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Evaluation of safety, immunogenicity and potency of live attenuated *Vero*cell adapted sheep pox vaccine in sheep.

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Abstract
Sheep pox(SP) disease is one of the priorities and high-impact animal diseases affecting sheep in many developing countries including India. As we know that prophylaxis using attenuated vaccines is the choice of control measure as the immunity is long lasting. In this study, we evaluated safety, immunogenicity and potency of a live attenuated Sheep pox vaccine prepared in a freeze-dried form containing vaccine virus titer of $10^{3.75}CCID_{50}$/dose by BBPL. The vaccine safety was tested in sheep using single dose and ten times the recommended field dose. In the safety trial, none of the vaccinated animals showed any deviation from physiological norms or fever, in-appetence or local/ generalized skin reactions. In the immunogenicity study of the SPV vaccine administered by intramuscular (I/M) or subcutaneous (S/C) route, all vaccinated sheep developed virus-neutralizing antibodies with a geometric meantiter of 1.79 $log_{10}$and 2.00 log_{10}respectively at 21 days post-vaccination. Marginal differencein sero-conversion was found in sheep vaccinated with SPV by I/M or S/C route. In potency study, all the vaccinated animals resisted challenge with virulent sheep pox virus on day 21 post vaccination and demonstrated full protection, while unvaccinated control sheep showed characteristic clinical signs of sheep pox disease. The mean protective index for SP vaccine was 3.46 and 3.41by intramuscular and subcutaneous routes respectively. Overall, the live attenuated sheep pox vaccine was found to be safe, immunogenicand potent as evident from sero-conversion as well as challenge studies in sheep.
Keywords:*Sheeppox (SP) disease; Sheep pox vaccine (SPV); safety; immunogenicity; potency; challenge; sero-conversion*

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I. Introduction

The World Organization for Animal Health (OIE) listed sheep pox as a notifiable disease. It is a highly contagious and devastating disease of sheep. The sheep pox virus belongs to the *Capripox*genus of the subfamily *Chordopoxvirinae* of the *Poxviridae* family. The virus is commonly transmitted by aerosol route, close contact with infected animals and mechanically by biting (stable) flies [1]. The pox virus infections are host specific for either sheep or goats, though cross infection has been reported [2]. The sheep pox disease occurs in Asia (India, China, Bangladesh, etc.) [3, 4, 5, 6]and Africa commonly and causes severe economic losses to sheep rearing farmers. The disease has been eradicated from most European countries [7]. Overall, among the pox viral diseases of domestic animals, sheep pox is highly infectious with considerable morbidity and mortality (10 – 50%). For sheep pox virus, sheep are the primary host and it infects all age groups, but the severity of infection and deaths occur mainly in young lambs, occasionally in yearlings and rarely in adults[6]. Severe economic losses are due to high mortality, abortion, mastitis, skin condemnation and loss of wool and mutton, besides restricting the export potential of the meat, wool and skin [8, 9, 10]. Importantly now a days the sheep pox and goat pox are re-emerging and expanding their territory since the recent outbreaks reported in Vietnam, Mongolia and Greece have beenreported [11].

India has the second largest population of goats (148.88 million) and fourth largest population of sheep (74.26 million) increased by 14.1% and 10.1%, respectively, over the previous census [12]. The disease is endemic in India and outbreaks have been reported regularly from almost all the states of India [13, 14]. Sheep farming is an important source of family income for poor farmersin rural areas, but it is substantially compromised by this disease [15]. Commonly inactivated vaccines were used for sheep pox in many states are

being used at present, which gives a short duration of immunity and requires booster vaccination for good and strong immunity [16].

The present study was undertaken to study the safety, immunogenicity and potency of live attenuated sheep pox vaccine made from sheep pox virus (SPV) Srinagar 38/00 strain propagated on *Vero* cells for the prevention and eradication of infection in sheep.

II. Materials and methods

2.1 Sheep

Healthy sheep of 6-12 month old were procured from the market and maintained at Brilliant BioPharmaPrivate Limited (BBPL) farm for different studies. Sheep were screened for SPV antibodies by serum neutralization test (SNT). Sero-negative sheep for SPV antibodies were used for carrying out safety, immunogenicity and potency studies of SPV vaccine following guidelines for care and handling of experimental animals as per the Committee for Purpose of Control and Supervision of Experiments on Animals (CPCSEA) and the CPCSEA protocol approval number is 25/9/2019-CPCSEA dated on 07-06-2019.

2.2 Virus isolation and attenuation

The attenuated Srinagar 38/00 strain of SPV was procured from Indian Veterinary Research Institute (IVRI), Mukteswar by Technology transfer agreement. However, the complete characterizations of this strain from isolation to attenuation was carried out by IVRI, Mukteswar. The SPVstrain was attenuated by passaging in *Vero*celllinefor 38 passages.

