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Original Research Article

Assessment of Immunogenicity of *Brucella abortus* S19 Phage Lysate in Mice

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Bruella abortus S19, Immunogenicity, Phage Lysate, Splenic Index

A B S T R A C T

This study aimed to evaluate immunogenicity of *Brucella abortus* S19 Phage lyaste in mice. *Brucella abortus* S19 phage lysate in three different doses were used in comparison to *Brucella abortus* S19 vaccine in five groups of mice. 1st, 2nd and 3rd group was inoculated S/C with 20µl, 40 µl, 60 µl of phage lysate respectively, 4th group was vaccinated with *Brucella abortus* S19 vaccine at recommended dose S/C where as 5th control group was inoculated with PBS S/C. Serological responses were measured by iELISA tests which indicated that phage lysate give significant antibody responses in a group inoculated with 60 µl phage lysate. All groups of animals were challenge with *Brucella abortus* S544 and spleen count was determined 15 days post infection. The immunogenic activity of phage lysate was 2.7 while that of *Brucella abortus* S19 was 2.3. In conclusion *Brucella abortus* S19 vaccine is of higher potency than phage lysate but both level of immunogenicity do not differ significantly.

Introduction

Brucellosis is a highly contagious, zoonotic and economically important bacterial disease of bovine worldwide, characterised by abortion in third trimester of pregnancy with retained placenta, infertility and reduced milk production (Kiros *et al*., 2016). Disease is mainly caused by *Brucella abortus* in bovine, which is facultative intracellular, Gram negative coccobacillus (Cutler *et al*., 2005). Brucellosis is widely prevalent in India among the bovine population both in organized farms and in the villages (Isloor *et al*., 1998 and Ramesh *et al*., 2013). It causes heavy economic loss to the animal industry through abortion, delayed conception, temporary or permanent infertility in the affected animals (Pal *et al*., 2017). Brucellosis in livestock is responsible for a median loss of US $ 3.4 billion (Singh *et al*., 2015) which can easily be avoided by initiating control measures.

In India live attenuated *Brucella abortus* strains 19 vaccine is only approved vaccine for the control of brucellosis in bovines. Although the protective immune response generated by S19 vaccination is both efficacious and sustained, there is persistence of a bacteria within target host, the possibility of reversion to virulence, interfere in diagnosis and abortion in pregnant animals, (Schurig *et al*., 2002). Inactivation or antigen extraction that does not or minimally alter the antigenic moieties

of the organism is the key to developing a cross-protective immunizing preparation. Previous research on bacteriophage (Larkum, 1929 and Compton, 1928) suggested that phage lyses preparation were indeed very good immunizing agent whose protection effect was stronger than that of regular heat and chemical inactivation of bacteria. Brucella phage possesses certain preventive properties against experimental challenge in experimentally challenged mice (Parnas, 1960; Parnas and Burdzy, 1961). Present study carried out to assesses the vaccine potency of *Brucella abortus* S19 phage lysate by evaluating its immunogenicity in swiss albino mice.

Materials and Methods

Ethical approval

All the experimental protocols carried out on laboratory animals were approved by the Animals Ethics Committee (AEC) of Indian Veterinary Research Institute (IVRI), Izatnagar-243122 (India).

Bacterial culture

Brucella abortus S19 and *Brucella abortus* 544, were obtained from Brucella Referral Laboratory, Division of Veterinary Public Health, IVRI, Izatnagar and maintained in the laboratory during the experiment by repeated sub culturing.

Phage Lytic to *Brucella* spp

Phage lytic to *Brucella abortus* S19 obtained from Deptt of Veterinary Microbiology GADVASU, Ludhiana (Chachra *et al.*, 2012)

Preparation of phage lysate

A 500 ml quantity of NZCYM broth in a 1000 ml capacity Erlenmeyer flask was seeded with about 5 ml quantity of the *Brucella abortus* S19 harvested from 48 hr culture on brucella agar and incubated at 37^0C. A 10 ml of phage stock was directly added to the 12 hr culture following strict aseptic measures, so that the phage: bacteria ratio became equivalent to multiplicity of infection (MOI) (1: 50). The phage- bacteria mixture was incubated at 37^0C in a shaking incubator for about 24-30 h until the turbidity of the suspension was reduced to about 90%. The resultant lysate was centrifuged to remove residual bacteria, and filtered through pre-sterilized 0.45µ filtration assembly (Millipore). The filtrate was collected in strict aseptic conditions and stored at 4^0C.

