aJava

Asian Journal of Animal and Veterinary Advances

Academic
Journals Inc.

www.academicjournals.com

Asian Journal of Animal and Veterinary Advances 10 (8): 365-375, 2015
ISSN 1683-9919 / DOI: 10.3923/ajava.2015.365.375

Effect of Stabilization and Reconstitution on the Stability of a Novel Strain of Live Attenuated Orf Vaccine (ORFV MUK59/05)

[1,2]Durlav Prasad Bora, [1,3]Veerakyathappa Bhanuprakash, [1]Gnanavel Venkatesan, [1,4]Vinayagamurthy Balamurugan, [1,5]Manimuthu Prabhu and [1,4]Revanaiah Yogisharadhya

[1]Division of Virology, Indian Veterinary Research Institute, Mukteswar, 263138, Nainital District Uttarakhand, India

[2]Department of Microbiology, College of Veterinary Science, Assam Agricultural University, Khanapara, Guwahati, 781022, Assam, India

[3]FMD Laboratory, Indian Veterinary Research Institute, H.A. Farm, Hebbal, Bangalore, 560024, Karnataka, India

[4]National Institute of Veterinary Epidemiology and Disease Informatics, Hebbal, Bangalore, 560024, Karnataka, India

[5]Sheep Breeding Research Station, Sandynallah, 643237, Tamil Nadu Veterinary and Animal Science University, Nilgiris District, Tamil Nadu, India

Corresponding Author: Veerakyathappa Bhanuprakash, Division of Virology, Indian Veterinary Research Institute, Mukteswar, 263138, Nainital District, Uttarakhand, India Tel: +918023410908 Fax: +918023412509

ABSTRACT

The study describes the effect of stabilization on the stability of an indigenous live attenuated orf vaccine with stabilizers like LS, LHT and TAA after lyophilization and also reconstitution with NSS, PBS, distilled water and 1 M $MgSO_4$ exposed at different temperatures under established lyophilization conditions. The stability of the vaccine was assessed both in freeze-dried and reconstituted forms at different temperatures. The results indicated that the orf vaccine lyophilized with LS stabilizer at 25 and 45°C and LHT at 37°C found superior over others in stabilizing the keeping quality of the vaccine. Intrinsic thermo-stability studies revealed the rapid deterioration of the vaccine, when compared to the vaccine stabilized with the either of the stabilizers. Among the diluents used for reconstitution of the vaccine, quality in terms of infectivity titers of the virus was well preserved in vaccine diluted with 0.85% NSS compared to other diluents like PBS, DW and 1 M $MgSO_4$. The 1 M $MgSO_4$ found unsuitable for diluting the orf vaccine. The study suggests that the LS at 25 and 45°C and LHT at 37°C are the choice of stabilizers and 0.85% NSS is the choice of diluent for orf vaccine at all temperatures contemplated.

Key words: Orf virus, contagious ecthyma, vaccine, stabilizers, reconstitution, thermostability

INTRODUCTION

Orf is a non-systemic eruptive skin disease that occurs worldwide in sheep and goats (Mondal *et al*., 2006). Orf virus (ORFV) is a DNA virus belongs to the genus *Parapoxvirus*, sub family Chordopoxvirinae of Poxviridae family. The disease is zoonotic and globally prevalent. Orf is enzootic in Indian sheep and goats (Hosamani *et al*., 2007; Dey and Kundu, 2009; Venkatesan *et al*., 2011; Yogisharadhya *et al*., 2012; Bora *et al*., 2012). Orf is characterized by proliferative and often self-limiting skin lesions including papules, vesicles and rapidly growing scabs on the skin of lips, oral mucosa and around the nostrils. In severe cases, the skin of eye,

udder or vulva may be affected including secondary bacterial infections with or without myasis (Housawi and Abu Elzein, 2000). Orf is considered as economically important due to its high morbidity in adult animals, repeated infection of hosts by subverting the host immunity, cross infectivity to other species and its zoonotic potential (Hosamani *et al*., 2009). Orf can be controlled by vaccination and live vaccines are used in some parts of the world.

Cryopreservation by lyophilization has become the accepted method for the long term preservation of sensitive biomolecules like live vaccines. These vaccines suffer serious deterioration in vaccination campaigns conducted in tropical and subtropical environments. The cause in most cases is due to the difficulty of maintaining the cold chain resulting in the loss of potency. In a live vaccine, viability of the virus dictates the efficacy. The success of immunization banks on the stability of the viral vaccine. In turn, the stability of the vaccine depends on the thermostability of the virus, property of stabilizer, the pH of the vaccine and the vaccine container. Temperature is an environmental factor and found to have a profound effect on the quality of vaccine. A lot of emphasis has been given on the vaccine quality studies performed under real storage conditions, in real-time and other environmental factors. It is mandated to have vaccine stability data prior clinical trial of a vaccine (WHO., 2005, 2009). Further, one has to ensure that animal to be vaccinated receives a required dose of the vaccine. It is obvious that vaccines lose their potency over a period of time and the loss depends on temperature and other factors. Hence, heat resistant vaccine formulations have immense value during cold chain break-down (Levin *et al*., 2007). It is possible to prolong the shelf-life of most of the vaccines at lower temperatures (Chen and Kristensen, 2009). Thus, to determine the optimum conditions for storage of vaccine and its use in the field, the data on the stability of the vaccine is a requisite. Similarly, the maximum temperature and durations that can be tolerated need to be demonstrated. The other crucial factor for lyophilized vaccine is reconstitution. Lyophilized vaccines lose their potency rapidly after reconstitution. Hence, the selection of a suitable diluent is also in indigence.