2.3 Vaccine and vaccination

The attenuated Srinagar 38/00 strain of SPV has been propagated on *Vero* cells at BBPL and the attenuated live vaccine was manufactured by freeze drying the viral harvest to contain 100 doses per vial. Freeze-dried SPV vaccine was reconstituted in 100 mL chilled diluent (Normal saline). Sheep were randomly divided into different groups before immunization with SPV vaccine (Table 1). Three different groups were made, viz Group A1, A2 and A3. Group A-1 (n = 3) and Group A-2 (n = 3) animals were immunized with 1 mL of sheep pox vaccine ($10^{3.75}$ $CCID_{50}$/dose) by intramuscular and subcutaneous route respectively, while Group A-3 (n = 2) was kept as an unvaccinated in-contact control.

Table 1: Experimental design showing different groups of sheep immunized with sheep pox vaccineand challenged.

Group (no. of animals	Route of administration	Dose of vaccine virus per animal	Dose of challenge virus
A1(3)	I/M	$10^{3.75}$ $CCID_{50}$/mL	~$10^{6.5}$ $CCID_{50}$/Animal, Intradermally
A2(3)	S/C	$10^{3.75}$ $CCID_{50}$/mL	
A3(2)	-	-	

2.4 Safety of sheep pox vaccine

The *Vero*cell adapted attenuated live SPV vaccine manufactured by BBPL was tested for safety in sheep (Table 3). The safety of the vaccine was evaluated by giving 1 field dose and 10 field doses (one field dose = $10^{3.75}$ $CCID_{50}$/mL) of the vaccine contained in 1mL by S/C route. All the immunized animals were clinically monitored by regular observations and by recording daily rectal temperature. Animals were critically observed for the development of any Sheep Pox related signs following vaccination and rectal temperatures were recorded daily up to 14 days post vaccination (dpv) [16].

2.5 Serum sample collection

Blood samples were also collected on 0 and 21dpvfor serum antibody estimation. Serum was separated from all the blood samples and heat inactivated at 56^0C for 30 minutes and stored at -20°C for further studies. All the serum samples were submitted for *in vitro* testing to estimate antibody levels against sheep pox virus.

2.6 Serum Neutralization Test (SNT)

SNT was carried out using the method described by Golding *et al*., (1976) [17] with minor modifications. Sera samples were serially diluted in doubling dilution. The diluted sera were neutralised with 100 $CCID_{50}$ of SPV and incubated at 37°C for one hour. Subsequently 100 µL (micro litres) *Vero* cells were added (0.2 X 10^6 cells/mL) per well in microwell plates (Nunc, Thermo Scientific). The plates were incubated at

37°Cin CO_2incubatorfor 5 days to check for any CPE. The serum antibody titerwas calculated using the Spearman-Karbermethod [18].

2.7 Challenge Study

Virulent SPV obtained from IVRI, Mukteshwar laboratory and maintained in the laboratory was used for challenge studies in SP vaccinated and control sheep groups. The sheep were challenged intra-dermally by injecting 0.2mL of SPV challenge virus of different ten-fold dilutions. Animals were critically observed for 2 weeks for the development of any specific disease, any local reaction or clinical symptoms following challenge and rectal temperature was recorded daily up to 14 days post-challenge (pc). The observations were compared with the control group (Un-vaccinated) and interpreted the results. The titration of the virus was performed in control sheep (Table 2). Thechallenge virus titer was calculated as per the formula established by Reed and Muench, 1938 [19]. The obtained titer for each group was compared with the titer of the unvaccinated control animals and the difference between the two titers expressed in log to represent the Protection Index (PI)[20, 19].

Table 2: Format used for inoculation into sheep flank intradermally at the rate of 0.2 mL / site in 5 replicates.

Replicates	Challenge Virus dilutions						
	10^1	10^2	10^3	10^4	10^5	10^6	10^7
1	*	*	*	*	*	*	*
2	*	*	*	*	*	*	*
3	*	*	*	*	*	*	*
4	*	*	*	*	*	*	*
5	*	*	*	*	*	*	*

III. Results

The sheep pox virus grew very well in *Vero*cell line and demonstrated cytopathogenic effect (CPE) after 4–5 days of incubation and the titer of the harvested suspension was ~10^6CCID_{50}/mL. Sheep pox vaccine batch produced from this virus was tested for sterility, purity and identity according to the standardguidelines [21]. The infective titer per dose for SPV vaccine was found $10^{3.75}$ $CCID_{50}$/mL. Safety,immunogenicityand potency of the vaccinewas evaluated on animals as per the standard protocol and guidelines [21].

3.1 Safety of sheep pox vaccine

The parameters for the establishment of safety of live *Vero* cell adapted sheep pox vaccine were established by Chaudhary, *et al*., 2009 [22]. Accordingly, as per the standard guideline of Indian pharmacopoeia 2018, the safety of the vaccine tested in the present study (Table 3). All vaccinated animals with one or ten field doses remained healthy, without any effect on their appetite and behavior following 14 dpv. No abnormal reactions were reported. None of the vaccinated sheep showed any deep necrotic lesions and generalization. Also, no sign of illness or rise in body temperature was observed in any of the vaccinated sheep, hence the vaccine proved safe.

