

Soluble Brucella Proteins as a Vaccine for Controlling Brucellosis in Sheep

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Abstract: This study aimed to evaluate soluble brucella proteins as a safe vaccine for control of brucellosis in sheep. Three groups of ewes were used to measure serological responses following inoculation with soluble brucella protein (SBP) mixed with mentonide-206 in comparison to Rev-1 vaccine. 1st group was inoculated with 8 mg of SBP S/C, 2nd group was vaccinated with Rev-1 vaccine at recommended dose S/C where 3rd group was inoculated with saline S/C as control group. Serological responses were measured with Rose Bengal test, modified Rose Bengal Test, CFT and IELISA tests which indicated that SBP give significant antibody responses in a group inoculated with 8 mg SBP mixed with mentonide-206 in a ratio of (1:1) in comparison to Rev-1 vaccinated group. Protective activity of SBP mixed with mentonide-206 was measured against Egyptian isolate of *Brucella melitensis* biovar 3 in Balb/C mice where SBP gave significant protection in comparison with Rev-1 vaccine with respect of PBS inoculated (unvaccinated) group. The protective activity of SBP was 3.016 while that of Rev-1 was 2.944, accordingly the Rev-1 vaccine is of higher potency than SBP but both are potent.

Key words: SBP • Rev-1 Vaccine • Balb/C Mice • Mentonide-206

INTRODUCTION

Brucellosis, also known as “undulant fever”, “Mediterranean fever” or “Malta fever” is a zoonosis and the infection is almost invariably transmitted by direct or indirect contact with infected animals or their products. It affects people and animals of all age groups and of both sexes. Although there has been great progress in controlling the disease in many countries, there still remain regions where the infection persists in domestic animals and, consequently, transmission to the human population frequently occurs. It is an important human and animal's disease in many parts of the world especially in the Mediterranean countries of Europe, north and east Africa, the Middle East, south and central Asia and Central and South America and yet it is often unrecognized and frequently goes unreported. There are only a few countries in the world that are officially free of the disease although cases still occur in people returning from endemic countries [1].

In Egypt, the most common cause of brucellosis in all animals' species is *Brucella melitensis* biovar 3 [2]. Control of brucellosis in Egypt depend on test and

slaughter policy and vaccination of brucella free animals but this policy is not realistic in the majority of places where *B. melitensis* is endemic due to lack of financial resources needed for compensation. International agencies have, therefore, proposed that whole flock vaccination should precede any test and slaughter program, until disease prevalence is significantly reduced, this policy is called Mass Vaccination. Vaccination of sheep is carried out with living *Brucella melitensis* Rev-1 vaccine using the recommended subcutaneous dose 1-3 X 10⁹ CFU/dose [3-5].

There were many trials to use and evaluate sub-cellular [as OMP, LPS and soluble Brucella protein (SBP)] and killed rough vaccines (as S45/20) in mice and sheep to overcome disadvantages of living brucella melitensis (Rev-1) vaccine which may transfer infection to human and animals and cause abortion in pregnant ewes. These types of vaccines have given significant protection in mice with respect to unvaccinated groups [6].

In this study we tried to use soluble Brucella proteins (SBP) as a safe vaccine to be used in sheep for control of brucellosis in sheep by evaluating its protection in Balb/C mice in comparison to Rev-1 vaccine.

MATERIALS AND METHODS

Strains: *Brucella abortus* S19 (CZ Veterinaria, S.A., Spain) and *Brucella melitensis* Rev-1 vaccine (CZ Veterinaria, S.A., Spain) were used.

Field isolate was isolated from aborted ewe (Egyptian isolate) and identified biochemically, serologically [7] and by using multiplex PCR [8] as *Brucella melitensis* biovar 3.

Preparation of Soluble Brucella Proteins (SBP): SBP was prepared as described by Yifan *et al.* [9] as follow:

Viable *B. abortus* (S19) organisms (5 ml of a suspension of 10^{10} /ml) were added to 800 ml of sterile trypticase soy broth (BBL Microbiology Systems, Cockeysville, Md.) and incubated at 37°C on a shaker platform for 48 h. Bacteria were harvested and washed once with saline. Hot saline extracts were obtained by suspending organisms in saline and autoclaving at 121°C for 15 min. The autoclaved suspension was centrifuged at 12,000 xg for 15 min and the supernatant was collected and precipitated with ammonium sulfate (50% saturation). After centrifugation at 8,000 xg for 15 min, the precipitate was dissolved in 0.01 M phosphate buffered saline (PBS) (pH 7.2) and dialyzed against 0.01 M phosphate buffer (pH 7.2) for 48 h. This preparation was designated SBP50. The supernatant from 50% ammonium sulfate precipitation was further precipitated with ammonium sulfate to 70% saturation. The resultant pellets were dissolved in PBS and dialyzed as described above. This preparation was termed SBP70. Both preparations were centrifuged at 12,000 xg to remove insoluble material and sterilized by filtration. The protein content was determined by A²⁸⁰ [10].

