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PROFILES OF SEROLOGICAL REACTIVITY AGAINST CYTOSOLUBLE PROTEINS OF BRUCELLA MELITENSIS IN INFECTED AND VACCINATED SHEEP

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SUMMARY

Sera from vaccinated lambs with *Brucella melitensis* Rev.1 and naturally infected sheep with *Brucella melitensis* biovar 3 as well as Brucella free sheep, were examined using standard serological tests.

No great difference in standard serological tests results between vaccinated and infected animals. These sera were also analyzed for their serological reactivity against whole killed cell (WKC) and cytosoluble protein extract (CPE) antigens using ELISA.

The present study showed that ELISA with WKC was unable to differentiate infected sheep from those vaccinated with Rev.1 while ELISA with cytosoluble proteins antigen may able to differentiate antibody response of infected animals from vaccinated ones.

Using immunoblot technique, sera from naturally infected animals showed strong antibody reactivity to 28.48KDa and variable reactions to 58.80, 49.70, 39.60, 32.47 and 18.00 KDa.

However sera from Rev.1 vaccinated animals showed less intense antibody reactivity which only observed against proteins with molecular masses of 49.70 and 39.60 KDa.

It is likely that cytosoluble proteins may provide useful serological reagent for differentiation between infected and vaccinated sheep.

INTRODUCTION

Brucellosis is an infectious disease of animal that is caused by a number of host - adapted species

of the gram negative intracellular bacteria of the genus Brucella(Ocholi et al 2005.) Among the Brucella species *Brucella melitensis* may cause abortion in sheep and goats which results in hugs economic losses, particularly in Mediterranean countries (Zygmunt et al 1994).

Brucella melitensis inapparent infection is however common and is important source of transmission of the disease Acha and Szyfres 1980.

Brucella melitensis infection of sheep can not be eradicated in heavily infected countries by testing and slaughtering alone and that a vaccination programme is necessary to stop spread of the disease (Polmmet 1992).

Brucella melitensis Rev.1 strain is actually the best vaccine available in ovine brucellosis (Schurig et al 2002).

Since this strain induce a long - lasting serological response against lipopolysaccharide (S-LPS) standard serological tests (Rose Bengal plate test, agglutination and complement fixation test) which principally measure antibody to smooth Brucella LPS have been used routinely for diagnosis but the most difficult task has been to distinguish antibodies of infected animals from those of vaccinated animals Dubray 1985.

These techniques do not also distinguish clearly between different stages of the infection and present cross reactions with other infectious agents such as *Yersinia enterocolitica*, *Francisella tularensis* and *Pseudomonas maltophilia* (Mittal and Tizard 1981).

Several authors in response to these problems have focused on the identification of immunogenic Brucella outer membrane proteins (OMP) in human (Goldbaum et al 1991), cattle (Belzer et al 1991), dog (Carmichael et al 1989), sheep (Zygmunt et al 1994) and goat (Zygmunt et al 1990).

However, antibody responses to OMP in *B. melitensis* infected sheep were low and heterogeneous (Zygmunt et al 1993).

Therefore we have focused in this research on identification of immunogenic cytosoluble proteins of *B. melitensis* field strain.

In addition try to differentiate between *B. melitensis* infected sheep and *B. melitensis* Rev.1 vaccinated sheep by using this antigen.

MATERIAL AND METHODS

Bacterial strain and growth condition:-

B. melitensis biovar3 field strain was isolated in Brucella Department Animal Health Research Institute, culture were grown at 37°C for 48 hours on tryptic soy broth supplemented with 0.1% yeast extract.

Antigen preparations:-

1) Whole cell heat killed antigen: (WCK antigen) Araj et al 1986.

The stock suspensions of bacteria were heated at 100°c for 30 min and washed three times in saline. The whole bacteria used as antigen in ELISA were standardized to optical density of 0.2 0 at 540 nm.

2) Cytosoluble antigen: (CPE antigen) *B. melitensis* biovar3 cells were grown as described above were harvested in the logarithmic phase of growth and washed once with cold saline.

A thick cell suspension prepared in 10 mM PBS pH 7.2 was supplemented with DNase and RNase and then lysed. (Chin and Turner 1990).

After incubation for 4h at 37°C with magnetic stirring the cell envelopes were sedimented (80.000 xg, 2h at 4°C) and the supernatant was held at 4°c for 24h before being ultracentrifuged again under the same condition.

The new supernatant (cytosoluble) was dialyzed against 10 mM sodium Phosphate buffer pH 7.2 and freeze dried (Blasco et al 1994).

