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ORIGINAL ARTICLE

Development of a process for upscaling and production of thermotolerant Peste-des-petits ruminants vaccine

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Abstract Vaccination is the most effective means of preventing Peste-des-petits-ruminants (PPR), an important disease of small ruminant population. The thermolabile nature of PPR vaccine poses a major constraint in shipping, storage and its successful application. In view of limited thermotolerance of PPR virus and ongoing global PPR control and eradication program, development of a thermotolerant PPR vaccine was tried using a novel lyophilization protocol and improved thermostabilization. A lyophilization cycle of 16 h (h) using 200 µl of PPR vaccine virus (stock titre 5.8 $\log_{10}$ $TCID_{50}$/vial in 200 µl) was developed. For this, five stabilizer formulations were selected out of ten formulations based on the stability of liquid vaccine at 37 °C and three freeze–thaw cycles. Improved thermostabilization of PPR vaccines was obtained by inclusion of 5% trehalose and 0.5% gelatine to Lactalbumin hydrolysate-sucrose (LS) formulations which significantly improved the stability of lyophilized vaccines with a shelf-life of at least 1305.3 days at 2–8 °C, 23.68 days at 25 °C, 20.88 days at 37 °C, 5.01 days at 40 °C and 3.22 days at 45 °C which qualifies the standards of a thermotolerant PPR vaccine as defined by the FAO and OIE. In reconstituted vaccines, the combination of LS, trehalose and gelatin (LSTG) provided a shelf-life of 1.77 days at 37 °C, 22.41 h at 40 °C and 10.05 h at 45 °C. The study suggested that use of the short lyophilization protocol standardized with 200 µl of lyophilized PPR vaccine stabilized with LSTG formulation, can be used to develop and upscale thermotolerant PPR vaccines during national and global PPR control and eradication as targeted by the FAO and OIE by 2030.

Keywords Peste-des-petits ruminants · Vaccine · Lyophilization · Upscaling · Thermostability

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Introduction

Peste-des-petits ruminants (PPR), affects about 80% of the global small ruminant population causing morbidity, mortality and significant economic impact to livestock economy affecting livelihood security of developing nations [9]. As per recent statistics of the World Organization for Animal Health (OIE) and the Food and Agricultural Organization (FAO) of the United Nations, the current expenditure on PPR vaccination have been estimated to be between US$ 270 and US$ 380 million per year [9]. Due to its devastating effects on the global food security and economic growth, the OIE and FAO launched 'PPR Global Control and Eradication Strategy' in 2015 with an objective to control and eradicate PPR globally by the year 2030. For the control of PPR, very effective and safe live attenuated vaccines are available. The first successful homologous PPR vaccine developed using an African strain, Nigeria 75/1 of lineage II origin, being widely used in African countries [7]. Another homologous vaccine against PPR was developed using an Indian strain, Sungri/96 of lineage IV origin [26] and is currently extensively used to control the disease in the Indian-subcontinent, Middle-East and South-Asia [18].While vaccination is considered as an

effective way to control PPR, thermal stability of such live attenuated vaccines remains a major concern in areas with tropical climatic conditions where optimal storage conditions are difficult to maintain. Since PPR virus (PPRV) is sensitive to high temperatures, a break in the cold chain during shipment to remote areas can cause substantial drop in vaccine efficacy or potency [23]. The constraint of maintaining the cold chain can be reduced through development and use of effective thermotolerant PPR vaccines which can be used under field settings [4].

Thermotolerance can be described as the ability of a vaccine and/or the parent virus/strain to retain a level of infectivity after exposure to heat, that is, the delayed heat degradation of the virus [17]. Thermotolerance can be achieved through a variety of means such as development of novel vaccines, improved formulation of stabilizers, improved manufacturing processes including the use of thermotolerant vaccine strains [10]. A thermotolerant PPR vaccine should maintain a shelf-life of 2 years at 2–8 °C, 10 days at 25 °C and 5 days at 40 °C as per the criteria established during the PPR-Global Eradication Programme Thermotolerant PPR Vaccines Workshop, 2017 [10]. In view of PPR global control and eradication strategy, developing thermotolerant vaccines alone is not sufficient to control the disease. Development and manufacture of economically priced vaccines and innovative approaches for large scale production of thermotolerant PPR vaccines will be important to enable effective responses and meet the global demand of vaccine doses for execution of successful vaccination strategies [3, 4].

Considering the demand of safe and efficacious vaccine in view of global strategy of PPR control and eradication, the present study was aimed to develop a process for thermotolerant PPR vaccine with simplified upscaling. To achieve this, synergistic effect of various stabilizer combinations over and above Lactalbumin hydrolysate-sucrose (LS) formulations were evaluated in combination with a short lyophilization cycle using low volume of high titred vaccine virus.

Materials and methods

Cells and viruses

Vero cells (ATCC® CCL-81) were cultivated using Eagle's Minimum Essential Media (EMEM, Sigma) supplemented with 10% Fetal bovine serum (FBS, Gibco), 200 mM L-glutamine and100mM sodium pyruvate (Himedia). For maintaining the cells, EMEM with 2% FBS was used as maintenance medium. Vero cell based live attenuated PPR vaccine virus of lineage IV origin (PPR Sungri/96, Seed 2, Passage 60) was used as a parent virus for this study [26]. This strain was maintained in Division of Biological Products, ICAR-Indian Veterinary Research Institute and stored at − 80 °C using standard protocols.

Vaccine virus propagation and storage

Vero cells were seeded into T-300 flask (TPP® 300 cm^2) with an average cell density of 1.8×10^7 cells in EMEM stabilized with 25 mM HEPES (Sigma Aldrich). The seeded cells were infected with PPR vaccine virus at a multiplicity of infection of 0.02. Infected cells were incubated at 37 °C and monitored routinely. After 5 days post infection (dpi), viral harvests were made when 80–90% of cytopathic effect was evident. The virus harvest was immediately preserved at − 80 °C until lyophilization.