Temperature is not uniform across all places. Thus, maintenance of shelf-life of the vaccine is very difficult and needs cold chain. Like any other lyophilized live vaccines, live orf vaccine is not an exception in losing its potency on thermal effect. The vaccine requires cold chain for storage and transport. As a result, the cost of production and transportation are high and user's expenses are unavoidable, especially in developing tropical and semitropical countries. Therefore, evaluation of the vaccines for their thermo-stability with different stabilizers is much demanding in order to maintain the effectiveness of immunization through reduction of potency due to heat damage, reduce cold chain costs and logistical requirements. Therefore, the present manuscript describes the identification of a suitable stabilizer and a diluent for stabilization and reconstitution of live orf vaccine.

MATERIALS AND METHODS

Cells and vaccine virus: The seed virus (orf virus isolate, Muk 59/05) isolated from the scab of orf infected goat, serially passaged (P49) in primary/secondary lamb testes (PLT/SLT) and maintained in the pox virus laboratory was used for preparation of the vaccine. The vaccine virus was propagated and assayed on PLT cells. The PLT cells were cultivated in Eagle's Minimum Essential Medium (EMEM) supplemented with 10% Fetal Calf Serum (FBS, HyClone) with antibiotics (Penicillin, 100 IU mL^{-1} and streptomycin, 100 μg mL^{-1}) and maintained in EMEM with 2% FBS.

Vaccine stabilizers: The stabilizers used for the preparation of orf vaccine were LS [5% lactalbumin hydrolysate (LAH) and 10% sucrose in Hank's Balanced Salt Solution (HBSS), pH 7.2] (Mariner *et al*., 1990), LHT and TAA (Adebayo *et al*., 1998) were prepared following established protocols. The composition of LHT stabilizer was same as that of LS stabilizer except 10% trehalose dihydrate instead of sucrose with pH 7.2. The TAA stabilizer was prepared which contained trehalose dihydrate along with amino acids L-Alanine and L-Histidine with a pH of 7.4 (Adebayo *et al*., 1998). Three different stabilizers and orf vaccine batches produced were tested for their sterility before lyophilization as described earlier (Sarkar *et al*., 2003; Riyesh *et al*., 2011). The different diluents tested were, 1X PBS (phosphate buffered saline, pH 7.4), 0.85% NaCl (Sodium chloride), distilled water and 1 M $MgSO_4$ (Magnesium sulphate). Each stabilizer formulation was tested separately with aforementioned diluents.

Preparation of orf vaccine: Primary or secondary lamb testes cells were seeded into roller culture bottles (1700 cm^2) at a concentration of 2.5×10^7 cells and incubated. The roller bottles with confluent PLT/SLT monolayers were infected with vaccine virus at 0.01 Multiplicity of Infection (MOI). Infected bottles were incubated in a roller apparatus and examined daily for CPE. After 2-3 days post infection (dpi) when more than 80-90% CPE was observed, virus was harvested from the infected cells by repeated cycles of freezing (-80°C overnight) and thawing (at ambinet temperature for 3-4 h). To maintain uniform virus titre, virus hervests from all the roller bottles were pooled after first cycle of freeze-thawing, aliquoted and preserved at -80°C until lyophilized.

Lyophilization was carried out using Edwards Modulyo 4K freeze-dryer as described earlier (Riyesh *et al*., 2011). Vaccine and stabilizers each were mixed in equal volumes. Taking sterile precautions, 1.0 mL of vaccine with stabilizer was dispensed into sterile freeze-drying vials using an automatic dispenser. The vials were half-closed with sterile rubber stopper to provide a vent during drying of the vaccine. The half-closed vials were then kept at -80°C for pre-freezing overnight. The vaccine vials were lyophilized at a condenser temperature of -60°C and a vacuum of 0.06 mbar. After 48 h, vials were rubber-stoppard under vacuum and tightly sealed with an aluminum cap under normal air pressure. A batch of vaccine containing different stabilizer formulations were lyophilized simultaneously under identical conditions to compare the quality of the vaccines in terms of Residual Moisture (RM) and loss in titre during lyophilization. To evaluate the intrinsic stability of vaccine virus, 1 mL of vaccine without any stabilizer was also freeze-dried under similar conditions.

Virus titration: After each exposure at a specified temperature, the freeze-dried vaccine virus was re-hydrated and virus infectivity titer was quantified in PLT/SLT cells by estimating the 50% Tissue Culture Infectivity Doses ($TCID_{50}$) in a microtitre plate using standard protocols. Briefly, 10-fold dilutions of vaccine were made in EMEM and titrated in cell monolayer of PLT/SLT grown in 96-well micro-titer plates using four replicates per dilution (100 μL $well^{-1}$). The inoculated plates were incubated at 37°C in a humidified atmosphere in the presence of 5% CO_2 and observed for CPE. Virus infectivity was quantified by estimating the 50% tissue culture infectivity doses ($TCID_{50}$) and end points were calculated (Reed and Muench, 1932).

Estimation of residual moisture: Thermo-gravimetric method was followed for estimation of Residual Moisture (RM) of the lyophilized vaccines (Worrall *et al*., 2000). Accordingly, the mean weight of 10 vials from each vaccine batch was taken and then dried at 80°C for 20 h and the weight of water loss from the dried vaccine was expressed as percentage.