Sodium dodecyle sulphate electrophoresis (SDS-PAGE):-

The *B.melitensis* biovar3 cytosoluble proteins (CPE) were solubilized by boiling CPE (3mg/ml) in the modified laemmli buffer (Laemmli, 1970) for 5 minutes.

Four hundred µl of solubilized antigen were subjected to 14% sodium dodecyl sulfate-polyacrylamide gel electrophoresis with a stacking gel of 5% acrylamide and electrophoresed at 40m A per gel for 4 hours.

Molecular mass. Marker used ranfrom 6.50 to 97.40 KDa (Bio-Rad Low PM., SA, Paris, France).

Gel were coommassie blue stained for proteins as previously described by (Lugtenberg et al 1975).

Sera:-

The first group of sere was collected from 30 lambs which previously subcutaneously (S/CU) vaccinated at the age of 3-6 months, with the recommended dose (1.2 x 10^9 cfu) of *B. melitensis* Rev.1 vaccine.

They were bled at 2 weeks post vaccination and every 2 weeks until 12 weeks post vaccination.

The second group of sera was collected from 23 naturally infected non vaccinated flocks in governmental farm where *B. melitensis* is endemic. These ewes had a history of abortion and *B. melitensis* biovar3 isolated from their aborted foeti.

Negative control sera were taken from 20 Brucellosis free ewes from a farm proved to be free

from Brucellosis.

Standard serological tests:-

Rose Bengal plate test (RBPT), Rivanol test (Riv.T), Standard tube agglutination test (TAT) and Mercaptoethanol test (MET) were carried out according to Alton et al 1975.

ELISA:-

Sera were tested for antibody reactivity in an indirect ELISA. Wells of polysteren plates coated by 200µl of whole bacterial antigen or 20µl/ml of cytosoluble antigen (100µl per well) diluted in PBS pH 7.2 for 18h at room temperature (Salih-Alj Debbarh et al., 1996).

After washing with 0.15 NaCl tween 20 (NaCl-T), the non specific binding sites of wells were blocked with PBS-T and 0.5% gelatin and washed with MNaCl-T.

One hundred µl of diluted sera (1:100) in PBS-T were applied. Following an incubation for 90min at 37°C the wells were again washed and then 100µl of diluted horseradish Peroxidase-Labeled rabbit antisheep IgG was added.

After incubation for 60 min at room temperature, the wells were washed with NaCl-T and 100µl of subtrate solution containing 1 mM ABTS plus 4m MH_2O_2 in 50 mM sod citrate (PH4.2) was added. The plates were then shaken continuously at room temperature for 1hour optical density (OD) values were read at 415 nm with ELISA reader.

Immunoblot analysis:-

Electrophoretic blotting on nitrocellulose membranes was performed at 160 m A for 1hour in a transblot apparatus (Towbin and Gordon 1984).

The nitrocellulose blots were shaken for 1.5 hour at 37°C in blocking buffer. The blots were then incubated for 2hour at room temperature with examined sera diluted 1:100 in blocking buffer washed at room temperature by shaking with four changes of PBS containing 0.3 BSA. Then shaken for 1.5 hour at room temp. in peroxidase conjugated rabbit antisheep and washed. The antigen antibody complexes were visualized by addition of substrate solution.

RESULTS

In this study the humoral response in serum of lambs vaccinated with *Brucella melitensis* Rev.1 vaccine using RBPT, Riv T, TAT and MET (Table 1) revealed that agglutinins were quite evidenced 2 weeks post vaccination ,reached their peak 4 weeks post vaccination.

Twelve weeks post vaccination only 6 (20%), 4 (13.3%), 7 (23.3%) and 5 (16.7) were reactor for RBPT, Riv T, TAT and MET respectively.

The sera from examined animals were tested in IELISA using whole killed cell (WKC) or cyto-

soluble (CPE) as a coating antigen.

The time course of the antibody responses against WKC for Rev.1 vaccinated, naturally infected and Brucella free animals are shown in Table 2 Rev.1 vaccinated animals showed whole killed cell antigen antibody responses which increased till 4 weeks post vaccination. This response declined thereafter till reach the lowest level at the end of test period. As well as all natural infected animals showed higher antibody response against WKC antigen (OD 0.702), five Brucella free animals also react with WKC antigen (OD 0.314).

With the CPE, all the sera from naturally infected animals showed higher antibody reactivities (OD 0.689) (Table 2).