Preparation of stabilizer formulations

To improve the stability of the existing PPR vaccine, ten different combinations of stabilizers were designed containing 5% Lactalbumin-hydrolysate (LAH) and 10% Sucrose (S), combinedly LS, as a common constituent. All the excipients used in preparation of stabilizer combinations were selected based on the information available [8, 11, 12, 19, 20, 23, 24, 28]. The excipients were prepared in Hank's Balanced Salt Solution (Sigma, USA) by keeping the concentration twice of its original value so that the final concentration of the excipients when mixed with the vaccine falls in the ratio of 1:1. All the excipients used in the present study were purchased from Sigma (USA). The formulation codes, components and final concentration of stabilizers are represented in Table 1.

Thermostability and effect of freeze–thaw on liquid PPR vaccine

The formulations were evaluated for stabilizing efficacy on liquid vaccine initially in order to screen the potential stabilizers. The vaccine aliquots were mixed thoroughly with respective stabilizer formulations and exposed to 37 °C and titrated at 0, 8, 16, 24, 30, 42, 48, 56 and 64 h intervals. The virus preparations, stabilized with various formulations which showed maximum shelf-lives were further selected for extensive thermostability studies of lyophilized as well as reconstituted vaccines. The effect of freeze–thaw on liquid vaccine with the selected stabilizer formulations was also used to screen the formulations. Freeze–thaw was performed by freezing 200 µl of virus aliquots (stock titre 6.5 $\log_{10}$ $TCID_{50}$/ml) at -20 °C for 3 h and then thawed at 20–25 °C for a total of three cycles. The infectivity titres were determined using Reed and Muench formula [21].

Table 1 Formulations used for evaluation of initial stability of PPR vaccine. Formulation X* was added later to study the thermostability of lyophilized and reconstituted vaccines as gelatine was found to improve the stability during initial screening of formulations

Formulation code	Composition	pH
A	LS	6.5–7.0
B	LS + 5% trehalose	7.0
C	LS + 5% trehalose + 0.5% gelatine	6.5–7.0
D	LS + 5% trehalose + 0.5% gelatine + 1% arginine	7.0–7.5
E	LS + 10% sorbitol	6.5–7.0
F	LS + 10% sorbitol + 10^{-6} M PEG-8000	7.0
G	LS + 1% PVP + 1% sodium glutamate	6.5–7.0
H	LS + 1% PVP + 50 mM L-glutamine	7.0
I	LS + 20 mM Tris–HCl + 0.02% tween 80 + 2 mM EDTA + 1 M trehalose	7.5
J	LS + 5% DMEM + 10% FBS	7.0
X*	LS + 0.5% gelatine	7.0

PEG polyethylene glycol, *PVP* polyvinyl pyrollidone, *Tris-HCL* Tris-hydrochloric acid, *EDTA* ethylenediaminetetraacetic acid, *DMEM* Dulbecco's modified eagle's medium, *FBS* Foetal bovine serum

Lyophilization of virus preparations

The five superior stabilizer formulations evaluated from the thermostability study of the liquid vaccine and the effect of freeze–thaw were included for lyophilization. Lyophilization of vaccines was carried out in an automated lyophilizer (Lyodryer, LT 5S) in the presence of LS as a baseline stabilizer. 200 µl aliquots of vaccine virus-stabilizer mix were added to 2 ml sterile lyophilization vials. A lyophilization program of 16 h of total run was designed, developed and optimized for the present study. The program consisted of a freezing cycle of 230 m (3.83 h), primary drying run of 680 m (11.33 h) and a secondary drying cycle of 30 m (0.5 h). The freezing cycle involved rapid freezing of the preparation at − 40 °C under vacuum pressure 400 mTorr. The primary drying was carried out at a shelf temperature of − 40 °C, − 35 °C, − 30 °C, − 25 °C, − 20 °C, − 15 °C, − 10 °C, − 5 °C and 0 °C for 60 m each followed by 5 °C for 40 m, 10 °C, 15 °C, 20 °C and 25 °C for 20 m each and 30 °C for 25 m. The secondary drying was done at a shelf temperature of 30 °C for 30 m under vacuum pressure of 100 mTorr. All the vials were finally sealed under vacuum following the lyophilization stage. A batch of vaccine containing different stabilizer formulations was lyophilized in a single run to compare the quality of finished product in terms of loss of titre, effect of temperature and residual moisture during and after lyophilization.

Measurement of residual moisture

Residual moisture (RM) in the lyophilized product was measured by thermogravimetric method described as by Worrall et al. [30]. The mean weight of 5 vials from each of the stabilizer formulations was taken in a high precision electronic balance (Aczet, Precision level 0.0000 g) and then dried for 20 h at 80 °C. The weight of the bound water loss from the dried vaccine was expressed as a percentage.

Thermostability testing of lyophilized vaccine

Stability test of the lyophilized vaccine virus for each stabilizer formulation were conducted at temperatures 2–8 °C, 25 °C, 37 °C, 40 °C and 45 °C. Lyophilized vial in triplicates were exposed at 2–8 °C in refrigerator and titrated at monthly intervals upto 6 months. The exposures of vaccines to 25 °C, 37 °C, 40 °C and 45 °C were done in incubators with periodic monitoring of temperatures. At temperature 25 °C and 37 °C, the sampling points were day 0, 1, 3, 7, 10, 14, 21, 25, 36 and 45. At temperature 40 °C and 45 °C, the lyophilized vaccines were sampled on 0, 8, 24, 40, 56, 72, 88, 104 and 120 h. The exposed vials were reconstituted with 200 µl of 0.85% sodium chloride (NaCl) solution, pooled and titrated in Vero cells. The infectivity titres of PPR vaccine with all the stabilizer formulations were assessed using Reed and Muench method and subjected to regression analysis.

Thermostability testing of reconstituted vaccine

For each stabilizer formulation, three lyophilized vaccine vials were taken and reconstituted separately in 200 µl of 0.85% NaCl solution, pooled and titrated immediately to assess the loss of titre on reconstitution. Sufficient number of reconstituted vials were exposed to 2–8 °C, 25 °C, 37 °C, 40 °C and 45 °C to study the effect of various

stabilizers upon storage of reconstituted vaccine upto 120 h. Samples from each temperature were taken out at 8, 24, 40, 56, 72, 88, 104 and 120 h and titrated on Vero cells.