Stability testing: The stability of live attenuated orf vaccine virus in the freeze-dried form was evaluated at 25, 37 and 45°C, whereas, following reconstitution stability was evaluated at 4, 25, 37 and 45°C temperatures.

Intrinsic thermo-stability: Orf vaccine virus was evaluated for its intrinsic stability at 37°C and 45°C temperatures. Six vaccine vials for each temperature without any extrinsic stabilizer were maintained at 37 and 45°C. The vaccine batches were sampled at an interval of 24 h for 15 days and reconstituted in 1 mL serum-free EMEM and the infectivity titers were calculated.

Impact of stabilizer on the quality of the vaccine after lyophilization process: There were four lots of freeze-dried vaccines with different stabilizers. The titer of each batch before and after lyophilization process was calculated to find out the loss in titer due to lyophilization process. Other physic-chemical properties including color, appearance and percentage of residual moisture attributing to the quality of vaccine were also assessed.

Thermo-stability of lyophilized vaccine: Ample number of freeze-dried vials of vaccine with each stabilizer was exposed at 25 and 37°C in incubators and 45°C in a dry oven. The vaccine vials were sampled from incubator (25°C) on monthly basis for 5 months; from 37°C on days 3, 5, 7, 10 and 14 and from 45°C at half-a-day basis (12 h) up to two days. Exposed samples were reconstituted with 1 mL of serum free EMEM and the infectivity titres of the vaccine virus in PLT/SLT cells were calculated. For each exposure, six samples (n = 6) were titrated and their log titers were averaged (Reed and Muench, 1932).

Thermo-stability of reconstituted vaccine: Freeze-dried vials of each stabilizer were reconstituted with four diluents viz. phosphate buffer saline (1X PBS), 1 M $MgSO_4$, Normal Saline Solution (NSS) and sterile Distilled Water (DW). For each stabilizer, three vials were taken and reconstituted separately with 1 mL of each diluent. Reconstituted vaccines were then exposed to 4°C (ice), 25, 37 and 45°C. The vaccine batches so incubated were sampled at 6 h basis for 48 h and the infectivity titers of the vaccine virus were calculated (Reed and Muench, 1932).

RESULTS

Vaccine quality: Adsorption method was followed for production of vaccine. The virus was identified based on B2L gene specific PCR and sequencing (Hosamani *et al*., 2007; Venkatesan *et al*., 2011). Different batches of orf vaccine containing LS (Batch I), LHT (Batch II) and TAA (Batch III) stabilizers were prepared. Freeze-dried vaccine was physically appeared from white to pink pellet depending on the stabilizer used. The vaccine batches were titrated before and after freeze-drying. The titers of each batch and other criteria attributing to the quality of vaccine are depicted in Table 1. The initial harvest titers of all batches of vaccines were more than 6.0 $\log_{10}$ $TCID_{50}$ mL^{-1}. The loss of virus during freeze-drying conditions for all the stabilizers was ranged from 0.45-0.80 $\log_{10}$ $TCID_{50}$. However, the vaccine stabilized with LS and TAA had least and highest RM, respectively (Table 1).

Intrinsic thermo-stability: In order to assess the stability of the vaccine in the absence of extrinsic stabilizer, the intrinsic thermo-stability of the orf vaccine was evaluated at 37 and 45°C.

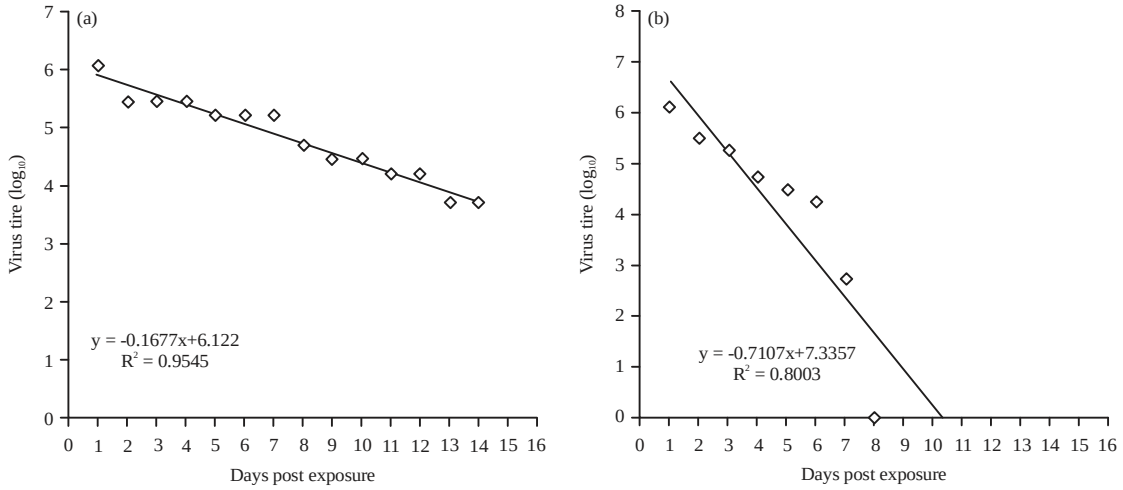

Fig. 1(a-b): Intrinsic thermo-stability of live orf vaccine exposed at (a) 37°C and (b) 45°C over a period of time

Table 1: Comparative quality of orf vaccine freeze dried with various stabilizers