Animal's Inoculation

Sheep Inoculation: Eight to 14 months old ewes were grouped into 3 groups; 1st group was inoculated S/C with 8000 ug (8 mg) of SBP mixed with mentonide-206 (Sepic, France) in ratio of 1:1 and 2nd group was vaccinated S/C with Rev-1 vaccine at the recommended dose “1-3 X 10^9 CFU/dose” [7 and pamphlet of produced company (CZ Veterinaria)] and 3rd group was inoculated S/C with saline. 2nd (Rev-1 vaccinated ewes) and 3rd (saline inoculated ewes) groups were used as positive and negative control groups respectively. Serum samples were collected every week over a period of 6 months and tested for humoral immune responses using IELISA (using SBP as coating antigen), CFT and Rose Bengal Test (RBT) according to Alton *et al.* [7] and MRBT according to Blasco *et al.* [11]. Complement fixation at a dilution of

3log2 (1:8), the level recommended by the Australian Bureau of Animal Health, was regarded as a positive reaction. Serum samples were titrated 1:4 to 1:128 in the CFT. Titers determined by the CFT were expressed as log2 of the reciprocal of the last dilution at which a positive reaction occurred [12, 13] while IELISA assay was carried out using SBP as coating antigen. For the animals vaccinated, the ELISA and the CFT were assessed by comparing the number of weeks after inoculation that each test was positive.

BALB/c Mice Inoculation: Three groups of six-week-old female BALB/c mice (Tuderbelharus Institute, Cairo) were used. 1st group was vaccinated subcutaneously with SBP (800 ug/ml) mixed with mentonide-206 at ratio of 1:1. 2nd group was vaccinated with Rev-1 vaccine at a dose of 1 X 10^5 CFU S/C [14] and 3rd group was inoculated with sterile 10 mM PBS (pH 6.85) S/C. The last 2 groups were used as positive and negative control groups respectively. Thirty days later, each mouse was challenged with *B. melitensis* biovar 3 strain isolated from infected sheep (Egyptian isolate) in a dose of 2-4 X 10^5 CFU intra-peritoneally [14]. Inoculums (either Rev-1 vaccine, SBP and challenge strain) were prepared in sterile 10 mM PBS (pH 6.85) and doses were adjusted to be in 0.1 ml to be administered to each mouse. Doses and time intervals were chosen on the basis of previous experiments with sub-cellular brucellosis vaccines in mice [14-16].

Splenic Growth of Challenge Strain in Balb/C Mice:

All mouse were euthanized 15 days post-challenge and spleens were removed aseptically, homogenized individually and three tenfold dilutions were done (1/10, 1/100 and 1/1000) in the PBS and 0.1 ml of each dilution was seeded in TSA plates. Plates were incubated at 37°C for 4 to 5 days to determine the CFU/spleen; Colonies of *Brucella* should be enumerated on the dilutions corresponding to plates showing fewer than 300 CFU. When no colony is seen in the plates corresponding to the 1/10 dilution, the spleen is considered to be infected with five bacteria. These numbers of *Brucella* per spleen are first recorded as X and expressed as Y, after the following transformation: $Y = \log(X/\log X)$ [17].

RESULTS AND DISCUSSION

In the present study, mean serum antibody responses of sheep vaccinated with soluble *Brucella* proteins (SBP) mixed with mentonide-206 began with high antibody titer (+++++) from 1st week post-vaccination when measured

with rose Bengal test. The antibody titer remained high till 19th week post-vaccination and the titer began to decrease slightly to (+++) till 20th week post-vaccination and (++) till the end of the study (24th weeks post-vaccination). These titers remained high from 1st week post-vaccination till the end of the study when modified Rose Bengal test (MRBT) was performed (Chart 1). Mean serum antibody responses of sheep vaccinated with Rev-1 vaccine at the recommended dose began with high antibody titer (+++++) at the 1st week post-vaccination when measured with rose Bengal test. The antibody remained high till the 9th week post-vaccination and the titer began to decrease slightly to (3.5+) till 11th week post-vaccination, (+++) till 13th week post-vaccination, (++) till 15th week post-vaccination and (+) till 19th week post-vaccination. Titer disappeared completely from 20th week post-vaccination. When MRBT was done, titers remained at high level (+++++) from 1st to 17th week post-vaccination then it decreased gradually till became (++) at the end of this study (Chart 2).