Virus titration

Virus titration of liquid, lyophilized and reconstituted vaccines was done sequentially as per the time points set up for various temperatures. Virus infectivity was quantified by estimating the 50% tissue culture infectivity dose ($TCID_{50}$) in 384 well microplates (BD Falcon). Briefly, 30 µl of Vero cells containing approximately 10^3 cells per well were seeded in 384 well microplates 24 h before titration and incubated at 37 °C in a humidified atmosphere of 5% CO_2. Ten-fold serial dilutions of different virus samples were prepared in EMEM containing 10% FBS. For each dilution, 50 µl of virus suspension being added in triplicate to the wells with preformed Vero cell monolayer. All the plates were incubated at 37 °C in presence of 5% CO_2 and checked for cytopathic effects. Titration were read on day 6 and the end point titres were calculated using Reed and Muench formula, simplified for calculation in MS Excel spreadsheet.

Statistical analysis

The viability of virus in liquid, lyophilized and reconstituted vaccines at different temperatures over the exposure period was carried out by regression analysis as described by Mariner et al. [14] in order to calculate the shelf and half-lives. The linear decay was analyzed by standard regression analysis to estimate the slope of the decay or degradation constant (k) and the intercept. The half-life was calculated as the time taken to lose 0.3 $\log_{10}$ $TCID_{50}$/vial based on the degradation constant (k) i.e. (0.3 $\log_{10}$ $TCID_{50}$)/k. Assuming a 100-dose pack size and a minimum required titre of 2.5 $\log_{10}TCID_{50}$ per dose, the required titre per vial will be 4.5 $\log_{10}TCID_{50}$. Therefore, the shelf life was calculated as the time taken to reach 4.5 $\log_{10}$ $TCID_{50}$/vial in a 100-dose vaccine preparation based on regression analysis. Thus, the formula for the corrected shelf-life was: Shelf-life = (Intercept—4.5 $\log_{10}$ $TCID_{50}$)/k. The two-factor analysis of variance was performed using JMP 9 software to evaluate the effect of stabilizers, temperature and time period on the stability of vaccines in liquid, lyophilized and reconstituted products.

Results

In the initial stage, stabilizer formulations (A to J) were screened for their enhanced stability by exposure of liquid vaccine at 37 °C for 0, 8, 16, 24, 30, 42, 48, 56 and 64 h followed by virus titration. The liquid vaccine stabilized with formulations A, B, C, D and E were found to maintain a superior shelf-life as compared to the formulations F, G, H, I and J (Fig. 1a). At 37 °C, vaccine stabilized with formulation C maintained the required infectivity i.e. 4.5 $\log_{10}TCID_{50}$/vial (100 doses) for a period of 46.88 h, followed by B (43.91 h), A (42.96 h), D (42.29 h), E (35.97 h), I (35.82 h), F (31.27 h), G (30.53 h), H (29.21 h) and J (24.07 h) (Table 2). Formulations containing 5% trehalose, 0.5% gelatine, 10% sorbitol and 1% arginine showed improved stability compared to the other excipients used in the stabilizer formulations. The formulations A, B, C, D and E were selected based on the regression analysis and the decay rate for further investigations.

The effect of three freeze–thaw cycles was assessed to mimic what a multi-dose vaccine vial might encounter during storage and to evaluate its stability with different stabilizer formulations. Comparative analysis of the stabilizer combinations at cycle 0, 1, 2 and 3 indicated that formulations C and D are equally superior to other formulations and a loss of only 0.25 $\log_{10}$ $TCID_{50}$ was observed up to two freeze–thaw cycles. Formulation C and D were found to represent significant differences with that of the rest of the combinations at p 0.05 level of significance ($p < 0.05$) indicating its superiority above all (Fig. 1b).

Based on the stability analysis of liquid vaccine and freeze–thaw exposure, the formulations B, C, D and E were found to enhance the stability when compared to the baseline stabilizer i.e. formulation A which consisted of LS alone. Though formulation D showed an improvement in stability similar to formulation C, however, addition of 1% arginine did not seem to improve the stability. Therefore, formulation C has been selected for further evaluation in substitute of formulation D. Addition of 0.5% gelatine has shown to increase the overall stability of the liquid vaccine exposed to 37°C. Therefore, we included an additional combination X, combining LS and 0.5% gelatine to observe the effect of gelatine alone. The final formulations A, B, C, E and X were evaluated to study their effects on stability of lyophilized and reconstituted PPR vaccines.

The PPR vaccine with an initial infectivity titre of 6.5 $\log_{10}$ $TCID_{50}$/ml was lyophilized with stabilizer formulations A, B, C, E and X using the developed 16 h lyophilization protocol in 200 µl volume (5.8 $\log_{10}TCID_{50}$/vial) in a single run. After lyophilization, no loss of virus titre was observed with formulation C which retained a titre of 5.8 $\log_{10}TCID_{50}$/vial, whereas formulation A, B, E and X induced a loss of 0.25 $\log_{10}$ $TCID_{50}$/vial and retaining a virus titre of 5.55 $\log_{10}$ $TCID_{50}$/vial. The loss on lyophilization and the residual moisture content obtained with different stabilizer formulations is represented in Table 3.