Orf vaccine with stabilizer	Titer ($log_{10}TCID_{50}$ mL^{-1}) Before lyophilization	After lyophilization	Loss during lyophilization ($log_{10}TCID_{50}$ mL^{-1})	Residual moisture (%)	Appearance
LS	6.15	5.70	0.45	4.26	White pellet
LHT	6.15	5.40	0.75	5.15	White pellet
TAA	6.15	5.35	0.80	6.20	Pale pink pellet

LS: Stabilizer lactalbumin hydrolysate and sucrose, LHT: Stabilizer lactalbumin hydrolysate plus trehalose dihydrate, TAA: Stabilizer trehalose with amino acids and divalent cations

The results indicate that the orf vaccine was found to have a recommended titer of 5.0 log_{10} $TCID_{50}$ (in a 100 dose vial) at 37°C for a period of 6.72 days, whilst at 45°C, this titer could be maintained only for 3.29 days with an initial vaccine virus tire of 6.10 log_{10} $TCID_{50}$ mL^{-1}. However, the half-life of the vaccine without any stabilizer was 1.70 and 0.42 days at 37 and 45°C, respectively. The respective degradation curves for intrinsic thermostability of orf vaccine at 37°C and 45°C are depicted (Fig. 1).

Thermo-stability of freeze-dried vaccine: Ample number of lyophilized vaccine vials was exposed at different temperature over different periods. The infectivity titers of all the vaccine combinations were assessed and subjected to regression analysis. The results indicate that the reduction in virus titer was not significant at all temperatures studied with respect to all the stabilizers ($p<0.05$). At 25°C, the infectivity titres of the freeze-dried vaccines were assessed for 5 months at monthly intervals and the shelf-life and half-life for each vaccine with different stabilizers with their degradation equations are depicted (Table 2). Among the three stabilizers, only LS stabilizer could maintain the required infectivity titer in a 100 dose vial over entire sampling period (5 months), however, the LHT and TAA stabilizers could maintain the required titer less than 5 months. The shelf-life and half-life was also more in case of LS stabilizer compared to other two, indicating its superiority. At 37°C, vaccine stabilized with LHT found to maintain the required titer for a period more than 24.23 days followed by LS (22.03 days) and TAA (7.37 days).

Table 2: Comparison of degradation values of orf vaccine lyophilized with different stabilizers at various temperatures

Temperature (°C) and stabilizer	Initial titre (log_{10} $TCID_{50}$ mL^{-1})	Sample size (N)	Regression equation	Student's t-test (p-value*)	Shelf-life[a]	Half life[b]
25						
LS	5.70	6	y = -0.0960x+5.8033	p<0.166015	8.36 months	1.71 months
LHT	5.40	6	y = -0.1746x+5.5881	p<0.175659	3.37 months	1.27 months
TAA	5.35	6	y = -0.2357x+5.5243	p<0.096485	2.22 months	08.52 days
37						
LS	5.70	6	y = -0.0352x+5.7756	p<0.100461	22.03 days	15.96 days
LHT	5.40	6	y = -0.0188x+5.4556	p<0.185198	24.23 days	05.26 days
TAA	5.35	6	y = -0.0570x+5.4203	p<0.062449	07.37 days	
45						
LS	5.70	6	y = -0.0200x+5.7000	p<0.067693	35.00 h	15.00 h
LHT	5.40	6	y = -0.0207x+5.7320	p<0.197658	35.36 h	14.49 h
TAA	5.35	6	y = -0.0131x+5.3880	p<0.137115	29.61 h	22.90 h

[a]LS: Stabilizer lactalbumin hydrolysate and sucrose, LHT: Stabilizer lactalbumin hydrolysate plus trehalose dihydrate, TAA: Stabilizer trehalose with amino acids and divalent cations, N: Number of vials, [a]Time required to reach 5.0 log_{10} $TCID_{50}$ in a 100 dose vaccine preparation, calculated from the regression equation ,[b]Time required for loss of half the original titre, i.e., 0.30 log_{10} $TCID_{50}$ based on the degradation constant, *Not significant

Table 3: Thermo-stability of orf vaccine stabilized with various stabilizers at 4°C after reconstitution with different diluents

	Titer ($log_{10}TCID_{50}$)											
	LS				LHT				TAA			
Parameters	PBS	NSS	$MgSO_4$	DW	PBS	NSS	$MgSO_4$	DW	PBS	NSS	$MgSO_4$	DW
Undiluted titer[a]	5.70	5.70	5.70	5.70	5.40	5.40	5.40	5.40	5.35	5.35	5.35	5.35
Loss on dilution	-	-	-	-	-	-	-	-	-	-	-	-
Time (h)[b]												
6	5.67	5.70	5.64	5.67	5.40	5.40	5.40	5.40	5.35	5.35	5.35	5.35
12	5.70	5.68	5.54	5.60	5.40	5.40	5.40	5.40	5.35	5.35	5.35	5.35
18	5.64	5.65	5.32	5.58	5.40	5.40	5.40	5.40	5.35	5.22	5.35	5.30
24	5.60	5.65	5.12	5.62	5.26	5.35	4.75	5.40	5.16	5.12	4.60	5.35
30	5.65	5.65	4.85	5.50	5.15	5.35	5.36	5.24	4.65	4.85	4.12	5.15
36	5.43	5.62	4.76	5.32	5.15	5.20	5.24	5.12	4.50	4.70	4.12	4.66
42	5.30	5.60	4.50	5.30	5.20	5.12	5.12	5.36	3.70	4.15	3.26	3.76
48	5.23	5.54	4.25	5.42	5.25	5.35	4.72	5.15	3.12	3.50	2.90	3.22
Degradation constant (k)[c]	NSD	NSD	NSD	NSD	NSD	NSD	NSD	NSD	NSD	NSD	NSD	NSD

