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Effect of chemical stabilizers on pellet profile and stability of lyophilized Peste-Des-Petits ruminants, sheep pox and goat pox vaccines at different temperatures

Mousumi Bora, Sahzad, Sunil Shebannavar, TVS Rao and GS Reddy

Abstract
Lactalbumin hydrolysate-sucrose (LS) as base formulations are commonly used as stabilizers for lyophilization of live viral vaccines. In the present study, the effect of gelatin hydrolysate along with LS (Stabilizer A) on the pellet profile and stability of lyophilized Peste-des-petits ruminants' virus (PPRV) and Sheep pox (SPV) vaccines and gelatin hydrolysate, sodium glutamate and LS (Stabilizer B) on pellet profile and stability of Goatpox vaccine (GPV) have been assessed. Both the stabilizers along with LS as base formulations, were found to be effective with respect to consistence cake appearance, texture and dissolution time of ≤ 10±5 seconds of the lyophilized pellet. The vaccines with modified stabilizers were also tested for their efficacy in stabilizing lyophilized PPRV, SPV and GPV vaccines under real time (5±3°C) and accelerated storage conditions (25±1°C and 36±1°C). Both the stabilizer formulations were found to be effective in extending the shelf-lives of vaccines up to at least 15.82, 21.55 and 13.32 days for PPRV, SPV and GPV vaccines respectively, at 36±1°C. At 25±1°C, SPV vaccine maintained a superior shelf-life (35.65 days) when to compared to PPRV (26.51 days) and GPV vaccine (28.1 days). All the vaccine batches maintained the minimum infectious titre in their lyophilized form (2.5 $\log_{10}CCID_{50}$/dose for PPR and 3 $\log_{10}CCID_{50}$/dose for SPV and GPV) at the end of 6 months of exposure period under real time conditions (5±3°C) thus providing an estimated shelf-life of at-least two years based on regression analysis. Thus, the stability evaluation of vaccines in the present study revealed that these vaccines can be effectively used for mass immunization programs in tropical field situations or during temperature excursion due to cold chain failures by delivering the required recommended dose in animals as they are found to be stable both at ambient as well as higher temperatures.

Keywords: peste-des-petits ruminants, sheep pox, goat pox, lyophilization, thermostability

1. Introduction
Livestock diseases cause heavy losses in terms of economic, trade and food security importance for a considerable number of countries (FAO, 2016) [3]. Even though many infectious diseases are successfully controlled with vaccine, diseases like Peste-des-petits ruminants (PPR) and Capripox viral diseases (Sheep and Goat pox) continues to emerge as important transboundary animal pathogens in recent times resulting in considerable disruption to global livestock trade (Babiuk *et al*., 2008; FAO 2016; Oludairo *et al.*, 2016) [1, 3, 11]. This finding can be correlated with the difficulty to distribute vaccines in remote rural areas, lack of cold chain infrastructure and often to weak thermostability of live attenuated vaccines (Hansen *et al*., 2015; Bora *et al*., 2018) [4, 2]. Vaccine instability during distribution, storage and administration remains a major hurdle in areas with tropical climatic conditions and requires the maintenance of a continuous cold chain to retain its potency (Kumru *et al.*, 2014) [8]. As vaccines employed in developing countries where optimal storage conditions are difficult to maintain, there is a great need to develop methods by which the ability of the vaccine composition to elicit a protective immune response in animals is not diminished over time, or is least diminished as little as possible. The current vaccines against PPR containing Sungri/96 strain is susceptible to thermal degradation (Sarkar *et al*., 2003) [14] and remains a major cause for vaccine failures (Singh and Bandyopadhyay, 2015) [15]. Though Capripox viral vaccines are generally considered to be thermo-resistant, the thermal degradation profile is important when these vaccines are to be used under conditions of high temperature exposure. Therefore, increasing the stability of the existing vaccines against PPR and Capripox viral diseases with improved stabilizer formulation is needed to preserve the adequate quality of vaccine during

storage and after accidental exposure to higher temperatures until the vaccines are administered.

Stabilizer components such as lactalbumin hydrolysate and sucrose (LS) have been widely used for PPR vaccines to preserve the virus activity over an extended period (Sarkar *et al*., 2003; Riyesh *et al*., 2011; Mariner *et al.*, 2017) [14, 13, 9]. However, selection of excipients that significantly improve the vaccine quality remains an important parameter with the increasing demand of quality live attenuated vaccines. Use of gelatin has been described as an effective stabilizer in the preparation of freeze-dried MMR vaccines (WHO, 2014) [18], duck viral hepatitis vaccine (Kang *et al*., 2010) [6] and liquid formulations of influenza vaccines (White *et al*., 2016) [17] thus extending the shelf-life under accelerated temperature conditions. Likewise, in an attempt to enhance the stability of existing PPR and Capripox viral vaccines (Sheep pox and Goat pox), the present study was designed to evaluate the effect of gelatin hydrolysate in combination with LS on the pellet profile and stability of lyophilized PPR, Sheep pox and Goat pox vaccines.

2. Material and Methods

2.1 Cells and Vaccine virus

Vero cells (ATCC®, CCL-81) available at Brilliant Biopharma Private Limited, were revived and routinely sub cultured in Minimum Essential Medium (MEM) with alpha modifications (Sigma Aldrich) supplemented with 10% Fetal Bovine Serum (FBS; Himedia)., MEM with 2% FBS was used as maintenance medium for maintaining the infected cells.