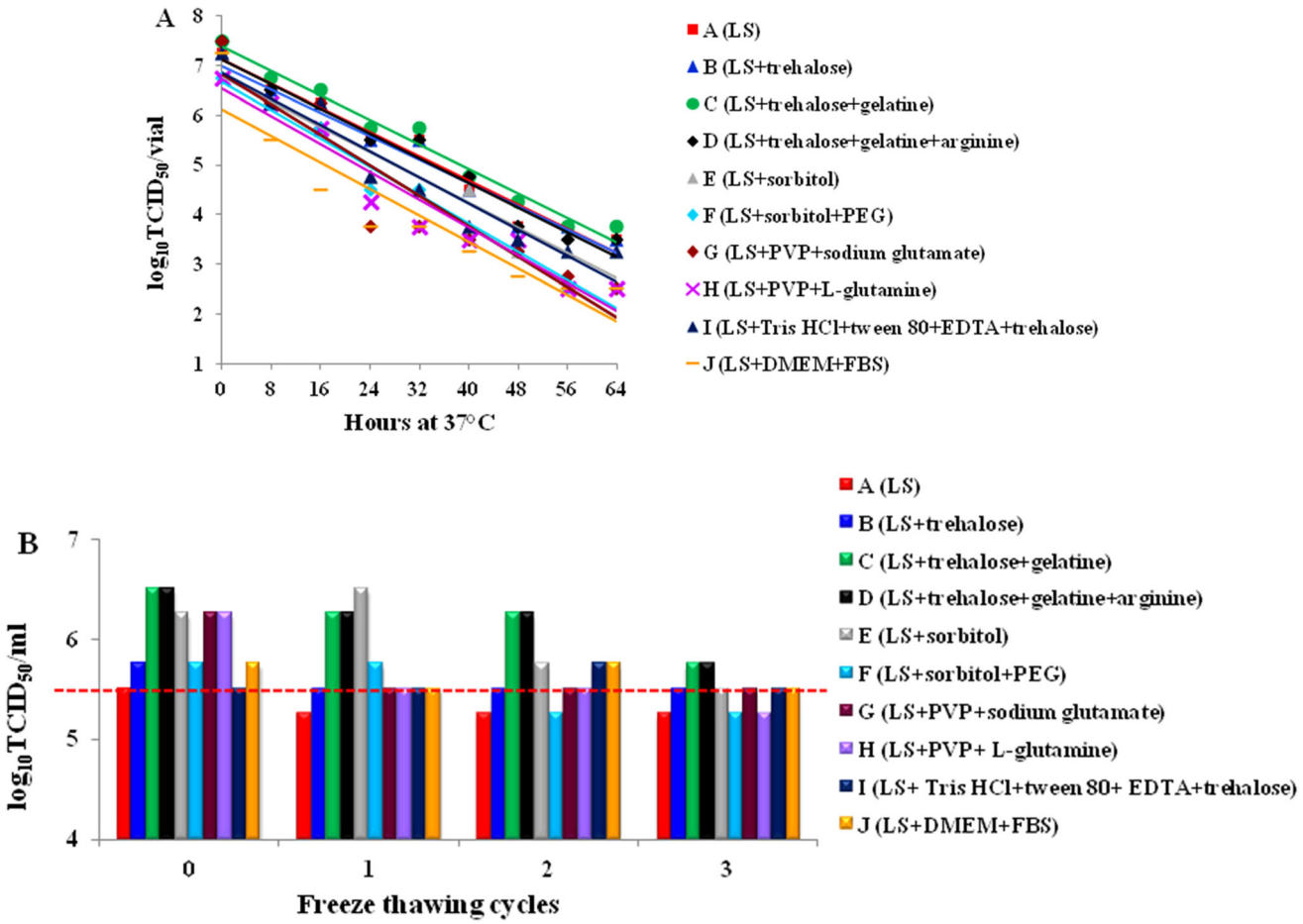

Fig. 1 **a** Screening of stabilizer formulation/combinations for liquid PPR virus stability by exposure at 37 °C. **b** Screening of stabilizer formulation/combinations for liquid PPR virus stability with three cycles of freeze–thaw. Note that stabilizer combinations C and D are equally superior to all others both for exposure at 37 °C and freeze–thaw cycles

Table 2 Comparison of degradation values of liquid PPR vaccine exposed to 37 °C with different stabilizer formulations

Stabilizers	Initial titre (log_{10} $TCID_{50}$/ml)	Sample size	Regression equation	Student's t-test		Shelf -life (h)	Half-life (h)
				t -Ratio	Probability		
A	7.25	9	y = −0.0609x + 7.1167	43.74	< 0.0001	42.96	4.92
B	7.25	9	y = −0.0589x + 6.9944	34.28	< 0.0001	43.91	5.09
C	7.50	9	y = −0.0615x + 7.3833	52.19	< 0.0001	46.88	4.87
D	7.50	9	y = −0.0620x + 7.1222	35.10	< 0.0001	42.29	4.83
E	7.25	9	y = −0.0630x + 6.7667	27.47	< 0.0001	35.97	4.76
F	7.25	9	y = −0.0755x + 6.8611	32.59	< 0.0001	31.27	3.97
G	7.50	9	y = −0.0766x + 6.8389	17.21	< 0.0001	30.53	3.91
H	6.75	9	y = −0.0698x + 6.5389	25.00	< 0.0001	29.21	4.29
I	7.25	9	y = −0.0656x + 6.8500	25.50	< 0.0001	35.82	4.57
J	7.25	9	y = −0.0667x + 6.1055	15.88	< 0.0001	24.07	4.49

Shelf-life-Time required to reach 4.5 log_{10} $TCID_{50}$ in a 100-dose presentation calculated from the regression equation

Half-life-Time required for loss of half of the original titre, i.e. 0.30 log_{10} $TCID_{50}$ based on degradation constant

The results of the stability of lyophilized vaccine sample at temperatures 2–8 °C, 25 °C, 37 °C, 40 °C and 45 °C indicated that stabilizer formulation C is superior to rest of the combinations with a shelf-life of 1305.3 days at 2–8 °C, 23.68 days at 25 °C, 20.88 days at 37 °C, 5.01 days at 40 °C and 3.22 days at 45 °C. The shelf and

Table 3 Comparison of loss during lyophilization and residual moisture content obtained in the 16 h lyophilization protocol. The loss in virus titre during lyophilization is expressed as titre before lyophilization minus titre after lyophilization. The residual moisture is represented in percentage (%) with respect to the stabilizer formulations

Stabilizer formulations	Virus quantity before lyophilisation (log_{10} $TCID_{50}$/vial)	Volume of virus-stabilizer mix for lyophilisation (µl)	Virus quantity after lyophilisation ($log_{10}TCID_{50}$/vial)	Loss ($log_{10}TCID_{50}$/vial)	Residual moisture (%)
A (LS)	5.8	200	5.55	0.25	2.88
B (LS + trehalose)	5.8	200	5.55	0.25	2.97
C (LS + trehalose + gelatine)	5.8	200	5.8	0.00	2.03
E (LS + sorbitol)	5.8	200	5.55	0.25	2.35
X (LS + gelatine)	5.8	200	5.55	0.25	1.49

half-lives are represented in Table 4 and the degradation curve for each stabilizer formulation is illustrated in Fig. 2.