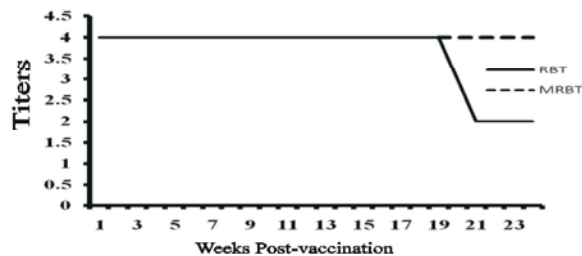


Chart 1: Mean serum antibody responses of sheep vaccinated with soluble Brucella proteins (SBP) mixed with mentonide-206 measured by the Rose Bengal test

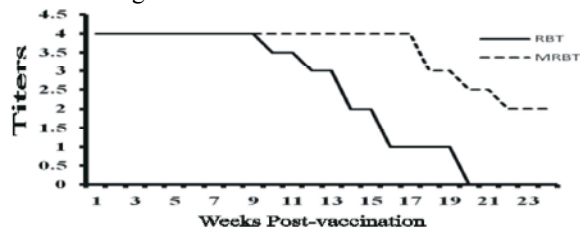


Chart 2: Mean serum antibody responses of sheep vaccinated with Rev-1 vaccine measured by the Rose Bengal test

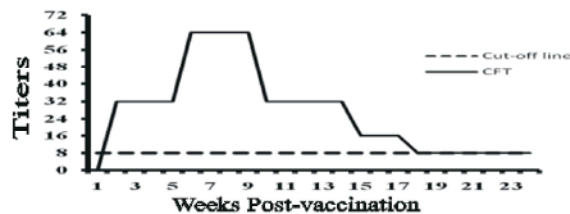


Chart 3: Mean serum antibody responses of sheep vaccinated with soluble Brucella proteins (SBP) mixed with mentonide-206 measured by CFT

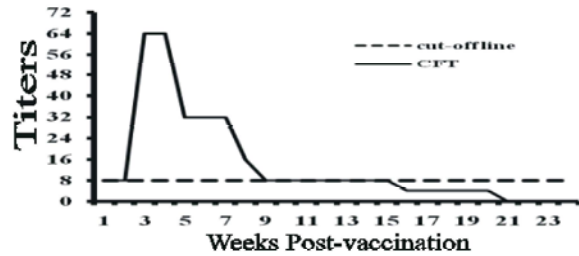


Chart 4: Mean serum antibody responses of sheep vaccinated with Rev-1 vaccine measured by CFT

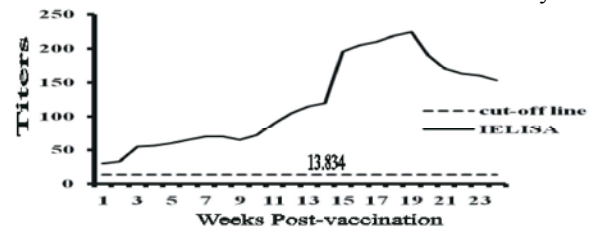


Chart 5: Mean serum antibody responses of sheep vaccinated with soluble Brucella proteins (SBP) mixed with mentonide-206 measured by IELISA using SBP as a coating antigen

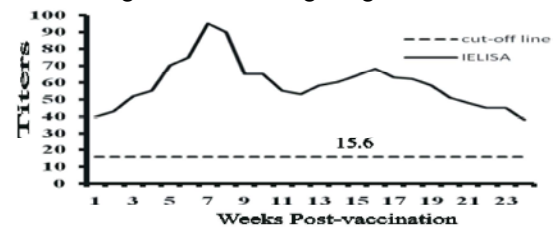


Chart 6: Mean serum antibody responses of sheep vaccinated with soluble Rev-1 vaccine measured by IELISA using SBP as a coating antigen

When CFT was done on the same serum samples according to Alton *et al.* [7] and judged according to Australian Bureau of Animal Health [12, 13] with cut off line at $3\log_2$ (1:8), antibodies responses of animals vaccinated with SBP mixed with mentonide-206 was absent in 1st week post-vaccination, these antibodies responses began to appear and became satisfactory from 2nd week post-vaccination and reached the peak at 6th to 9th week post-vaccination and it began to decline gradually from 10th week post-vaccination till the end of the study but the titers remained satisfactory ($3\log_2$, 1:8) (Chart 3). On other hand, the immune responses of ewes vaccinated with Rev-1 vaccine were satisfactory from 1st week post-vaccination and reached the peak at 3rd and 4th week post-vaccination it began to decline gradually from 5th week post-vaccination till it became unsatisfactory from 16th week post-vaccination and disappeared completely at 21st week post-vaccination (Chart 4).