-: No loss in titre immediately after reconstitution with 100 mL of respective diluents at 4°C, Titres are expressed as $log_{10}TCID_{50}$, [a]Titre after dilution in 1 mL serum free EMEM, [b]Elapsed time after reconstitution, [c]Degradation constant calculated from regression equation (log_{10} $TCID_{50}$ 6 h^{-1}), NSD: No significant degradation over the sampling period, LS: Stabilizer lactalbumin hydrolysate and sucrose, LHT: Stabilizer lactalbumin hydrolysate plus trehalose dihydrate, TAA: Stabilizer trehalose with amino acids and divalent cations, PBS: Phosphate buffered saline, NSS: Normal saline, DW: Distilled water, $MgSO_4$: Magnesium sulphate

Similarly, the half-life of the vaccine is more for the LHT stabilized vaccine than that stabilized with other two stabilizers. However at 45°C, there was a reduction in the titre of virus with all stabilizers and respective half-life and shelf-life are depicted in Table 2.

Thermo-stability of reconstituted vaccine: Freeze-dried vaccines stabilized with different stabilizers were reconstituted in 1 mL of each of the four diluents to maintain a shelf-life of 100 doses ($10^{5.0}TCID_{50}$ mL^{-1})/vial and exposed at 4, 25, 37 and 45°C for 48 h. Infectivity titers for each sample in different stabilizers are shown in Table 3-6. Results revealed that the vaccines with LS stabilizer, could maintain the required infectivity titer with all the diluents throughout the sampling period under the temperatures studied except 1 M $MgSO_4$ and at 45°C. At higher temperatures (37 and 45°C), LS stabilized vaccines diluted with NSS showed better stability over other three diluents. The results of vaccine stabilized with LHT stabilizer were comparable with

that of LS stabilized vaccines. The required titer was almost maintained with all diluents over the sampling period at lower temperatures (4 and 25°C), while, at higher temperatures, it was maintained only for 36 h. The 1 M $MgSO_4$ was found to be a poor diluent in both LS as well as LHT stabilized vaccines, but worked well with TAA stabilized vaccines at 37°C compared to other diluents. Vaccines stabilized with TAA could not maintain the required titer in the vaccines for more than 24 h with any of the diluents used. Overall, vaccines stabilized with LS stabilizer diluted with normal saline solution showed better stability, followed by LHT with PBS or NSS. The TAA stabilizer was found to be a poor stabilizer for orf vaccine in all the temperature studied.

Table 4: Thermo-stability of orf vaccine stabilized with various stabilizers at 25°C after reconstitution with different diluents

	Titer ($log_{10}TCID_{50}$)											
	LS				LHT				TAA			
Parameters	PBS	NSS	$MgSO_4$	DW	PBS	NSS	$MgSO_4$	DW	PBS	NSS	$MgSO_4$	DW
Undiluted titer[a]	5.70	5.70	5.70	5.70	5.40	5.40	5.40	5.40	5.35	5.35	5.35	5.35
Loss on dilution	-	-	-	-	-	-	-	-	-	-	-	-
Time (h)[b]												
6	5.70	5.70	5.70	5.70	5.40	5.40	5.40	5.35	5.35	5.35	5.35	5.35
12	5.70	5.70	5.70	5.70	5.40	5.40	5.40	5.35	5.35	5.35	5.20	5.35
18	5.50	5.65	5.24	5.46	5.25	5.40	4.85	5.25	4.75	5.16	4.80	5.25
24	5.52	5.60	5.21	5.40	5.12	5.40	4.60	5.12	4.75	5.05	4.52	5.12
30	5.30	5.56	5.30	5.40	5.12	5.26	4.75	4.75	4.15	4.62	4.15	4.75
36	5.42	5.50	5.12	5.30	5.36	5.12	4.70	3.84	3.52	3.76	3.35	3.84
42	5.12	5.32	4.76	5.12	4.62	4.75	4.85	3.75	2.52	3.16	3.25	3.75
48	5.35	5.16	5.12	5.12	5.12	5.25	4.85	3.80	2.25	2.75	2.76	3.80
Degradation constant (k)[c]	NSD	NSD	NSD	NSD	NSD	NSD	NSD	NSD	NSD	NSD	NSD	NSD

-: No loss in titre immediately after reconstitution with 100 mL of respective diluents at 4°C, Titres are expressed as $log_{10}TCID_{50}$, [a]Titre after dilution in 1 mL serum free EMEM, [b]Elapsed time after reconstitution, [c]Degradation constant calculated from regression equation (log_{10} $TCID_{50}$ 6 h^{-1}), NSD: No significant degradation over the sampling period, LS: Stabilizer lactalbumin hydrolysate and sucrose, LHT: Stabilizer lactalbumin hydralysate plus trehalose dihydrate, TAA: Stabilizer trehalose with amino acids and divalent cations, PBS: phosphate buffered saline, NSS: Normal saline, DW: Distilled water, $MgSO_4$: Magnesium sulphate

Table 5: Thermo-stability of orf vaccine stabilized with various stabilizers at 37°C after reconstitution with different diluents