Production of hyper immune sera

Two healthy rabbit were used for production of hyperimmune sera against the prepared sterile phage lysate adjuvanted with freund complete adjuvant following the recommended schedule. Rabbits were bled through ear vein, serum was collected and stores at -20 C till further uses.

Immunoblotting of phage lysate

The immunoblotting was done to assess the immunoreactivity of various protein fractions in phage lysate. The procedure of Towbin *et al*. (1979) was followed with little modifications.

Estimation of carbohydrate, protein and endotoxin content of phage lysate

Total protein and carbohydrate concentrations and the endotoxin content of phage lysate preparation was determined by methods describe by Bradford, 1976; Dubois *et al*., 1956 and Limulus Amoebocyte Lysate Assay using E Toxate Kit (Sigma) as

per the manufacturer's protocol respectively.

Swiss Albino mice inoculation

Five groups of six-week-old female swis albino mice were used. 1st, 2nd, 3rd group was vaccinated subcutaneously with 20µl (0.094 mg total protein and 0.06 µg total carbohydrate), 40µl (0.18 mg total protein and 0.12 µg total carbohydrate), 60µl (0.282 mg total protein 0.24 µg total carbohydrate) phage lysate respectively. 4th group was vaccinated with S19 vaccine at a dose of 1 X 10^5 cfu in 0.1 ml PBS S/C (Nielsen and Ewalt, 2008) and 5th group was inoculated with sterile 100 µl PBS (pH 6.8) S/C. Thirty days later, each mouse was challenged with *B. abortus* S544 in a dose of 2 X 10^5 cfu in 0.1ml PBS intra-peritoneal (OIE, 2008). Serum was collected from each mouse by bleeding inner canthus at an interval of 1 week for 28 day and titre of antibrucella IgG antibodies was determined by Indirect ELISA as per protocol of Alton *et al*., 1988 using sonicated *Brucella abortus* S19 as a coating antigen.

Splenic growth of challenge strain in swiss Albino mice

Mice were killed by cervical dislocation 15 days post-challenge and spleens were removed and weighted aseptically. Each spleen was homogenized individually in 9 time to weight of spleen in balanced salt solution (BSS) (pH 6.8) and three tenfold dilutions were done (1/10, 1/100 and 1/1000) in the BBS and 0.2 ml of each dilution was seeded in Brucella agar media (BAM) plates (4 plate for each dilution, 2 kept aerobically and 2 kept in 5 % CO_2). 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) (Bosseray, 1993).

Statistical analysis

The data were analysed by one-way analysis of variance (ANOVA) following Least square test using an error of 0.05 using SPSS Statistics software.

Results and Discussion

The laboratory mice has been the most widely used for the brucellosis model **(**Grillo *et al***.,** 2012). Brucella have tendency to localized in spleen of mice and replicate result in enlargement of spleen which is prevented by administration of vaccine strain of S19 (Grillo *et al*., 2012). Ability of different phage lysate preparation and brucella vaccine to prevent enlargement of mice spleen was measured in term of splenic index [(spleen wt/ body wt) x100] to reduce the effect of body wt on spleen and it was found that phage lysate (20µl, 40µl and 60µl prevent the enlargement of spleen in comparison to unvaccinated mice (Table 1) (Figure 1).