The lyophilized vaccine vials with stabilizer A, B, C, E and X were reconstituted with 200 µl of 0.85% NaCl and exposed at 2–8 °C, 25 °C, 37 °C, 40 °C and 45 °C for 120 h. At 2–8 °C, no loss of titre was noticed in all the formulations, except for stabilizer A where an initial loss was obtained on 8 h post exposure. At 25 °C, formulations

Table 4 Comparison of degradation values of lyophilized PPR vaccine (Sungri/96) exposed to 2–8 °C, 25 °C, 37 °C, 40 °C and 45 °C at different time points

Temperature (°C)	Stabilizers	Initial titer ($log_{10}TCID_{50}$/vial)	Sample size	Regression equation	Student's t test		Shelf-life (Days/h)	Half-life (Days/h)
					t Ratio	Probability		
2–8	A	5.55	6	y = −0.0536x + 5.2821	47.37	< 0.0001	437.7 days	167.7 days
	B	5.55	6	y = 0.000x + 5.55	NS	NS	ND	ND
	C	5.8	6	y = −0.0268x + 5.6661	101.62	< 0.0001	1305.3 days	335.7 days
	E	5.55	6	y = −0.1071x + 5.6571	56.72	< 0.0001	324 days	84 days
	X	5.55	6	y = −0.0893x + 5.4607	48.97	< 0.0001	322.8 days	100.8 days
25	A	5.55	10	y = −0.0573x + 5.3545	42.77	< 0.0001	14.91 days	5.24 days
	B	5.55	10	y = −0.0331x + 5.2359	46.26	< 0.0001	22.23 days	9.06 days
	C	5.8	10	y = −0.0368x + 5.3713	48.57	< 0.0001	23.68 days	8.15 days
	E	5.55	10	y = −0.0445x + 5.4214	42.71	< 0.0001	20.71 days	6.74 days
	X	5.55	10	y = −0.0426x + 5.1897	38.86	< 0.0001	16.19 days	7.04 days
37	A	5.55	10	y = −0.0590x + 5.2048	49.96	< 0.0001	11.95 days	5.08 days
	B	5.55	10	y = −0.0483x + 5.4318	55.47	< 0.0001	19.29 days	6.21 days
	C	5.8	10	y = −0.0481x + 5.5042	47.36	< 0.0001	20.88 days	6.24 days
	E	5.55	10	y = −0.0610x + 5.4626	52.58	< 0.0001	15.78 days	4.92 days
	X	5.55	10	y = −0.0582x + 5.2936	77.92	< 0.0001	13.64 days	5.15 days
40	A	5.55	9	y = −0.0170x + 5.6290	82.41	< 0.0001	2.76 days	17.65 h
	B	5.55	9	y = −0.0114x + 5.6696	59.07	< 0.0001	4.27 days	26.32 h
	C	5.8	9	y = −0.0111x + 5.8464	80.05	< 0.0001	5.01 days	27.03 h
	E	5.55	9	y = −0.0128x + 5.8345	55.11	< 0.0001	4.34 days	23.44 h
	X	5.55	9	y = −0.0148x + 5.6172	50.74	< 0.0001	3.14 days	20.27 h
45	A	5.55	9	y = −0.0190x + 5.3257	38.54	< 0.0001	1.8 days	15.79 h
	B	5.55	9	y = −0.0149x + 5.7032	66.74	< 0.0001	3.36 days	20.13 h
	C	5.8	9	y = −0.0161x + 5.7456	61.18	< 0.0001	3.22 days	18.63 h
	E	5.55	9	y = −0.0185x + 5.4094	61.07	< 0.0001	2.04 days	16.22 h
	X	5.55	9	y = −0.0201x + 5.4462	46.83	< 0.0001	1.96 days	14.93 h

ND not determined, *NS* not significant

Shelf-life- Time required to reach 4.5 log_{10} $TCID_{50}$ in a 100-dose presentation calculated from the regression equation

Half-life- Time required for loss of half of the original titre, i.e. 0.30 log_{10} $TCID_{50}$ based on degradation constant

