic proteins of *Br. melitensis* could constitute a specific and sensitive test for diagnosis of bovine brucellosis **(Goldbaum, 1992).**

Also **Baldi*et al.* (1996)** reported that no cross reactivity reported to take place between *Br. melitensis* cytoplasmic proteins and proteins from other genera.

From the results showed in Table (7), it is clear that indirect ELISA using LPS cannot able to differentiate S_{19} vaccinated animals from infected ones.

Table (7): Ability of different serological test to differentiate S_{19} vaccinated animals (ADV) at different intervals post vaccination.

Time of examination (weeks)	IELISA	
	LPS IELTSA	CPE IELISA
2	22	100
4	0	95.8
6	0.2	96.8
8	22.3	99.1
10	34	100
12	60	100
16	72.3	100
20	91.3	100
24	97.7	100

In similar approach **Fensterbank*et al.* (1982)** showed that S_{19} vaccination produce an intense serological response as measured by standard serological tests which do not permit distinction between vaccinated and infected animals**. Jacques *et al.* (2007)** has also reported that S_{19} vaccine is known to induce antibody response in vaccinated animals indistinguishable by current conventional serological tests from those observed in challenge animals.

Conventional serological methods such as agglutination tests and primary binding assays such as ELISA, principally measure antibody S-LPS either as presented on intact bacterium or immobilized 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 they have a limited ability to discriminate vaccinated from naturally infected animals **(Crasta*et al.* 2008).**

When Brucella infection is diagnosed by measuring anti LPS antibodies, at least two interfering groups exist: animals recently vaccinated with S_{19} and those infected with crossly reacted bacteria. Several authors have proposed the use of antigenic components different from LPS as means to improve the diagnosis of bovine brucellosis **(Cloekaert*et al.* 1992; Gold baum*et al*,. 1994 and Hemmen*et al.*, 1995).**

In the present study we have been performed to measure the humoral immune response of vaccinated cattle against CPE.

As**Limet*et al.* (1988)** suggested that antigens not present in S_{19} should be used to differentiating vaccinated from infected cattle. Another possibility however, is that because of transient exposure to brucella antigens in S_{19} vaccinated cattle, poorly immunogenic component fails to significantly impact the immune system.

In deed ELISA with CPE antigen able to differentiate antibody responses of S_{19} vaccinated cow from those of suspected animals Table (7). This suggest that the antibody of cow infected with virulent *Brucellamelitensis* differ qualitatively from those of S_{19} vaccinated cows by their specificity for cytosoluble proteins antigen **(Debbrah*et al.*, 1995).**

As far as, we know in this study is applied to follow up the humoral immune response of vaccinated to cytoplasmic proteins of brucella. As shown here, this antiprotein response is measured by immunoblot or ELISA, could be useful for differentiating vaccinated from infected animals. In contrast detecting anti-LPS IgG antibodies would not be useful for testing cattle post vaccination.

While S_{19} vaccinated animals developed anti-LPS IgG level that overlapped with those developed by brucella infected cattle, a significant anti-18-KDa-protein antibody response was detected only in brucella infected cattle.

In conclusion, the antiprotein(especially 18-kd protein) humoral reactivity could be potentially useful for differentiation of Brucella infected cattle from those vaccinated with S_{19}.

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References

Almuneef, M and Memish, Z.A.(2003): Prevalence of Brucella antibodies after acute brucellosis. J. Chemother. 15:148.

Bassairi, M.; Ahmed, S.; Giavendoni, L.; John-Satiki, J. T.; Mebus, J. and Yilma, T. (1993): Immunological response to baculovirus expressed F and H. J. Virol., 76:1255- 1261.

Bogard, W. C.; Gunn, D. L. and Abernethy, T. (1987): Isolation and characterization of murine monoclonal antibodies specific for gram negative bacteria lipopolysaccharaides associated of cross gens reactivity with lipid a specificity. Infect. Immun., 4:899-908.

Baldi, P. C.; Araj, G. F.; Racaro, G. C.; Wallach, J. C. and Fossati, C. A. (1999): Detection of antibodies to brucella cytoplasmic proteins in cerebrospinal fluid of patients with neurobrucellosis. Clin. Diag. Lab. Immuno., 6:756-759.

Baldi, P.C.; Giambartolomei, G. H.; Goldbaum, F. A.; Abdón, L. P.; Velikovsky, C. A.; Kittelberger, R. and Fossati, C. A. (1996): Humoral immune response against lipopolysaccharide and cytoplasmic proteins of Brucellaabortus in cattle vaccinated with *Br. abortus* S_{19} or experimentally infected with Yersinia enterocolitica serotype 0:9. ClinDiagn Lab Immunol.;3(4): 472–476.

Chin, J.C. And Turner, B. P.(1990): Profiles of Serological Reactivity against Cytosoluble Antigens of *Brucellaovis* in Experimentally Infected Rams. J.ClinMicrobiol.; 28(12): 2647–2652.

