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Immunogenicity of a combined vaccine containing foot and mouth disease virus antigen and *Pasteurella multocida* antigen in bovines

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Abstract
In the present study, humoral immune response in calves was evaluated by monitoring the serum antibody titres against Foot and Mouth Disease (FMD) virus in serum samples obtained from different animals at 0 and 28 days post vaccination (dpv), inoculated with a combined vaccine containing FMD virus and *Pasteurella multocida* (*P. multocida*) antigens. Serum neutralizating antibody titres for the vaccinated calves against FMD virus indicated protective immune response at 28 dpv. Evaluation of protective index (PI) against *P. multocida* in the combined vaccine showed protection with a PI of $10^{4.8}$ in mice. The combined vaccine containing blend of FMD virus and *P. multocida* antigens complies to the standards laid down by Indian Pharmacopeia and can be used effectively to prevent FMD and Haemorrhagic septicaemia in bovines in endemic areas.

Keywords: Foot and mouth disease, haemorrhagic septicaemia, FMD+HS combined vaccine, immunogenicity

Introduction
Veterinary vaccines are important for animal health and welfare and continues to play a major role in terms of agricultural development and food security of developing nations (Meeusen *et al*. 2007) [10]. There is a strong demand for combined vaccines in areas where infectious diseases like Foot and Mouth disease (FMD), Black Quater (BQ) and Haemorrhagic septicaemia (HS) persists endemically (Srinivasan *et al*. 2001) [16]. In India, outbreaks of FMD can be observed throughout the year and a total of 472 outbreaks/cases have been recorded during 2013-2014 (Project Directorate Foot and Mouth Disease, PD-FMD, Annual Report, 2013-2014) [14]. The scenario of such wide prevalence of FMD outbreaks in India, could be due to huge population of susceptible animals, unrestricted animal movement, circulating virus serotypes and strains, inapparent infection in small ruminants and limited vaccination (Biswal *et al*. 2012) [1]. On several occasions, cases of death in cattle and buffalo were observed following FMD due to secondary bacterial infections revealing post mortem lesions indicative of HS (PD-FMD, Annual Report, 2013-2014) [14]. Under such situations, prophylactic vaccination of animals against FMD and HS has become an important input with respect to livestock security and economic significance of the agricultural production system in India (Srinivasan *et al*. 2001) [16].

Combined vaccines or adminstration of different vaccines simultaneously are becoming increasingly important in areas where diseases like FMD and HS persists endemically (Palanisamy *et al*. 1992, Srinivasan *et al*., 2001, Chhabra *et al*., 2004) [12, 16, 2]. However, development of a combined vaccine requires critical consideration of each combination of antigens or live microorganisms in terms of quality, safety and efficacy (EMEA, 2000) [4].The serological response of simultaneous administration of FMD, HS and BQ antigens in cattle revealed that combined administration of these antigens did not differ from the response of vaccines administered separately (Reddy *et al*., 1997) [16]. Considering the tremendous economic importance of FMD and HS in India, a combined vaccine against the two diseases is likely to be useful to protect animals as well as in terms of cost and convenience (Palanisamy *et al*., 1992) [12]. The present study was undertaken to evaluate the efficacy of combined vaccine containing FMD and HS antigens and its immunological response in bovines.

2. Materials and Methods

2.1 Vaccine strains

FMD virus (FMDV) reference serotypes O, A and Asia 1 (procured from Central Laboratory of Project Directorate on FMD; IVRI, Mukteshwar-Kumaon, Uttarakhand, India) grown on bioreactors containing Baby Hamster Kidney (BHK-21) suspension cell cultures using Glasgow's minimum essential medium (GMEM) supplemented with sodium bicarbonate and antibiotics (Penicillin @100 IU/ml, Streptomycin @ 100µg/ml and Kanamycin 100µg/ml).The growth and maintenance media were supplemented with 8% and 2% adult bovine serum (ABS), respectively.

Pasteurella multocida (P-52) vaccine strain procured from Indian Veterinary Research Institute (ICAR-IVRI) was used for the study. The vaccine seed was grown on HS fermenter media containing sodium chloride, glucose, bacterial peptone, yeast extract, L-cysteine, potassium dihydrogen phosphate, sodium sulphate and sucrose and incubated for 20 hours at 37 ℃ under constant stirring before inactivation.