	Titer ($log_{10}TCID_{50}$)											
	LS				LHT				TAA			
Parameters	PBS	NSS	$MgSO_4$	DW	PBS	NSS	$MgSO_4$	DW	PBS	NSS	$MgSO_4$	DW
Undiluted titer[a]	5.70	5.70	5.70	5.70	5.40	5.40	5.40	5.40	5.35	5.35	5.35	5.35
Loss on dilution	-	-	-	-	-	-	-	-	-	-	-	-
Time (h)[b]												
6	5.70	5.70	5.70	5.70	5.40	5.40	5.40	5.40	5.35	5.35	5.35	5.35
12	5.70	5.70	5.70	5.70	5.40	5.40	5.40	5.40	5.20	5.26	5.15	5.25
18	5.60	5.70	5.50	5.70	5.26	5.35	5.15	5.26	4.80	5.15	4.76	5.12
24	5.60	5.62	5.16	5.65	5.15	5.30	4.65	5.14	4.52	4.75	4.85	4.70
30	5.50	5.6	5.16	5.60	5.15	5.15	4.75	5.12	4.15	4.32	4.50	4.15
36	5.45	4.85	4.65	5.60	5.26	5.35	4.75	4.62	3.35	4.12	4.50	3.60
42	5.40	4.85	4.25	5.35	5.15	5.25	4.50	4.75	3.25	3.75	4.36	3.60
48	5.62	5.62	4.30	5.52	5.15	5.25	4.12	4.80	2.76	3.25	3.75	3.12
Degradation constant (k)[c]	NSD	NSD	NSD	NSD	NSD	NSD	NSD	NSD	NSD	NSD	NSD	NSD

-: No loss in titre immediately after reconstitution with 100 mL of respective diluents at 4°C, Titres are expressed as $log_{10}TCID_{50}$, [a]Titre after dilution in 1 mL serum free EMEM, [b]Elapsed time after reconstitution, [c]Degradation constant calculated from regression equation (log_{10} $TCID_{50}$ 6 h^{-1}), NSD: No significant degradation over the sampling period, LS: Stabilizer lactalbumin hydrolysate and sucrose, LHT: Stabilizer lactalbumin hydralysate plus trehalose dihydrate, TAA: Stabilizer trehalose with amino acids and divalent cations, PBS: phosphate buffered saline, NSS: Normal saline, DW: Distilled water, $MgSO_4$: Magnesium sulphate

Table 6: Thermo-stability of orf vaccine stabilized with various stabilizers at 45°C after reconstitution with different diluents

	Titer ($log_{10}TCID_{50}$)											
	LS				LHT				TAA			
Parameters	PBS	NSS	$MgSO_4$	DW	PBS	NSS	$MgSO_4$	DW	PBS	NSS	$MgSO_4$	DW
Undiluted titer[a]	5.70	5.70	5.70	5.70	5.40	5.40	5.40	5.40	5.35	5.35	5.35	5.35
Loss on dilution	-	-	-	-	-	-	-	-	-	-	-	-
Time (h)[b]												
6	5.70	5.70	5.70	5.70	5.40	5.40	5.40	5.40	5.23	5.35	5.35	5.30
12	5.70	5.70	5.70	5.70	5.36	5.40	5.30	5.35	5.16	5.20	5.12	5.24
18	5.35	5.60	5.25	5.50	5.36	5.38	5.16	5.28	4.75	5.15	4.66	4.70
24	5.15	5.25	4.85	5.23	5.15	5.24	4.85	5.12	4.16	4.35	3.72	4.22
30	5.26	5.15	4.85	5.20	5.26	5.30	4.50	4.80	3.45	3.75	3.32	3.65
36	5.12	5.35	4.35	4.50	5.25	5.15	3.76	4.52	3.12	3.50	3.16	3.15
42	4.85	5.30	3.20	4.45	4.76	5.20	3.80	4.76	3.12	3.50	3.12	3.15
48	4.72	5.15	3.16	4.60	4.85	4.95	3.75	4.50	2.16	2.56	2.20	2.25
Degradation constant (k)[c]	NSD	NSD	NSD	NSD	NSD	NSD	NSD	NSD	NSD	NSD	NSD	NSD

-: No loss in titre immediately after reconstitution with 100 mL of respective diluents at 4°C, Titres are expressed as $log_{10}TCID_{50}$, [a]Titre after dilution in 1 mL serum free EMEM, [b]Elapsed time after reconstitution, [c]Degradation constant calculated from regression equation (log_{10} $TCID_{50}$ 6 h^{-1}), NSD: No significant degradation over the sampling period, LS: Stabilizer lactalbumin hydrolysate and sucrose, LHT: Stabilizer lactalbumin hydralysate plus trehalose dihydrate, TAA: Stabilizer trehalose with amino acids and divalent cations, PBS: Phosphate buffered saline, NSS: Normal saline, DW: Distilled water, $MgSO_4$: Magnesium sulphate