The sera of Rev.1, vaccinated animals did not react with CPE at any time after vaccination except for 2 and1 vaccinated animals which showed positive but low antibody response (OD 0.301)4 week post vaccination and (OD 0.297) 6 weeks post vaccination respectively (Fig1).

Only one serum from brucella free animals showed antibody reaction with an optical density 0.205 (Table 3).

The subunit polypeptide compositions of CPE analyzed by SDS-PAGE revealed 8 major protein bands ranging from 58.80 to 18.00 KDa were identified by coomassie brilliant blue staining with major bands at 58.80, 49.70, 39.60, 36.34, 34.60, 32.47, 28.48 and 18.00 KDa (Fig2). Pooled sera of naturally infected animals showed a strong response to a proteic band of 28.48 KDa wherease the response to the number of detectable bands of 58.80, 49.70, 39.60, 32.47 and 18.00 KDa was variable (Fig 3).

Table (1) Result of standard serological tests of vaccinated and infected sheep:

Animal examined	Time of examination (weeks)	RBPT		Riv.T		TAT		MET	
		No. of reactor	%	No. of reactor	%	No. of reactor	%	No. of reactor	%
Vaccinated (30)	0	0	0	0	0	0	0	0	0
	2	25	83.3	24	80.0	26	86.7	20	66.7
	4	29	96.7	27	90.0	30	100.0	27	90.0
	6	20	66.7	18	60.0	25	83.3	21	70.0
	8	19	63.3	16	53.0	20	66.7	18	60.0
	10	17	56.7	15	50.0	18	60.0	16	53.0
	12	6	20.0	4	13.3	7	23.3	5	16.7
Infected (23)		23	100	23	100	23	100	23	100
Control (20)		2	10	0	0	4	20	0	0

( ) = No of animals.

Table (2) Antibody reactivity of sera from vaccinated and naturally infected sheep against WKC in indirect ELISA

Animal examined	Time of examination (weeks)	Positive for ELISA			Negative for ELISA		
		No.	%	Mean optical density	No.	%	Mean optical density
Vaccinated (30)	0	0	0	--	30	100	0.139
	2	28	93.3	0.365	2	6.7	0.098
	4	30	100	0.523	0	0	--
	6	26	86.7	0.396	4	13.3	0.122
	8	21	70	0.309	9	30	0.103
	10	18	60	0.299	12	40	0.096
	12	6	20	0.261	24	80	0.116
Infected (23)		23	100	0.702	0	0	--
Control (20)		5	25	0.314	15	75	0.119

( ) = No. of examined animals

Optical density ≥ 0.2 consider positive

Table (3) Antibody reactivity of sera from vaccinated and naturally infected sheep against CPE in indirect ELISA

Animal examined	Time of examination (weeks)	Positive for ELISA			Negative for ELISA		
		No.	%	Mean optical density	No.	%	Mean optical density
Vaccinated (30)	0	0	0	--	30	100	0.151
	2	0	0	--	30	100	0.142
	4	2	6.7	0.301	30	93.3	0.136
	6	1	3.3	0.297	29	96.7	0.179
	8	0	0	-	30	100	0.158
	10	0	0	-	30	100	0.173
	12	0	0	-	30	100	0.164
Infected (23)		23	100	0.689	0	0	--
Control (20)		1	5	0.205	19	95	0.170