2.2 Purification and inactivation of FMDV and *Pasteurella multocida* vaccine harvest

Twenty four hours after infection, the FMDV viruses were inactivated by binary ethyleneimine (BEI), concentrated and purified by ultra filtration (20 fold concentration).

The culture containing *P. multocida* vaccine strain was formalin inactivated, concentrated and purified by ultrafiltration.

2.3 Preparation of vaccine blends

The concentrated O, A and Asia 1 antigens were diluted with Phosphate Buffer Diluent (PBD; pH 7.6) with suitable payloads of antigen per dose. The concentrated *P. multocida* antigen was added to the FMD antigen pool. The antigen suspension was then added to adjuvant Montanide Oil ISA 50V2. The ratio of the adjuvant to the total volume was 50:50. The mixture was emulsified using a homoginizer to form a water-in-oil blend and filled in 100 ml of polypropylene botlles and stored at 2-8 ℃.

2.4 Immunization and challenge

Immunization and challenge experiments were done as per Indian Pharmacopoeia (2018) [8] with prior approval from Committee for the purpose of Control and Supervision of Experiments on Animals (CPCSEA).

2.4.1 Potency of FMD in cattle

A total of 10 calves of 8-12 month old, seronegative for FMD, maintained at Brilliant Bio Pharma Private Limited, Hyderabad were randomly selected. Animals were dewormed 14 days before immunization. The animals were divided into 2 experimental groups containing 5 calves each. The first group served as control, second group was immunized with 2 ml of oil adjuvant FMD + HS combined vaccine deep intra muscularly (I/M). The animals were kept together under same managemental conditions. They were fed on green fodder and wheat bhusa (adlibitum), and concentrate mixture @ 0.75 kg/animal during whole period of study (Chhabra *et al*., 2004) [2]. Serum neutralization test (SNT) has been carried out for quantitative estimation of neutralizing antibodies against FMDV on the sera collected on the day of vaccination (Day 0) and at 28 days post vaccination.

2.4.2 Serum neutralization test

The serological response was measured by micro-serum neutralization as described by Golding *et al.* test using BHK-21 monolayer cells (Golding *et al.*, 1976) [5]. Titres were calculated as per the method described by Spearman-Karber (1931) [9] and expressed as $\log_{10} SN_{50}$ values.

2.4.3 Potency of HS in mice

The vaccine blend was tested for HS potency in mice. A total of 100 Swiss albino mice weighing 18-20g were divided into two groups, each group containing 50 mice. Fifty (50) mice of one group were injected with the first dose of the oil adjuvanted vaccine @ 0.2 ml intramuscularly. The dose was repeated after 14 dpv. After 7 days of the second dose, the vaccinated mice were divided into 10 groups of 5 mice each. Fifty mice of the same stock were taken as control animals and has been divided similarly into 10 groups of 5 each. The animal groups were then challenged with serially diluted cultures (10^{-1} to 10^{-10} dilutions) of virulent *P. multocida* P-52 strain on 21st dpv by inoculating @ 0.2 ml ranging from 10^{-1} to 10^{-10} dilutions through subcutaneously route. The animals from the vaccinated and control group were observed for any untoward reaction and mortality for 5 days. The 50 percent median lethal dose of the challenge organism for vaccinated and control mice was calculated by Spearman-Karber method (1931) [9].

2.4.4 Protective Index

The median lethal dose (LD_{50}) for each of the vaccine blends including unvaccinated controls was calculated as the amount of a given substance required to kill 50% of a test population after 5 days using Spearman-Karber method (1931) [9]. The protective index (PI) was calculated as:

PI=LD_{50} in control mice-LD_{50} in vaccinated mice

LD_{50} = Reciprocal of 50% endpoint dilution

Log_{10} 50% end point dilution = Log_{10} of dilution showing a mortality next above 50% - (Proportionate distance x logarithm of dilution factor)

Proportionate distance = [(mortality at dilution next above 50%)-50%]/[(mortality next above 50%) - (mortality next below 50%)]