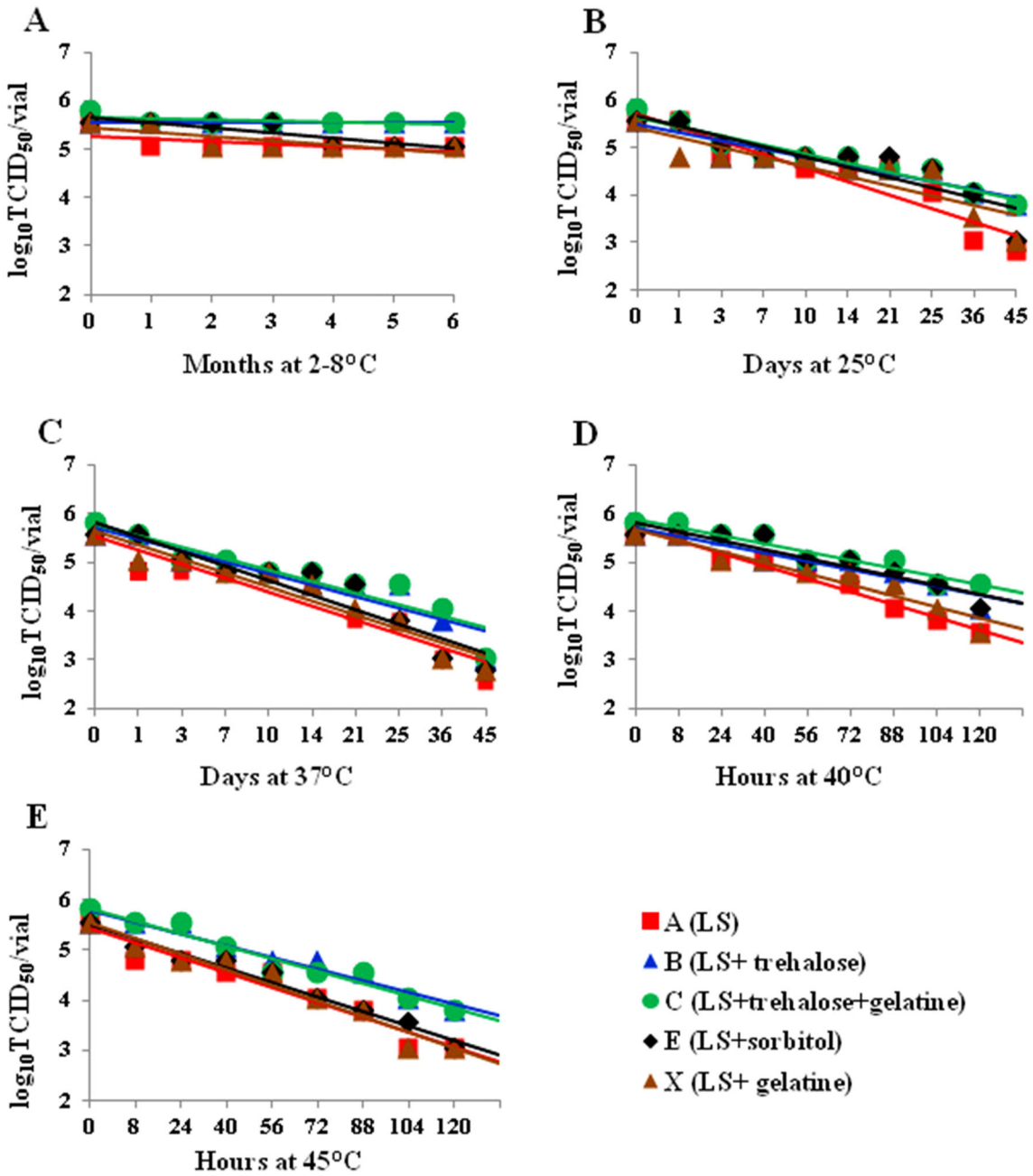

Fig. 2 Degradation curves for lyophilized PPR vaccine stabilized with formulation A, B, C, E and X at different temperature and time points. The vaccine formulations were stored at 2–8 °C (**a**), 25 °C (**b**), 37 °C (**c**), 40 °C (**d**) and 45 °C (**e**), and their infectivity titres were measured as $TCID_{50}$/vial

B and E were superior to C with a shelf-life of 10.13 days. Formulation C performed superior at higher temperatures and maintained a shelf-life of 1.77 days at 37 °C, 22.41 h at 40 °C and 10.05 h at 45 °C. The results of the stability test of reconstituted vaccine at temperatures 2–8 °C, 25 °C, 37 °C, 40 °C and 45 °C are represented in Table 5 and the degradation curve for each stabilizer formulation is illustrated in Fig. 3.

Discussion

The present study focuses to identify stable formulations for PPR vaccine which could withstand hot climatic environment and maintain its stability in regions with poor infrastructure and cold chain maintenance. The work initially identified four different combinations of stable formulations, namely B, C, D and E out of ten (A–J) which conferred improved stability to liquid vaccine exposed at

Table 5 Comparison of degradation values of reconstituted PPR vaccine (Sungri/96) exposed to 2–8 °C, 25 °C, 37 °C, 40 °C and 45 °C at different time points

Temperature (°C)	Stabilizers	Initial titer ($log_{10}TCID_{50}$/vial)	Sample size	Regression equation	Student's t test		Shelf-life (Days/h)	Half-life (Days/h)
					t Ratio	Probability		
2–8	A	5.55	9	y = 0.002 x + 5.218	58.46	< 0.0001	14.95 days	6.25 days
	B	5.55	9	y = 0.0000x + 5.5500	NS	NS	ND	ND
	C	5.8	9	y = 0.0000x + 5.8000	NS	NS	ND	ND
	E	5.55	9	y = 0.0000x + 5.5500	NS	NS	ND	ND
	X	5.55	9	y = 0.0000x + 5.5500	NS	NS	ND	ND
25	A	5.55	9	y = −0.005x + 5.3059	54.48	< 0.0001	6.71 days	2.5 days
	B	5.55	9	y = −0.0049x + 5.6913	48.26	< 0.0001	10.13 days	2.55 days
	C	5.8	9	y = −0.0076x + 5.9848	51.23	< 0.0001	8.14 days	1.64 days
	E	5.55	9	y = −0.0049x + 5.6913	48.26	< 0.0001	10.13 days	2.55 days
	X	5.55	9	y = −0.0073x + 5.6903	66.37	< 0.0001	6.79 days	1.71 days
37	A	5.55	9	y = −0.0353x + 5.4709	74.43	< 0.0001	1.14 days	8.5 h
	B	5.55	9	y = −0.0338x + 5.8351	49.43	< 0.0001	1.64 days	8.87 h
	C	5.8	9	y = −0.0351x + 5.9922	59.30	< 0.0001	1.77 days	8.54 h
	E	5.55	9	y = −0.0371x + 5.9102	36.16	< 0.0001	1.58 days	8.08 h
	X	5.55	9	y = −0.0364x + 5.6481	45.01	< 0.0001	1.31 days	8.24 h
40	A	5.55	9	y = −0.0433x + 5.0326	27.53	< 0.0001	12.24 h	6.92 h
	B	5.55	9	y = −0.0397x + 5.3283	25.22	< 0.0001	20.64 h	7.55 h
	C	5.8	9	y = −0.0407x + 5.4123	23.75	< 0.0001	22.41 h	7.37 h
	E	5.55	9	y = −0.0418x + 5.3115	40.96	< 0.0001	19.41 h	7.17 h
	X	5.55	9	y = −0.0148x + 5.3115	40.96	< 0.0001	19.41 h	7.17 h
45	A	5.55	9	y = −0.0434x + 4.3637	11.07	< 0.0001	3.14 h	6.91 h
	B	5.55	9	y = −0.0434x + 4.8644	21.87	< 0.0001	8.40 h	6.91 h
	C	5.8	9	y = −0.0449x + 4.9514	18.67	< 0.0001	10.05 h	6.68 h
	E	5.55	9	y = −0.045x + 4.7313	14.92	< 0.0001	5.14 h	6.67 h
	X	5.55	9	y = −0.0452x + 4.6616	14.26	< 0.0001	3.58 h	6.64 h

ND not determined, *NS* not significant

Shelf-life- Time required to reach 4.5 log_{10} $TCID_{50}$ in a 100-dose presentation calculated from the regression equation