Cloeckaert, A.; Zygmunt, M. S.; Wergifosse de P.; Dubray, G. and Limet, J. N.(1992): Demonstration of peptidoglycan associated brucella outer membrane protiens by use of monoclonal antibodies. J. Gen. Microbiol., 138: 1543-1550.

Crasta, O.R.; Folkerts, O.; Fei, Z.; Mane, S.P.; Evans, C.; Martino-Catt, s.; Bricker, B.; Yu,G.; Du, L. and Sorbral, B. W.(2008): Genome Sequence of *Brucellaabortus* Vaccine Strain S19 Compared to

Virulent Strains Yields Candidate Virulence Genes.PLoS ONE 3(5):e2193.

De Klerk, E. and Anderson, R. (1985): Comparative evaluation of the enzyme-linked munosorbent assay in the laboratory diagnosis of brucellosis. J. Clin. Microbiol.21, 381.

Debbarh, H.S.; Cloechaert, H.; Bezard, A.; Dubray, G. and Zygmunt, M. S. (1996): Enzyme linked immunosorbent assay with partially purified cytosoluble 28 KDa protein for serological differentiation between *Brucellamelitensis* infected and *Brucellamelitensis* Rev.1vaccinated sheep. Clin. Diag. Lab. Immun., 3(3):305-308.

Debbarh, H.S.; Cloeckaert, A.; Zygmunt, M. S.andDubray, G. (1995): Identification of sero-reactive *Brucellamelitensis*cytosoluble proteins which discriminate between antibodies elicited by infection and Rev.1 vaccination in sheep. Vet. Microbiol.,;44(1):37-48.

Fenstarbank, R.; Pardon, P. and Marly, J. (1982): Comparison between subcutaneous and conjunctival route of vaccination with Rev 1 strain against *Brucellamelitensis*infection in ewes. Ann. Rech. Vet., 13:295-301.

Galanose, G.; Luderitz, O. and Westphal, O. (1969): New method for the extraction of R-lipopolysaccharide. Euro J. Bioch., 9: 245-249.

Garin-Bastuji, B.; Bowden, R. A.; Dubray, G. andLimet, J. N. (1990): Sodium dodecyl sulfate-polyacrylamide gel electrophoresis and immunoblotting analysis of smooth-lipopolysaccharide heterogeneity among *Brucellabiovars* related to A and M specificities. J. Clin. Micro., Oct;28(10):2169-74.

Goldbaum, F. A.; Rubbi, C. P. and Fossati, C. A. (1994): Removal of LPS from brucella cytoplasm fraction by affinity chromatography with an anti-LPS monoclonal antibody as immunosorbent. J. Med. Microbiol., 40:174-178.

Goldbaum, F. A.; Rubbi, C. P.; Wallach, J. C. Miguel, S. E.; Baldi, P. C. and Fossati, C. A. (1992): Differentiation between active and in active human brucellosis by measuring antiproteinhumoral immune responses. J. Clin. Microbiol., 30:604-607.

Hemmen, F.; Weynants,V.; Scarcez,T.; Letesson, J. J. and Saman, E.(1995): Cloning and sequence analysis of a newly identified *Brucellaabourtus* gene and serological evaluation of the 17 kilodalton antigen that it encodes. ClinDiagn Lab Immunol. 2(3):263–267.

Jacques, T.; Verger, J. M.; Laroucau, K.; Grayon, M.; Vizcaino, N.; Pei, X. A.; Cutode, F.; Carreras, F. and Guilloteau, L. A. (2007): Immunological responses and protective efficacy against *Brucellamelitensis* induced by bp26 and OMP 31 *Brucellamelitensis* Rev. 1 deletion mutant in sheep. Vaccine 15, 25(5): 294-305.

Jimenez de Bagues, M. P.; Marin, C. M.; Blasco, J. M.; Moiyon, I.andGamazo, C. (1992): An ELISA with brucella; lipopolysaccharides antigen for the diagnosis of *Br. melitensis*infectionin sheep and for the evaluation of serological responses following subentaneous or conjunctival *Brucellamelitensis* strain Rev. 1 vaccination. Vet. Microbiol., 30:233-241.

Kittelberger, R.; Bundesen, P. G.; Cloeckaert, A.; Greiser-Wilke, I. and Letesson, J. J. (1998): Serological cross-reactivity between Brucellaabortus and Yersiniaenterocolitica 0:9 IV. Evaluation of the M- and C- epitope antibody response for the specific detection of B. abortus infections.Vet. Microbiol. 15; 60 (1): 45-57.

Laemmli, U. K. (1970): Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nat. (London) 227: 680 - 685.