DISCUSSION

The present study was envisaged to compare the efficacy of three different stabilizers such as LS, LHT and TAA in preserving the infectivity of the lyophilized novel orf vaccine (ORFV Muk 59/05) at various temperatures and also the effect of reconstitution of the vaccine with diluents like NSS, PBS, distilled water and 1 M $MgSO_4$. The vaccines were freeze-dried in batches using aforesaid stabilizers. There is no literature on the thermo-stability of orf vaccine; therefore, the current results are compared with two morbillivirus vaccines (Mariner *et al*., 1990; Sarkar *et al*., 2003). The loss of titre under these freeze-drying conditions was ranged from 0.45-0.80 log_{10} $TCID_{50}$ and was more (0.80 log_{10} $TCID_{50}$ mL^{-1}) with TAA stabilized vaccine. This is in accordance with the findings for Tissue Culture Rinderpest (TCRP) (Mariner *et al*., 1990) and PPR vaccines (Sarkar *et al*., 2003) stabilized with BUGS stabilizer. This loss could have been obviated, if the recommended freeze-drying conditions (Mariner *et al*., 1990) were espoused. It has been recommended to use trehalose dihydrate (non-reducing disaccharide) under precise control. However, here it was used under conventional freeze-drying conditions along with amino acids (L-Alanine and L-Histidine). Further, residual moisture is an additional factor contributing to the keeping quality of freeze-dried vaccines (Precausta *et al*., 1982). Most of the live vaccines lose their potency rapidly at high RM and therefore, any freeze-dried vaccine is expected to have RM less than 3% (Burke *et al*., 1999). In the current investigation, RM ranged from 4.26-6.20%, which is relatively high and it could be due to the absence of heating phase of the vaccine vials during secondary drying and the results are in conformity with the earlier ones (Sarkar *et al*., 2003).

Though, the ORFV is stable at ambient temperature, the data on the stability of it at various conditions are not available. In order to assess the stability of the vaccine virus at 37 and 45°C without any stabilizer, the intrinsic thermo-stability of the vaccine was undertaken. This is useful in the application of the vaccine at the field level. On comparison of the keeping quality (half-life and shelf-life) of the vaccine with and without stabilizers, it is observed that the fall in virus titer of the vaccine was relatively faster without a stabilizer than in the presence of a stabilizer as normally seen with any of the other live vaccines.

The major disadvantage of live attenuated vaccine is the maintenance of 'cold-chain', a costly affair. Therefore, it is desirable for an ideal stabilizer to maintain recommended titer in a vaccine vial for an extended period at a given temperature. Thermo-stability studies on different viral vaccines have been exhaustively conducted (Plowright and Ferris, 1962; De Boer and Barber, 1964; Plowright *et al*., 1970; Bansal *et al*., 1976; Sarkar *et al*., 2003; Riyesh *et al*., 2011). The infectivity titers obtained in this study were analyzed by regression. The decrease in titre was not significant at all temperature with all stabilizer combinations. This is possibly due to short duration of study at higher temperatures (14 days at 37°C and 48 h at 47°C). At 25°C, LS stabilizer found better in maintaining the quality of the vaccine in terms of shelf-life and half-life compared to LHT and TAA. However at 37°C, orf vaccine stabilized with LHT stabilizer showed better stability than that of LS and TAA. Earlier studies have indicated that the increased concentration of trehalose dihydrate prolonged the stability of TCRP and PPR vaccines (Worrall *et al*., 2000). It is known that the trehalose protects cell membrane, protein and other molecules from drying and also preserve the natural biological activity of the molecules. It forms protective layer around the protein and other biomolecules protecting biological/s under adverse conditions like extreme temperatures, hypertonia and dehydration. Trehalose has become nature's dessiprotectant (Worrall *et al*., 2000). In a desiccating environment, trehalose dries as a transparent glass and results in vitrification. This prevents the expansion of fluids and cells from disruption. Trehalose provides a micro scaffold and supports the tertiary structural integrity of biomolecules and reduces degrading molecular reactions to insignificant levels (Levin *et al*., 2007). It is inert, non-toxic, nonhygroscopic and re-dissolves exhibiting solubility. In the present study, possibly one of these effects might had a role in preserving the quality of vaccine at 37°C, though not contemplated in this case. At this temperature, the vaccines stabilized with LS with a titer of 5.7 $\log_{10}TCID_{50}$ mL^{-1} should be utilized within 22.03 days, if 100 doses are destined to be used with a protective titer of 3.0 $\log_{10}$TCID50/dose, whilst, TAA stabilized vaccines should be utilized within 7.37 days. However, TAA stabilizer found unsuitable for orf vaccine at this temperature. At 45°C, all three stabilizer combinations showed stability up to 29 h only after which the titers were drastically receded. At this temperature, both LS and LHT found advantageous over the other.

The findings in respect of thermo-stability of reconstituted vaccine were interesting. Except in few cases, the recommended titer for a 100 dose vial (5 $\log_{10}TCID_{50}$) was maintained up to 48 h at 4 and 25°C with all the four diluents used. But at 37 and 45°C, NSS and PBS performed well for 42 h, but thereafter, the titer receded rapidly, whereas, 1 M $MgSO_4$ diluent found unsuitable even at lower temperatures. The vaccine reconstituted with NSS can be used at ambient temperatures even up to 42 h and possibly more.

CONCLUSION

To sum up, three different stabilizer formulations were compared in terms of their thermo-protective effect on a novel live attenuated orf vaccine virus (ORFV Muk 59/05) under conventional lyophilization conditions. The LS stabilizer found superior in maintaining the quality of orf vaccine for an extended period at 25°C followed by LHT and TAA. However at 37°C, LHT was found better over others and at 45°C, both LS and LHT performed well under conventional lyophilization conditions. Among the diluents, NSS was a diluent of choice for reconstitution of the orf vaccine.

ACKNOWLEDGMENTS

The authors thank the Director, Indian Veterinary Research Institute for providing necessary facilities to carry out this work and the staff of Pox Virus Laboratory for providing technical assistance. The financial assistance received under the project Niche Area of Excellence: Development of veterinary immunodiagnostics and immunoprophylactics by Indian Council of Agricultural Research, New Delhi is gratefully acknowledged. Further, the first author acknowledges IVRI for financial support in terms of fellowship to carry out the PhD program.