Table 3: Experimental design showing different groups of sheep used for evaluating safety of sheep pox vaccine (SPV).

Sr. No.	Group (no. of animals)	Animal No	Dose of vaccine (S/C)	Critical sign observed	Rise in body temp.	Pox / deep Necrotic lesion observed
1	A (n=03)	12-14	1 field dose	No	Normal	Deep necrotic lesion and generalization was not observed
2	B (n=03)	15-17	10 times field dose	No	Normal	
S/C: Subcutaneous route						

3.2 Determination of antibody titer (Immunogenicity)

Serological response after vaccination was evaluated by SNT in *Vero* cells. Very goodSPV antibody response was obtained post vaccination by I/M and S/C routes. Thegeometric meanserum neutralizationtiterobtained by I/M and S/C route was 1.79 $\log_{10}$ and 2.00$\log_{10}$ respectively (Table 4). Overall satisfactory antibody response was observed by both routes, whereas control animal antibody titer observed was <0.30 on 21 dpv. Importantly, no antibody response in control animals indicated that there was no shedding of vaccine virus from the vaccinated animals. Thesefindingscoincide with the findings of previously published report [23].

3.3 Results of vaccine potency:

Challenge studies: Both vaccinated and unvaccinated control animals were challenged on 21 dpv with the virulent sheep pox virus. For challenge study, different groups were made as shown in Table 4. All vaccinated animals in Group A1 and A2 showedno abnormal rise in body temperature (body temp. range 101.8-102.8°F) after challenge with virulent sheep pox virus during the observation period of 14 days, whereas the animals from control group A3 demonstrated high body temperatures (102.2-104.8^{0}F), extensive local reactions (pock lesions) and other associated clinical signs of sheep pox. Overall, all animals from vaccinated groups A1 and A2 were protected after challenge withvirulent virus and showedgood antibody titer; however none of the control animal from in Group A3 showed protection after challenge. The mean Protective Indexof vaccinated animal groups by I/M or S/C routes was 3.46 and 3.41 respectively, whereasthe control animal group (N=2)showed the signs of disease indicating no protection from challenge virus(Table 4).

Table 4: Results of neutralizing SPV antibody response after vaccination and protection of vaccinated sheep after challenge by SP virulent strain (I/dermal route) on day 21

Group (no. of animals	Animal No	Route of administration	Days post vaccination (Serum neutralizing antibody titer inlog_{10})		Geometric mean antibody titer	No of animals challenged/protected	Mean Log_{10} challenge virus titer	*Protective Index
			Day 0	Day 21				
A1(3)	1	I/M	<0.30	1.82	1.79	3/3	2.83	3.46
	2		<0.30	2.14				
	3		<0.30	1.48				
A2(3)	4	S/C	<0.30	1.80	2.00	3/3	2.88	3.41
	5		<0.30	1.82				
	6		<0.30	2.46				
A3(2)	7	-	<0.30	<0.30	<0.30	2/0	6.29	NA
	8		<0.30	<0.30				

I/M: Intra-muscular, S/C: Subcutaneous, I/d: Intradermal, NA: Not Applicable
*: Protective Index = Difference of Log10 affective (challenge virus) titer for the vaccinated and control group > 2.5 is considered as evidence of protection (as per IP, 2018)

IV. Discussion

In the present study, sheep pox virus was adapted to*Vero* cell line and resulted in attenuation and considerable titer which helps to produce good potent and safe vaccine. Also, in the present study the challenge SPV had a considerable titer in *in-vivo* models, as reported by the earlier workers [24]. This showed that the challenge virus used in the present study was highly virulent and pathogenic for sheep. As like other workers, who used various cell lines for propagation and adaptation sheep pox virus, we also attenuated and propagated the SPV virus in *Vero*cell line[24, 25, 26].

In the safety study, the current SPV vaccine did not exhibit any considerable reaction at the site of inoculation. All vaccinated animals, even at tenfold field dose, remained healthy without any clinical sign of illness and rise in body temperature during the 14 days period following vaccination. This indicated that the virus was sufficiently attenuated to be used as a vaccine at the field level.

Satisfactory serum neutralizing antibodies were elicitedby the SP vaccine on 21dpv. Importantly earlier studies [27, 28] also indicated high level of neutralizing antibody by 21 day post vaccination.As reported by earlier workers[26, 29], challenge study, being the method of choice to assess the protection provided by SPV vaccines, was conducted in the present study. All vaccinated animals were protected without any clinical symptoms of disease, whereas the control animals succumbed to challenge and shown specific signs of sheep pox disease.

V. Conclusion

The present study showed that the live attenuated SPV vaccine is safe, immunogenic and potent to protect vaccinated animals against the virulent sheep pox virus challenge. This suggests that the present SPV vaccine could be a suitable for use under field conditions to prevent outbreaks of sheep pox disease in India.

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