Letesson, J.J.: Tibor, A.: van Eynde, G.; Wansard, V.; Weynants, V.;Denoel, P.andSaman, E. (1997): Humoral immune responses of Brucella-infected cattle, sheep, and goats to eight purified recombinant Brucellaproteins in an indirect enzyme-linked immunosorbent assay. Clin.Diagn. Lab. Immunol. 4, 556.

Limet, J. N.; Kerkhofs, P.; Wijffels, R. and Dekeyser, P. (1988): Le diagnostic serologque de la brucellse bovine par ELISA. Ann. Med. Vet., 132:565-575.

Lowry, O. H.; Rosebrough, N. T.; Farr, A. L. and Randall, R. J. (1951): Protein measurement with the folin phenol reagent. J. Biol. Chem., 193:265.

Magee, J.T. (1980): An enzyme-labelledimmunosorbent assay for *Brucellaabortus*antibodies. J. Med. Microbiol. 13, 167.

Marin, C. M.; Moreno, E.; Moriyon, I.; Diaz, R. and Blasco, J. M. (1999): Performance of competitive and indirect enzyme-linked immunosorbent assay, gel immunoprecipitation with native hapetn polysaccharide and standard serological tests in diagnosis of sheep brucellosis. Clin. Diag. Lab. Immun.; 6(2): 269-272.

Nielsen, K., and Duncan, J. R. (1982): Demonstration that nonspecific bovine *Brucellaabortus* agglutinin is EDTA-labile and not calcium-dependent. J. Immunol. 129:366-369.

Nielsen, K. and Gall, D. (1994): Advances in the diagnosis of bovine brucellosis use of enzyme immunoassaya. Gen. Eng. Biotechnol., 14:25-39.

Nielsen, K. and Kowk, A. (1995): Resue of polystyrene 96 well plate for indirect enzyme immunoassay for detection of antibody to *Brucellaabortus*. Arch. Med. Vet., 27:39-43.

Nielsen, K.; Cherwonogrodzky, J. W.; Duncan, J. R. and Bundle, D. R. (1989): Enzyme linked immunosorbent assay for differentiation of the antibody response of cattle naturally infected with *Brucellaabortus* or vaccinated with strain 19. Am. J. Vet. Res., 50:5-9.

Ocholi, R. A.; Kawaga, J. K. P. Ajogi, I. and Bale, J. O. (2005): Abortion due to *Brucellaabortus* in sheep. In Nigeria, Riv. Sci. Tech., Int, Epiz, 24(3):978-

Oncel, T. (2005): Sero-Prevalence of *Brucellacanis* infection in dogs in two provinces in Turkey. Turk. J. Anim. Sci. 29: 779-783.

Qureshi, N. and Takayama, K. (1982): Purification and structural determination of nontoxic lipid A obtained from lipopolysaccharide of *Salmonella typhinurium*. J. Biol. Chem. 2 (19) : 11808-11815.

Samartino, L.; Gall, D.; Gregoret, R.; Nielsen, K. (1999): Validation of enzyme-linked immunosorbent assays for the diagnosis of bovine. brucellosis. Vet. Microbiol.70:193.

Serikawa, T.; Iwaki, S.; Mori, M.; Muraguchi, T. and Yamada, J. (1989): Purification of *Brucellacains* cell wall antigen by using immunosorbent assay for specific diagnosis of canine brucellosis. J. Clin. Microbiol., 5(27):837-842.

Towbin, H.; Staehelin,T. and Gordon, J. (1979): Electrophoretic transfer of protein from Polyacrylamide gels to nitrocellulose sheets: Procedure and some applications. Proc. Natl. Acad. Sci. USA, 76: 4350- 4354.

Weyanants, V.; GTibor, A.; Denoel, P. A.; Saegerman, C.; Godfrroid, J.; Thiange, P. and Letesson, J. J. (1996): Infection of the cattle with Yersinia enterocolitica 0:9 a cause of false positive serological reactions in bovine brucellosis diagnostic tests. Vet. Microbiol. 48: 101-112.

Xavier, M. T. N.; Paixao, T. AF.P.; Large, A. P. And Santos, R. L.(2009): Pathological; immunohistochemical and bacteriological study of tissue and milk of cows and feuses experimentally infected with *Brucellaabortus*: J: co,p:Path: 2009. 140-149-197.

Zygmunt, M. S.; Debbarh, H. S.; Cloeckaert, A. and Dubray, G. (1994): Antibody response of *Br. melitensis* outer membrane antigens in naturally infected and Rev. 1 vaccinated sheep. Vet. Microbiol., 39:33-46.

Zygmunt, M. S.; Dubray, G.; Bundle, D. R. and Perry, M. P. (1988): Purified native heptens of Brucellaabortus and *Brucellamelitensis* 16 M reveal the lipopolysaccharide origin of the antigens. Ann. Inst. Pasteur/Microbiol.(Paris), 139: 421-433.

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