Table 1: Protective activity level of periplasmic proteins with mentonide 206 adjuvant after inoculation in female Balb/C mice

Mice No.	Spleen weight	Average spleen colony count (culturing 200 µl on each TSA plate)			Protective activity		
		Dilution 1/10	Dilution 1/100	Dilution 1/1000	Dilution 1/10	Dilution 1/100	Average
1	0.1112	50	11	N*	3.09	3.40	3.250
2	0.1632	56	8	N	2.99	3.12	3.060
3	0.1034	30	4	N	2.92	3.03	2.980
4	0.0808	47	6	N	3.19	3.28	3.240
5	0.1335	18	2	N	2.63	2.67	2.650
Mean results							3.036

N* = No growing colonies

Table 2: Protective activity level of Rev-1 vaccine after inoculation in female Balb/C mice

Mice No.	Spleen weight	Average spleen colony count (culturing 200 µl on each TSA plate)			Protective activity		
		Dilution 1/10	Dilution 1/100	Dilution 1/1000	Dilution 1/10	Dilution 1/100	Average
1	0.1444	52	7	N*	3.01	3.110	3.060
2	0.1231	31	5	N	2.87	3.050	2.960
3	0.0900	34	4	N	3.02	3.086	3.050
4	0.1200	22	7	N	2.75	3.190	2.970
5	0.1250	19	N	N	2.68	-	2.680
Mean results							2.944

N* = No growing colonies

Table 3: Protective activity level of PBS after inoculation in female Balb/C mice (unvaccinated group)

Mice No.	Spleen weight	Average spleen colony count (culturing 200 µl on each TSA plate)			Protective activity
		Dilution 1/10	Dilution 1/100	Dilution 1/1000	Dilution 1/1000
1	0.5225	U*	U	520	5.22
2	0.2258	U	U	588	5.61
3	0.4093	U	U	654	5.41
4	0.4327	U	U	608	5.36
5	0.5292	U	U	543	5.23
Mean results					5.36

U*= Uncountable

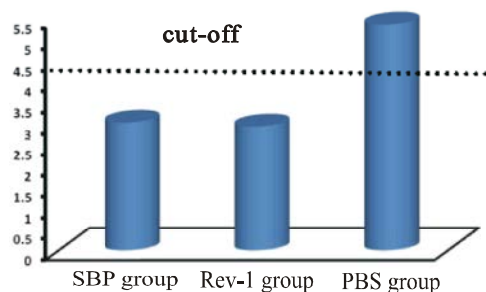


Fig. 1: Protective activity of soluble Brucella proteins, Rev-1 vaccine and PBS in inoculated groups of female Balb/C mice

IELISA was done according to Alton *et al.* [7] with using of SBP as a coating antigen. Cut-off line was calculated according to Colby [18]. Chart (5) shows that immune responses of ewes vaccinated with SBP mixed with mentonide-206 began weekly but satisfactory from 1st week post-vaccination till the 20th week post-vaccination where it began to decline gradually. On the other hand, mean immune responses of ewes vaccinated with Rev-1 vaccine began strongly from 1st week post-vaccination and remain high and fluctuated with the peak at 8th week post-vaccination. Mean immune responses decreased gradually from 16th week

post-vaccination (Chart 6). In general immune responses of 2 groups remained satisfactory along the entire period of the study.

Protection activity conferred by SBP against field isolate was measured in Balb/C mice using Rev-1 vaccinated and PBS inoculated groups as control groups. Fig. (1) shows that protection activity conferred by SBP and Rev-1 inoculated groups were significant with respect of PBS inoculated group.

Mean of protection activity of SBP group was 3.036 (Table 1) where in Rev-1 group was 2.944 (Table 2) and in PBS inoculated group was 5.36 (Table 3).

OIE consider a vaccine to be protective should give protective activity 2.5 and at least not more than 4.5 according to the results of vaccinated control groups and according to the dose of challenge and virulence of strain used in challenge. The protection activity conferred by SBP and Rev-1 were higher than 2.5 and this might be due to the higher dose of challenged isolates ($2-4 \times 10^5$ CFU) according to Bosseray [15] than that advised by OIE [17] (1×10^5 CFU).

The differences between the protection afforded by SBP or Rev-1 were not significant. It must be but in our consideration that the SBP used in this study was prepared from more than 1 year and kept at -20 C and this may reduce their efficacy and protective activity but anyhow results indicate the long durability of these proteins. These results agree with Bowden *et al.* [19], Cloeckart *et al.* [20, 21] and Phillips *et al.* [22] when they used sub-cellular vaccines against both rough and smooth brucella infections.

From these results we can recommend the use of SBP as Brucella vaccine but it needs more researches on large scale to establish the effect, duration of immunity and accurate dose of SBP in main host and to make potency test in the main host. SBP can be also used as immune-stimulant for Brucella vaccines especially in adult ewes which need reduced-reduced dose to decrease the possibility of abortion in pregnant ewes. Finally, we can recommend the use of SBP as a potent vaccine or immune-stimulant when inoculated in combination with oily adjuvant which will increase the duration of immune responses against these types of proteins.

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