Western blot analysis of phage lysate revealed that almost all the three class of outer membrane protein were present in phage lysate as describe by Verstreate and Winter, 1984. Protein band II(34-40 kDa) also known as porins were found to induce lymphocyte proliferation and strong delayed type- hypersensitivity (DTH) reaction in infected cattle (Winter, 1987). Lower MW proteins band which represent cytosolic ribosomal protein (17 kDa) is a immunodominant molecule which was

found immunoprotective and provoked strong delay type hypersensitive immunity in primed guinea pig (Bachrach *et al*., 1994). Indirect ELISA titer denoted as (p/n) ratio of OD which showed that immune responses of mice vaccinated with 60 µl phage lysate satisfactory from 1st week post-vaccination till the 4th week post vaccination, its level reaches maximum after 14 day and persist to 21 day DPI where it began to decline gradually. The phage lysate (20µl, 40µl) follow the same regime but the level of immune response are not significant. On the other hand, mean immune responses of mice vaccinated with *Brucella abortus* S19 vaccine began strongly from week post-vaccination and remain high at 4 week post-vaccination (Table 2).

Table.1 Mean splenic index of different groups of mice

	Mean splenic index				
Mice group	Control	S19	20µl PL	40µl PL	60µl PL
Mean ± SE	1.570 ±0.241[b]	0.463±0.074[a]	0.650±0.09[a]	0.545±0.074[a]	0.542±0.074[a]

Mean value bearing super script a and b in row differ significantly (P<0.01)

Table.2 Mean ELISA OD (p/n ratio) of different vaccinated mice group

	Titre post vaccination			
Mice group	7 day	14 day	21day	28 day
20µl PL	0.971±0.086[a]	1.392±0.164[a]	1.170±0.107[a]	0.998±0.089[a]
40 µl PL	1.294±0.210[a]	1.677±0.166[a]	1.420±0.172[a]	1.336±0.139[a]
60 µl PL	1.397±0.195[a]	2.064±0.225[a]	1.921±0.25[a]	1.742±0.249[a]
S19	1.530±0.437[a]	2.245±0.228[ab]	2.669±0.174[ab]	2.944±0.153[b]

Table.2 Mean CFU from spleen in 20µl Phage Lysate vaccinated mice

Mice No.	**Average total spleen count**			**Immunogenicity(Y): log (X/log X)**		**Average Immunogenicity (Y)= log (X/log X)**
	Dilution					
	1:10	**1:100**	**1:1000**	**1:10**	**1:100**	
1	7500	7000	N	3.286	3.260	3.273
2	8200	7000	N	3.321	3.260	3.290
3	10200	11000	2000	3.405	3.434	3.420
4	18000	105000	40000	3.626	4.320	3.973
5	4200	2000	N	3.064	2.782	2.923
6	9000	7000	N	3.357	3.260	3.308
					Mean	**3.364**
					SD	**±0.342**

Table.3 Mean CFU from spleen in 40µL Phage Lysate vaccinated mice

Mice No.	Average total spleen count			Immunogenicity(Y): log (X/log X)		Average Immunogenicity(Y)= log (X/log X)
	Dilution					
	1:10	1:100	1:1000	1:10	1:100	
1	2200	2000	N	2.818	2.782	2.800
2	1500	1000	N	2.674	2.522	2.598
3	900	1000	N	2.483	2.522	2.503
4	7300	5000	N	3.276	3.130	3.203
5	14000	11000	3000	3.528	3.434	3.481
6	8700	5000	N	3.344	3.130	3.237
					Mean	**2.970**
					SD	**±0.393**

Table.3 Mean CFU from spleen in 60µl Phage Lysate vaccinated mice

Mice No.	Average total spleen count			Immunogenicity: log (X/log X)		Average Immunogenicity(Y)= log (X/log X)
	Dilution					
	1:10	1:100	1:1000	1:10	1:100	
1	1400	200	N	2.648	1.939	2.293
2	8500	4200	N	3.335	3.064	3.199
3	6600	4600	N	3.237	3.098	3.168
4	9400	2400	N	3.373	2.852	3.112
5	2500	700	N	2.866	2.391	2.628
6	1000	200	N	2.522	1.939	2.230
					Mean	**2.772**
					SD	**±0.4078**