REFERENCES

Adebayo, A.A., J.W. Sim-Brandenburg, H. Emmel, D.O. Olaleye and M. Niedrig, 1998. Stability of 17D Yellow fever virus vaccine using different stabilizers. Biologicals, 26: 309-316.

Bansal, R.P., S.K. Chawla, R.C. Joshi and D.C. Shukla, 1976. Studies on attenuated rinderpest vaccine of tissue culture origin. Indian J. Anim. Sci., 41: 18-26.

Bora, D.P., N.N. Barman, S.K. Das, V. Bhanuprakash and R. Yogisharadhya *et al*., 2012. Identification and phylogenetic analysis of orf viruses isolated from outbreaks in goats of Assam, a northeastern state of India. Virus Genes, 45: 98-104.

Burke, C.J., T.A. Hsu and D.B. Volkin, 1999. Formulation, stability and delivery of live attenuated vaccines for human use. Crit. Rev. Therapeut. Drug Carrier Syst., 16: 83-83.

Chen, D. and D. Kristensen, 2009. Opportunities and challenges of developing thermostable vaccines. Expert Rev. Vaccines, 8: 547-557.

De Boer, D.J. and T.L. Barber, 1964. pH and thermal stability of rinderpest virus. Archiv fur die Gesamte Virusforschung, 15: 98-108.

Dey, A. and P. Kundu, 2009. Outbreak of contagious ecthyma in goats. Indian Vet. J., 86: 242-247.

Hosamani, M., S. Yadav, D.J. Kallesh, B. Mondal, V. Bhanuprakash and R.K. Singh, 2007. Isolation and characterization of an Indian Orf virus from goats. Zoonoses Public Health., 54: 204-208.

Hosamani, M., A. Scagliarini, V. Bhanuprakash, C.J. McInnes and R.K. Singh, 2009. Orf: An update on current research and future perspectives. Expert Rev. Anti-Infect. Ther., 7: 879-893.

Housawi, F.M. and E.M. Abu Elzein, 2000. Contagious ecthyma associated with myiasis in sheep. Rev. Scient. Tech., 19: 863-866.

Levin, A., C. Levin, D. Kristensen and D. Matthias, 2007. An economic evaluation of thermostable vaccines in Cambodia, Ghana and Bangladesh. Vaccine, 25: 6945-6957.

Mariner, J.C., M.C. van den Ende, J.A. House, C.A. Mebus, S. Salifou and C. Stem, 1990. The serological response to a thermostable Vero cell-adapted rinderpest vaccine under field conditions in Niger. Vet. Microbiol., 22: 119-127.

Mondal, B., A.K. Bera, M. Hosamani, P.A. Tembhurne and S.K. Bandyopadhyay, 2006. Detection of orf virus from an outbreak in goats and its genetic relation with other parapoxviruses. Vet. Res. Commun., 30: 531-539.

Plowright, W. and F.D. Ferris, 1962. Studies with rinderpest virus in tissue culture. III. The stability of cultured virus and its use in virus neutralization tests. Arch. Gesamte Virusforschung, 11: 516-533.

Plowright, W., C.S. Rampton, W.P. Taylor and K.A.J. Herniman, 1970. Studies on rinderpest culture vaccine. III. Stability of the lyophilised product. Res. Vet. Sci., 11: 71-81.

Precausta, P., J.P. Soulebot, M. Bugand, A. Brun and G. Chappuis, 1982. Modalities of production and immunity conferred by an inactivated rabies vaccine originating from cell culture. Comp. Immunol. Microbiol. Infect. Dis., 5: 217-226.

Reed, L.J. and H. Muench, 1932. A simple method of estimating fifty per cent endpoints. Am. J. Hyg., 27: 493-497.

Riyesh, T., V. Balamurugan, A. Sen, V. Bhanuprakash, G. Venkatesan, V. Yadav and R.K. Singh, 2011. Evaluation of efficacy of stabilizers on the thermostability of live attenuated thermo-adapted *Peste des petits ruminants* vaccines. Virologica Sinica, 26: 324-337.

Sarkar, J., B.P. Sreenivasa, R.P. Singh, P. Dhar and S.K. Bandyopadhyay, 2003. Comparative efficacy of various chemical stabilizers on the thermostability of a live-attenuated *peste des petits ruminants* (PPR) vaccine. Vaccine, 21: 4728-4735.

Venkatesan, G., V. Balamurugan, D.P. Bora, R. Yogisharadhya, M. Prabhu and V. Bhanuprakash, 2011. Sequence and phylogenetic analyses of an Indian isolate of orf virus from sheep. Veterinaria Italiana, 47 : 323-332.

WHO., 2005. Guidelines for nonclinical evaluation of vaccines. WHO Technical Report Series, No. 927, WHO Expert Committee on Biological Standardization, Fifty-Fourth Report, World Health Organization, Geneva, Switzerland.

WHO., 2009. Guidelines on stability evaluation of vaccines. Biologicals, 37: 424-434.

Worrall, E.E. J.K. Litamoi, B.M. Seck and G. Ayelet, 2000. Xerovac: An ultra rapid method for the dehydration and preservation of live attenuated rinderpest and peste des petits ruminants vaccines. Vaccine, 19: 834-839.

Yogisharadhya, R., V. Bhanuprakash, G. Venkatesan, V. Balamurugan, A.B. Pandey and S.B. Shivachandra, 2012. Comparative sequence analysis of poxvirus A32 gene encoded ATPase protein and carboxyl terminal heterogeneity of Indian orf viruses. Vet. Microbiol., 156: 72-80.