( ) = No. of examined animals

Optical density ≥ 0.2 consider positive

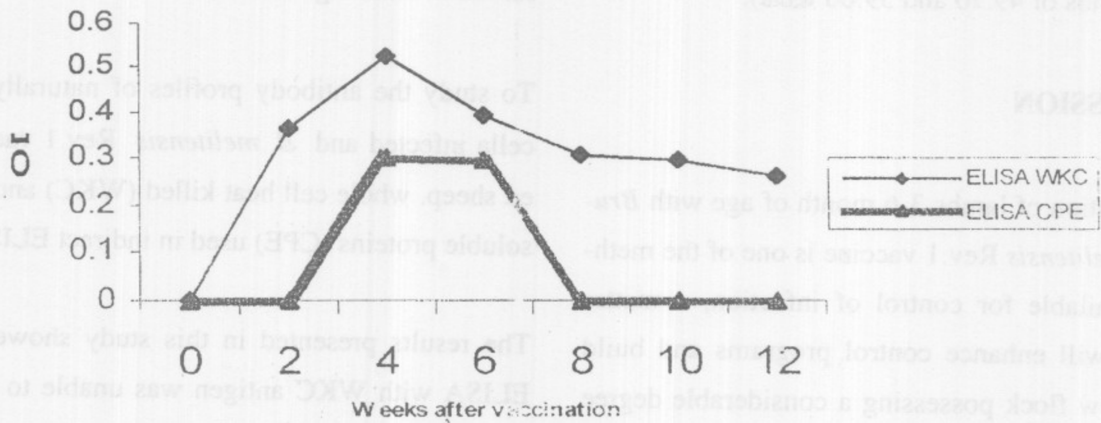
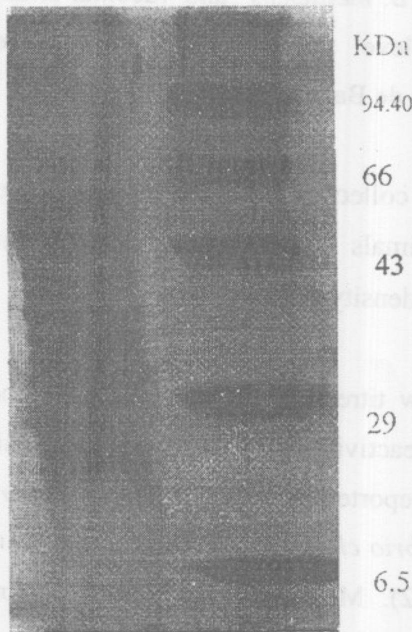
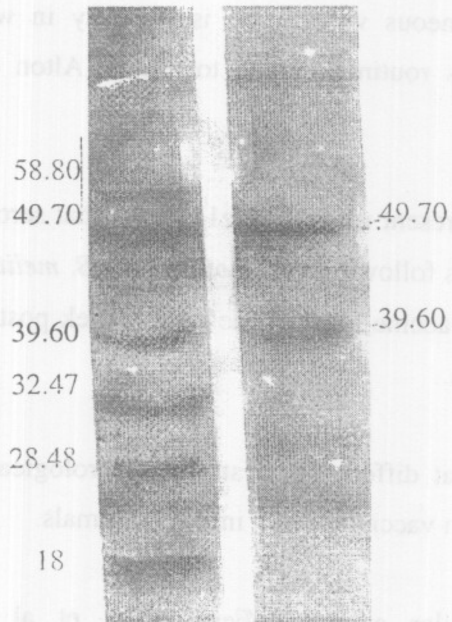


Figure (2):



SDS-PAGE of cytosoluble protein stained with commasic stain .
Lane 1 = marker .
Lane 2 = cytosoluble protein.

Figure (3):



Immunoblott analysis of infected and vaccinated animal sera against cytosoluble protein.
Lane 1 = vaccinated animals.
Lane 2 = infected animals

Development of IgG against individual polypeptide of CPE in vaccinated animals was also assessed by immunoblotting. Only two bands were observed with serum taken 4 week post vaccination (bands of 49.70 and 39.60 KDa).

DISCUSSION

Vaccination of lambs 3-6 month of age with *Brucella melitensis* Rev.1 vaccine is one of the methods available for control of infection, a matter which will enhance control programs and build up a new flock possessing a considerable degree of immunity against Brucella infection. (Bosseray 1985).

Subcutaneous vaccination is the way in which Rev.1 is routinely given to sheep (Alton et al 1988).

In the present work (Table1) shows that serological titers following vaccination with *B. melitensis* Rev.1 vaccine reached the peak 4week post vaccination.

No great difference in standard serological test between vaccinated and infected animals.

In similar approach Fensterbank et al 1982 showed that S/Cu vaccination produces 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 Rev.1 vaccine is known to induce antibody response in vaccinated animals indistinguishable by current conventional serological tests from those observed in challenge animals.

To study the antibody profiles of naturally Brucella infected and *B. melitensis* Rev.1 vaccinated sheep, whole cell heat killed (WKC) and cytosoluble proteins (CPE) used in indirect ELISA.

The results presented in this study showed that ELISA with WKC antigen was unable to differentiate infected sheep from those vaccinated with strain Rev.1. Because of the presence of a large amount of smooth LPS on WKC antigen, both virulent *B. melitensis* and vaccinal strain induce a high level of anti- LPS antibody response(Jimenez de Bagues et al 1992).

In sera collected from Brucella free sheep only five animals positive showed result with mean optical density 0.314.

The low titre in these samples could be due to cross reactivity with microorganisms similar to those reported between *Yersinia enterocolitica* O:9 *Vibrio cholerae* and *B. abortus* (Lindberg et al 1982). Moreover this low background titre could attribute to the presence of cross reactive polysaccharide in this antigen preparation.