3. Results

Immunity to FMD was assessed by serum neutralizing (SN) antibody test and estimation of the $\log_{10}SN_{50}$ values. The titre of these antibodies remained well above the protective level (1.5 $\log_{10}$ /ml for SNT) as described by OIE (OIE, 2010) [11]. All vaccinated cattle responded to vaccination, with an increase in the $\log_{10}$ antibody titre from 0.84 at day 0 to a range of 1.86-1.89 after four weeks of vaccination. The serum neutralization titres ($\log_{10}SN_{50}$ values) for FMDV O, A and Asia-1 at 28 dpv was found to be 1.86, 1.89 and 1.86 respestively which can be considered as protective titres when compared to the serum neutralization titres (≤ 0.84-0.86) on day 0. The serological response of calves to foot and mouth disease virus antigens O, A and Asia 1 in FMD vaccine is presented in Table 1.

Table 1: Serological response of calves to FMDV antigens ($log_{10}SN_{50}$ values)

Calf No	Dose	Antibody titres (SN_{50})					
		FMDV O		FMDV A		FMDV Asia 1	
		0 dpv	28 dpv	0 dpv	28 dpv	0 dpv	28 dpv
1	2 ml I/M	0.82	1.95	0.90	1.95	0.82	1.95
2		0.75	1.87	0.75	1.87	0.90	1.87
3		1.05	1.80	0.97	1.87	0.75	1.80
4		0.82	1.87	0.90	1.87	0.82	1.80
5		0.75	1.80	0.75	1.87	0.90	1.87
Mean		0.84	1.86	0.85	1.89	0.84	1.86

The potency of *Pasteurella multocida* antigens when tested in mice induced a protective index of $10^{4.8}$ which was above the recommended unit (10^4 Protective index) as described by Indian Pharmacopeia, 2018 [8]. The protective index for Haemorrhagic septicaemia vaccine against *Pasteurella multocida* was calculated based on the data presented in Table 2.Control mice started dying within 36 hrs of challenge (80% mortality), which continued till 48 hrs (85% mortality) followed by 72 hrs (100% mortality). Interestingly in the test groups the overall survival rate after 36 hrs was 95%, whereas 90% in 48 hrs and the observation was continued for 5 days with no further mortality in the test group. No animals showed any abnormal local or systemic reactions when combined FMDV and *Pasteurella multocida* antigens were used in the formulation.

Table 2: Mortality rate of mice challenged with *Pasteurella multocida* antigens in vaccinated and control group

Vaccinated group challenged with diluted cultures of *Pasteurella multocida* (No of mice 50)							
Dose (dilutions)	**Animal challenged**	**Animal live**	**Animal dead**	**Cumulative live**	**Cumulative dead**	**Total**	**% Mortality**
10^{-1}	5	0	5	0	17	17	100
10^{-2}	5	0	5	0	12	12	100
10^{-3}	5	1	4	1	7	8	88
10^{-4}	5	3	2	4	3	7	43
10^{-5}	5	4	1	8	1	9	11
10^{-6}	5	5	0	13	0	13	0
10^{-7}	5	5	0	18	0	18	0
10^{-8}	5	5	0	23	0	23	0
10^{-9}	5	5	0	28	0	28	0
10^{-10}	5	5	0	33	0	33	0
Control group challenged with diluted cultures of *Pasteurella multocida* (No of mice 50)							
Dose (dilutions)	**Animal challenged**	**Animal live**	**Animal dead**	**Cumulative live**	**Cumulative dead**	**Total**	**% Mortality**
10^{-1}	5	0	5	0	41	41	100
10^{-2}	5	0	5	0	36	36	100
10^{-3}	5	0	5	0	31	31	100
10^{-4}	5	0	5	0	26	26	100
10^{-5}	5	0	5	0	21	21	100
10^{-6}	5	0	5	0	16	16	100
10^{-7}	5	0	5	0	11	11	100
10^{-8}	5	2	3	2	6	8	75
10^{-9}	5	2	3	4	3	7	43
10^{-10}	5	5	0	9	0	9	0

Log_{10} 50% end point dilution of vaccinated group= 3.90
Log_{10} 50% end point dilution of control group= 8.70
Protective index (PI) = 4.80