Half-life- Time required for loss of half of the original titre, i.e. 0.30 log_{10} $TCID_{50}$ based on degradation constant

37 °C as compared to A which was considered as the baseline stabilizer. Evaluation of regression analysis demonstrated that vaccine stabilized with formulation C which consisted of LS, 5% trehalose and 0.5% gelatine showed a superior stability over the rest of the formulations. LS has already been proved as a stabilizer of choice for PPR vaccines as reported in previous studies [14, 22, 23], therefore our attempt was to get a superior combination over and above LS. The use of trehalose (2.5–5%) [14, 23] and gelatine in the range of 0.5–2% [12, 28] have proved as effective vaccine stabilizers in several other live attenuated vaccines. Therefore, a combination of LS-trehalose-gelatine was attempted which significantly improved the stability of liquid vaccine over stabilizer A, consisting of LS alone. Formulation B, a combination of LS and trehalose also offered better thermal protection in terms of shelf-life of liquid PPR vaccine. The combination of LS and sorbitol (Formulation E) offered a shelf-life of 35.97 h at 37 °C and based on the stability conferred by different stabilizers it can be kept next to formulation C and B. Sorbitol is recommended as an effective stabilizer for lyophilization of several viruses such as herpes simplex, adeno, chikungunya, cytomegalovirus, 17D yellow fever vaccine and respiratory syncytial virus [1, 19]. Combination of sorbitol and gelatine has been described in previous reports of stability of rinderpest [15] and measles virus [6].

Effect of repeated freeze-thawing cycles on liquid PPR vaccine was considered as another criteria for quick screening of stabilizer combinations. The results indicated that formulations C, D and E, mostly consisted of trehalose, gelatine and sorbitol, induced a loss of 0.75 log_{10} $TCID_{50}$/

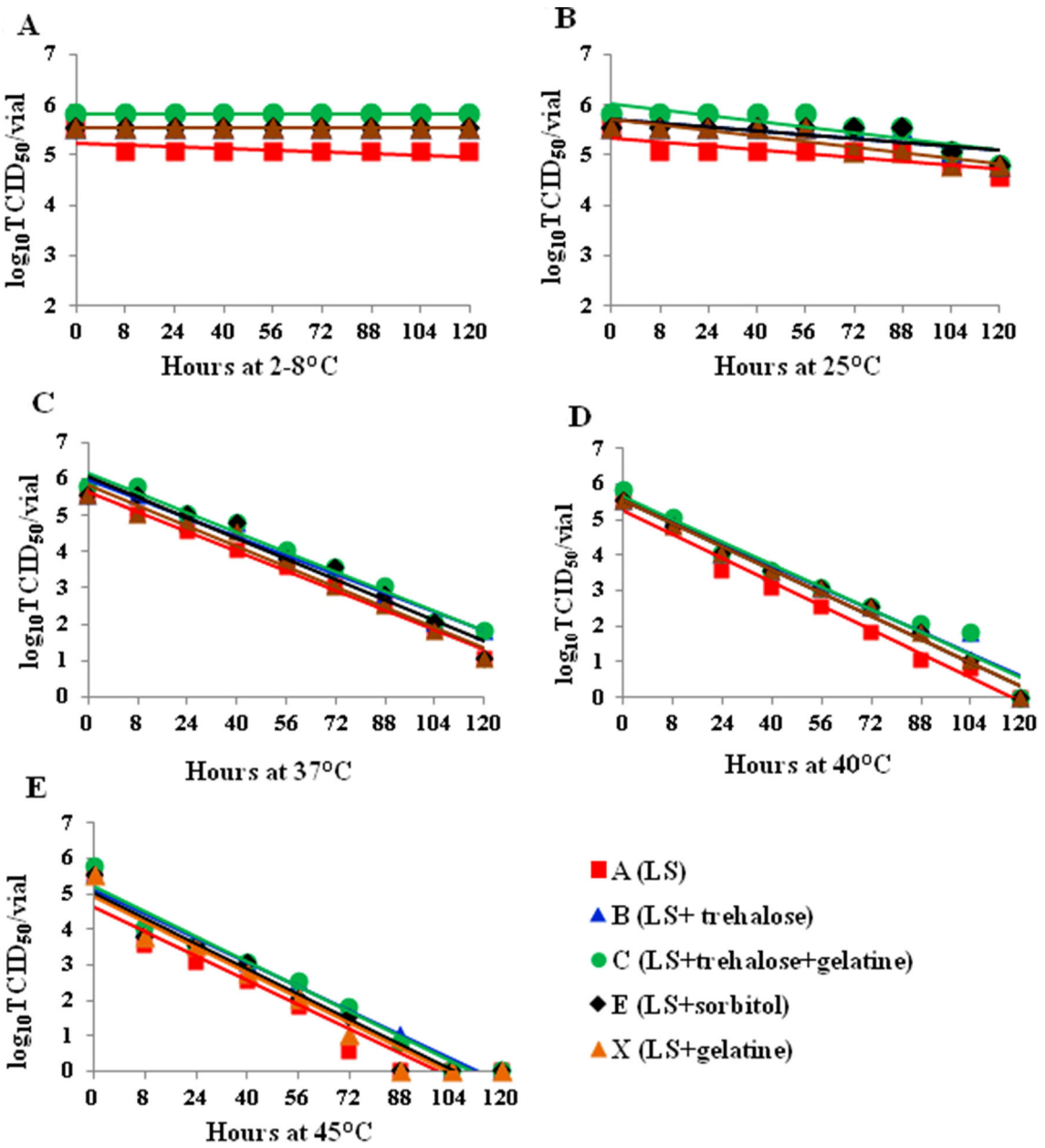

Fig. 3 Degradation curves for reconstituted PPR vaccine stabilized with formulation A, B, C, E and X at different temperature and time points. The vaccine formulations (200 µl/vial) were stored at 2–8 °C (**a**), 25 °C (**b**), 37 °C (**c**), 40 °C (**d**) and 45 °C (**e**) for 120 h and their infectivity titres were measured as $TCID_{50}$/vial

ml and performed better in stabilizing the vaccine at the end of three freeze-thawing cycles. Recent studies have proved trehalose as an effective protein stabilizer which helps to retain its activity in solution as well as in the lyophilized state [13, 16, 27]. Sorbitol and gelatine have been proved as effective cryoprotectants and stabilizing agent of proteins as per previous reports [5, 12, 25]. Overall, the performance of stabilizer formulation C and D in all the freeze–thaw cycles was superior to all combinations and represent significant differences at p 0.05 levels.