In deed ELISA with CPE antigen may able to dif-

ferentiate antibody responses of Rev.1 vaccinated sheep from those of naturally infected.

This suggest that the antibody of sheep infected with virulent *Brucella melitensis* differ qualitatively from those of *B. melitensis* Rev.1 vaccinated sheep by their specificity for cytosoluble proteins antigen (Salih-Alj Debbrah et al 1995).

The antibody response against cytosoluble proteins however was always less intense and more heterogeneous than antibody response against WKC antigen. In addition the IgG response against WKC preceded that against cytosoluble proteins in all infected sheep which confirmed that smooth LPS present in WKC antigen is the major immunodominant antigen in smooth *Brucella melitensis* infection (Zygmunt et al 1988).

In this study immunoblot technique was used to analyze ovine antibody responses to cytosoluble proteins of *Brucella melitensis* biovar 3 to determine whether there are antigen specific difference in serologic responses of strain Rev.1 subcutaneous vaccinated and naturally infected animals.

By using horseradish peroxidase labeled rabbit anti sheep IgG conjugate, sera from naturally infected lambs showed strong reactivity to band 28.48KDa and variable reactions to 58.80, 49.70, 39.60, 32.47 and 18.00KDa (Fig3).

However sera from Rev.1 vaccinated animals showed immunoglobulin reactivity which observed against 49.70 and 39.60KDa protein bands.

Immunoglobulin previously conducted by Salih Alj Debbrah et al 1995 showed that the 28KDa protein band was the immunodominant antigen in either natural or experimental infection with *Brucella melitensis* in sheep.

Based on these data, these proteins could also possibly useful to permit distinction between infected sheep and Rev.1 vaccinated ones.

Among these proteins, the 28KDa protein seem to be the most interesting since it is detectable earlier in infection and had a high frequency of reactivity in naturally infected lambs.

In similar approach, Belzer et al 1991 have shown by using a preparation B. abortus salt- extractable proteins that some proteins of which in majority group ranging in molecular weight from 51KDa to 45KDa differentiated antibody responses from *B.abortus* naturally infected cows from vaccinated with *B. abortus* vaccine strain.

The results of this study confirmed that cytosoluble proteins may provide useful reagent for differentiation between infected and vaccinated sheep.

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مظاهر التفاعل السيرولوجي للبروتين الخلوي الذائب للبروسيلا ميليتنسيس في الاغنام المصابة والمحصنة

د/ هدى محمد زكى

قسم البروسيلا معهد بحوث صحة الحيوان. الجيزة. الدقى

تم فحص سيرم من حملان محصنة بلقاح البروسيلا مليتنسيس ريف ١ ومن اغنام مصابة طبيعياً بالبروسيلا مليتنسيس النوع ٣ وكذلك من اغنام خالية من الاصابة بالبروسيلا باستخدام الاختبارات السيرولوجية المعتادة وقد ظهر انه لا يوجد فرق كبير فى النتائج بين الحيوانات المصابة والمحصنة.

كما تم فحص هذا السيرم سيرولوجياً ضد انتيجينات الخلية المقتولة (WKC) ومستخلص البروتين الخلوي الذائب (CPE) باستخدام الاليزا.

واوضحت النتائج الموجودة في هذه الدراسة ان الاليزا باستخدام الخلايا المقتولة لم تستطيع التفرقة بين الاغنام المصابة والمحصنة بالريف ١ ولكن الاليزا باستخدام مستخلص البروتين الخلوي الذائب يمكن ان يفرق بين رد الفعل المناعي للحيوانات المصابة والمحصنة.

وباستخدام الامينوبلوت وجد ان السيرم من الحيوانات المصابة طبيعياً تتفاعل بقوة مع شريحة البروتين ذات الوزن الجزيئي ٢٨,٤٨ كليودالتون وتتفاعل تفاعل متغير مع الشرائح ذات الوزن الجزيئي ٥٨,٨٠ و٤٩,٧٠ و٣٩,٦٠ و ٣٢,٤٧ و١٨ كليودالتون ووجد ان السيرم من الحيوانات المحصنة بلقاح الريف ١ اظهرت تفاعلاً اقل للاجسام المضادة مع ولوحظ فقط ضد الشرائح البروتينية ذات الوزن الجزيئى ٤٩,٧٠ و٣٩,٦٠ كليودالتون

وعلى ذلك يمكن اعتبار ان انتيجين البروتين الخلوي الذائب يمكن ان يفيد كمادة سيرولوجية للتفرقة بين الاغنام المصابة والمحصنة