4. Discussion

Foot-and-mouth Disease (FMD) is the most contagious transboundary animal diseases (TADs) primarily affecting cattle and buffalo. The causative agent FMD virus (FMDV) is antigenically diverse having seven distinct serotypes (O, A, C, Asia-1 and South African Territories SAT 1-3) (Pattnaik *et al*., 2012) [13]. Among the three serotypes (O, A, Asia-1), serotype O is the most dominating one followed by serotype Asia-1 and A. (PD FMD Annual Report, 2013-2014) [14] and continued to be most predominant one and was responsible for 98% of the incidences recorded during 2017-18 (PD FMD, Annual Report, 2017-2018) [14]. Likewise, Haemorrhagic septicaemia (HS) is considered economically to be the most important disease of cattle and water buffaloes and is endemic in India with most of the states reporting the disease (Venkataramanan *et al*., 2005 [18], Govindaraj *et al*., 2017) [7]. In most of the Southern states in India, because of lack of vaccination against HS before monsoon, several outbreaks of HS were reported following an outbreak of FMD (PD FMD Annual Report, 2013-2014) [14]. Simultaneous occurance of FMD and HS can result into fatal mortalities in the infected animals. In such circumstances, combination vaccines not only protect the animals from these co-existing diseases but also reduces the vaccination costs and manpower (Surya Prasad *et al*., 2019) [17].

In the present study, the immunogenicity and potency of a combined FMD-HS vaccine was evaluated. The study was conducted using calves and laboratory mice to assess the immunopotency of FMD and HS vaccine respectively. Results obtained in the present study indicated that the neutralizing antibodies remained well above the protective level (1.5 log_{10}/ml for SNT) for FMD as described by OIE (OIE, 2010) [11]. All vaccinated cattle responded well to vaccination, with an increase in the log_{10} antibody titre from 0.84 at day 0 to a range of 1.86-1.89 after four weeks of vaccination upon estimation of the $log_{10}SN_{50}$ values. The

result in the present study were in support of the findings of Srinivasan *et al*. (2001) [16] where they recorded the serum neutralization values above 1.61 for type O, 1.01 for type A and 1.41 for type Asia-1 respectively as protective titres for FMD vaccines (Srinivasan *et al*., 2001) [16]. El-Sayed and co-workers also recorded higher antibody titres (1.77 for type O and 1.55 for type A) in cattle on 4th week post vaccination with bivalent oil vaccine containing type O and A FMDV strains (El-Sayed *et al*., 2012) [3]. However, they recorded peak titres on 12th week post vaccination for both the types (El-Sayed *et al*., 2012) [3]. Reports of combined FMD+HS oil adjuvant vaccine conferring higher immune response and longer immunity in vaccinated animals has also been recorded suggesting the advantage of such combined vaccines to prevent pattern of combined outbreaks (Chhabra *et al*., 2004) [2].

The potency test conducted for HS vaccine (strain-P52) has shown good potency. A volume of 0.2 ml of broth dilution from 10^{-1} to 10^{-10} was injected into each mice, as dilution increased death rate decreased which indicated the minimum load of bacterium was required to induce infection. Our results are in support of the study conducted by Gowrakkal *et al*. (2014) [6] to evaluate the immuno efficiency of hemorrhagic septicemia vaccine strain in mice. A protective index of $10^{4.8}$ was obtained on estimation of LD_{50} which was above the recommended unit (10^4 Protective index) as described by IP that indicates that the HS vaccine was potent. Gowrakkal and co-workers (2014) [6] found similar observation while evaluating the potency of hemorrhagic septicemia vaccine in mice recording a protective index of $10^{6.47.}$ Therefore, the current finding indicated that the combined vaccine passed by acquiring the minimum required criteria for immunogenicity and can be used effectively to prevent the outbreaks of disease recorded every year.

5. Conclusion

The results of the present study indicated that combined vaccine containing *Foot and Mouth Disease virus* and *Pasteurella multocida* antigens prepared and formulated according to the described procedure was found to be safe and effective in cattle and mice respectively. The FMD-HS combined formulation will be an ideal vaccine in terms of cost-effectiveness and can be effectively used in developing countries to control the disease in endemic areas.

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