Table.4 Mean CFU from spleen *in B.abortus* S19 vaccinated mice

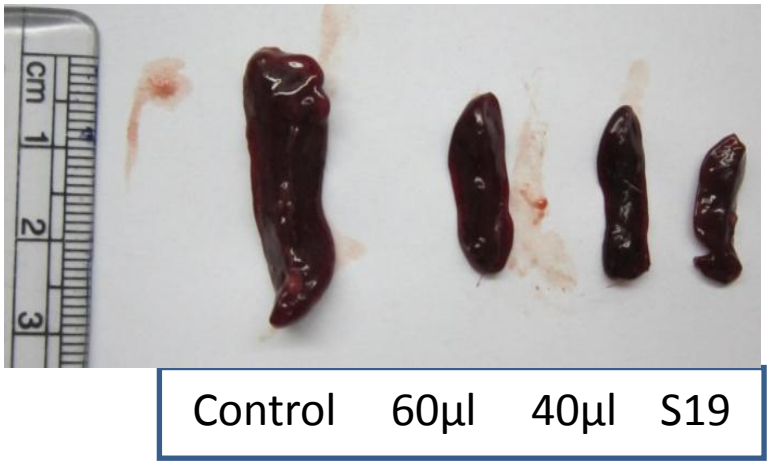
Mice No.	Average total spleen count			Immunogenicity(Y): log (X/log X)		Average Immunogenicity(Y) = log (X/log X)
	Dilution					
	1:10	1:100	1:1000	1:10	1:100	
1	600	500	N	2.334	2.267	2.301
2	220	800	N	1.972	2.440	2.206
3	1500	1800	N	2.674	2.742	2.708
4	1200	200	N	2.590	1.939	2.264
5	8000	200	N	3.311	1.939	2.625
6	200	700	N	1.939	2.391	2.1650
					Mean	**2.378**
					SD	**±0.229**

Table.6 Mean CFU from spleen in unvaccinated control mice

Mice No.	Average total spleen count			Immunogenicityy(Y)= log (X/log X)
	Dilution			
	1:10	1:100	1:1000	
1	U	145X 10^3	25X 10^4	4.665
2	U	230 X10^3	28X 10^4	4.710
3	U	U	35 X 10^4	4.800
4	U	U	12 X10^5	5.295
5	U	86 X10^4	6 X10^5	5.016
6	U	U	80 X10^4	5.132
			Mean	**4.936**
			SD	**±0.251**

N=No growing colony of *Brucella abortus* S544, sd= standard deviation, Y= log (X/logX) where X= total cfu/spleen, U=uncountable colony, PL= phage lysate

Figure.1 Enlargement of spleen in different group of mice



Immunogenic activity of phage lysate against *Brucella abortus* S19 was measured in Swiss albino mice using S19 vaccinated and PBS inoculated groups as control groups. Mouse strain CD-1, recommended by OIE, to test new vaccines or to study or compare classical vaccines but many workers also used swiss albino and BALB/c-mice (Cloeckaert *et al*., 2004). Mean of Immunogenic activity of phage lysate (20µl, 40µl and 60µl) group was (3.3, 2.9 and 2.7) (Table: 3, 4 and 5) where in S19 group and PBS inoculated group was 4.9 was 2.3 respectively (Table 6 and 7). It shows that immunogenic activity conferred by phage lysate (60µl) and S19 inoculated groups were significant with respect of PBS inoculated group and does not differ significantly to each other ($P<0.05$). OIE consider a vaccine to be protective should give immunogenic activity 2.5 and at least not more than 4.5. Statistical analysis of immunogenic value by Least Difference Square showed that the differences between the immunogenic activity elicited by Phage lysate (60µl) or S19 were not significant

differ but differ significantly with 20µl and 40 µl phage lysate. Immune response(IgG) of 60 µl phage lysate decrease at 30 DPI as compare to S19 vaccine yet the protection activity is found comparable with the S19 it might be due to therapeutic activity of phage which present in phage lysate. Prostetova (1959) showed that brucella phage is capable of persisting for a long time both in a healthy guinea pig (up to 30 days) and in a guinea pig infected with brucellosis (over 45 days).

Hence, concluded that the worldwide economic losses due to brucellosis are extensive not only in animal production but also in human health. Development of newer generation safe vaccine can help to control this crippling disease and reduce the economic losses. Present study found that Brucella phage lysate is protective and immunogenic to mice but it needs more researches on large scale to establish the effect, duration of immunity and accurate dose of phage lysate in main host.

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