During this study we developed a short lyophilization program with less volume of high titred virus that could replace the long conventional protocol of lyophilization in order to upscale PPR vaccines in sufficient quantities without any additional cost. The protocol, designed to be completed in 16 h was developed using 200 µl of vaccine preparation. The short lyophilization cycle optimized using LS as a stabilizer component induced a loss of 0.25 $\log_{10}$ $TCID_{50}$/vial and retained a RM level of 2.88% which was within the acceptable limits (0.5–3%) [29]. Similar losses during lyophilization were obtained with formulation B, E

and X with an exception of C where no loss of titre during lyophilization was observed. The RM contents were observed to be considerably high in previous reports of lyophilization of PPR vaccine using the conventional protocol [22, 23]. It was anticipated that the high moisture levels obtained with the conventional cycle might be due to absence of heating phase during the secondary drying cycle that retained maximum bound moisture. In the present study since low volumes (200 µl) of vaccine have been used, the short lyophilization program successfully reduced the RM levels without the use of heating phase. Therefore, the developed protocol can efficiently be used to upscale the vaccine batches using low volumes of high titred virus. Rapid lyophilization protocols have already been applied on several viruses to support large scale production and supply of vaccines [19].

The thermostability of lyophilized vaccine at 2–8 °C has shown that formulation B and C are undoubtedly superior and statistically highly significant ($p < 0.001$) to the rest of the formulations. The shelf-life of vaccine with formulation B at 2–8 °C could not be interpreted as the regression coefficient was positive indicating that the infectivity titre against time period has an increasing trend. Formulation C has maintained its stability for an extended period of time and resulted in a shelf-life of 1305.3 days. Sarkar et al. reported much longer shelf-life (2051 days) at refrigeration temperature using 5% trehalose and LS as individual stabilizer components [23]. However, the half-life of vaccine virus with stabilizer B was more (9.06 days) than that of formulation C (8.15 days) at 25 °C.

There was a gradual decrease of infectivity titres with respect to all the stabilizers at 25 °C. When the titres were plotted against the exposed time period, the lyophilized vaccine with formulations B, C and E exhibited a biphasic degradation where an initial sharp drop of titre was observed at day 3 indicating rapid initial loss following which a gradual reduction of titre was observed throughout the exposure period. Similar biphasic degradation curves were obtained with lyophilized PPR vaccines stabilized with LS and trehalose as stabilizer component [14]. In contrast, the degradation curves were more likely to be triphasic with formulation A and X where an initial loss was obtained on day 3 followed by a further significant loss by day 25–36 days after which the titre dropped to maximum levels.

The lyophilized vaccines with formulation B and C have superior shelf-lives at 37 °C, which was however contrary to the findings of Mariner et al. where the estimated shelf-lives at 37 °C of trehalose preparation with or without LAH was zero days [14]. Similar biphasic degradation curves were observed with formulations B, C and X indicating a rapid loss followed by a gradual linear decay.

At 40 and 45 °C, the degradation curves exhibited rapid linear decay of infectivity titres at each point of time. The shelf-life for lyophilized vaccine with formulation C was 5.01 days at 40 °C indicating that it could maintain its stability at a higher temperature or during a transient break in the cold chain. At 45 °C, formulation B and C showed superiority over A, E and X maintaining a shelf-life of 3.32 and 3.36 days respectively. Therefore, the formulations B and C enhanced the shelf-lives of lyophilized vaccines from hours to days both at 40 °C and 45 °C which will be useful for any breakage in cold chain during long term storage for mass vaccination camps or remote areas with insufficient infrastructures for vaccine storage. Further, if vaccination is carried out in cold seasons, these stabilizer combinations could be very useful.

The thermostability of reconstituted vaccine was evaluated to determine whether multi-dose reconstituted vaccines could be used over an extended period during consecutive vaccination programs in remote or temporary locations. Storage of reconstituted vaccines for a multidose presentation is a common practice in field due to unavailability of adequate vaccines. Since, no loss of titre were observed with stabilizers B, C, E and X on reconstitution and storage upto 120 h at 2–8 °C, the shelf and half-lives could not be extrapolated from the regression equation. This finding indicates that lyophilized vaccines once reconstituted can be used for the next day and might be stored for a longer period for mass vaccination if stored at refrigeration temperature (2–8 °C). The present study revealed that the reconstituted vaccine with stabilizer B, C and E is capable of withstanding storage at room temperature for at least 8 days. Formulation C maintained a shelf-life of 1.77 days at 37 °C and 22.41 h at 40 °C which was a significant improvement over the currently used stabilizer A. Reconstituted PPR vaccine comprising of formulation C provided a shelf-life of at least 22 h post dilution at 40 °C which will be useful during mass immunization programs where the stringency of maintaining the cold chain is limited. Exposure of vaccines to 45 °C significantly decreases the shelf-life of reconstituted vaccines as also observed in the previous studies [2, 15, 22, 23].

The present study identified a stable formulation for a 100-dose presentation of PPR vaccine consisting of LS in combination of trehalose and gelatin (formulation C). PPR vaccine lyophilized with formulation C is stable with a shelf-life of 1305.3 days at 2–8 °C, 23.68 days at 25 °C and 5.01 days at 40 °C and can be applied successfully in field settings for mass vaccination programs. This shelf-life can be further extended if these are presented at low doses (i.e. 25 or 50 doses/vial) in view of short lyophilization cycles and low volumes (200 µl/vial) of freeze-drying. We also evaluated the stabilizing potentiality of this stabilizer combination on reconstituted vaccine which can be stored

upto 8–10 days at 25 °C under proper storage conditions. This finding may be useful during extended program on vaccination in PPR endemic areas. The miniaturisation of lyophilization protocol which was successfully optimized in the present study, in combination with new combination of stabilizer formulation will provide an opportunity to upscale the existing vaccines without any extra cost.

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Compliance with ethical standards

Conflict of interest The authors declare no conflict of interest.

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