The three vaccine virus strains used in this study were PPRV Sungri/96, Sheep pox virus (SPV Srinagar 38/00) and Goat pox virus (GPV Thiruvarur strain). The vaccine strains stored at -80°C freezer were revived and propagated in Vero cells and titrated before scale up for bulk production and lyophilization.

2.2 Formulation of vaccine stabilizers

Two different stabilizers, A and B were tested in the study. For each stabilizer different concentration of excipients were prepared depending on data found in literature (Sarkar *et al*., 2003; Kang *et al*., 2010) [14, 6]. Stabilizer A consisted of 5.1 % lactalbumin hydrolysate (LAH; BD), 5.05 % sucrose (Himedia) and 0.63% gelatin hydrolysate (Himedia) in water for infection (WFI) and stabilizer B contained 5% LAH, 10% Sucrose, 1% sodium glutamate and 0.63% gelatin hydrolysate. Stabilizer A was used for lyophilization of PPR and Sheep pox vaccine virus whereas, Goat pox vaccine virus was lyophilized using Stabilizer B. The concentration of stabilizers mentioned here indicate the final strength of individual stabilizer in the vaccine preparation.

2.3 Vaccine virus propagation and storage

Vaccine batches containing PPRV, SPV and GPV were prepared for quality and stability of individual vaccine virus. Vero cells were seeded into T-175 cc flask (Nunc, USA) with an average cell density of 2 x 10^7 cells per flask. The seeded cells were co-infected with the respective vaccine viruses at a multiplicity of infection (MOI) of 0.01. The infected flasks were incubated at 36±1°C and harvested when around 70-80 % cytopathic effect (CPE) was evident. The stabilizers were added to the viral harvest and stored at -80°C deep freezer. To maintain uniform virus titre, the infected flasks were pooled and 1 ml of virus-stabilizer mix was dispensed in sterile 2 ml capacity lyophilization vials and partially sealed with vented rubber stoppers and stored overnight at -80°C freezer until lyophilization. One ml of aliquot from the respective vaccine virus batch was subjected to titration in order to estimate the infectious titre of the harvest stock.

2.4 Lyophilization

Lyophilization of vaccine viruses was carried out using an automated bench top freeze-dryer (Labocon, LFD-BT-102) with a total run of 18 hours. The vaccine vials were lyophilized with a vacuum pressure of not more than 150 Millitorr (mTorr) during the drying process. After completion of lyophilization, the vaccine vials were rubber stoppered under vacuum and sealed tightly with an aluminium cap under normal air pressure. Lyophilized vaccine vials from the individual vaccine batches comprising of PPRV, SPV and GPV vaccines were titrated immediately to estimate the loss on lyophilization by reconstituting the vials using 1 ml of diluent containing 0.85% sodium chloride (NaCl).

2.5 Evaluation of pellet profile of lyophilized vaccines

The evaluation of the lyophilized pellets for cake appearance and color, consistency, dissolution time and presence of particulate matter was assessed as an attribute of the manufacturing process. The dissolution time was determined as the time required to obtain a clear solution visually, when 1 ml of diluent was added to the vial containing the lyophilized cake.

2.6 Quality attributes of lyophilized vaccines

The quality of the vaccine viruses lyophilized with different stabilizers were evaluated based on the loss on lyophilization, vacuum packaging and residual moisture (RM) content. Loss on lyophilization was estimated by deducting the virus harvest titres to that of the lyophilized vaccine virus titres. The vaccine vials after lyophilization were tested for presence of vacuum using a vacuum spark tester (B type electric spark vacuum detector, Sanxing). The amount of moisture in the lyophilized vaccine samples was determined by the Karl Fischer compact tiltrotor (Mettler Toledo, V30S).

2.7 Stability of lyophilized vaccine viruses

The vaccine batches of PPRV and SPV lyophilized with Stabilizer A and GPV with Stabilizer B were subjected to real time stability at 5±3°C and accelerated stability test at 25±1°C and 36±1°C. At 5±3°C, the vaccine vials were stored up to 6 months and titrated at monthly intervals. At 25±1°C, the lyophilized vaccines were stored up to 30 days and titrated on day 0, 1, 3, 5, 7, 9, 12, 15, 18, 21, 24, 27 and 30. At 36±1°C, the vaccine vials were stored up to 15 days and titrated on day 0, 1, 3, 5, 7, 9, 12 and 15. The lyophilized vials from respective vaccine virus batches were reconstituted in 100 ml of diluent representing 100 doses of vaccine stored at respective stability temperatures.

2.8 Assay of virus titre

Virus titration was performed on 96 well tissue culture plate using 48 hours old Vero cell monolayer with an initial cell seeding rate of 0.3 x 10^6 cells/well. The lyophilized vaccine vials were reconstituted in 100 ml of sterile diluent considering 100 doses of vaccine. Serial ten-fold dilutions of reconstituted virus suspension were made in maintenance medium and titrated on Vero cell monolayers using four replicates per dilution (100µl/well). The plates were incubated

at 36±1℃ in presence of 5% CO_2 for 6 days with a change of media every alternate day. After observing the PPRV, SPV and GPV specific cytopathic effects on the sixth day, the virus infectivity was quantified by estimating the 50% cell culture infective dose ($CCID_{50}$) and the end points were calculated as per Spearman-Karber method (Karber 1931) [7].

2.9 Statistical analysis

The shelf-lives of the vaccine viruses was estimated by regression analysis of the infectivity titres generated from the real-time and accelerated stability test at different temperature over the exposure period. The shelf-life of PPR vaccines was calculated considering the minimum required infective titre i.e. 2.5 $\log_{10}$ cell culture infective dose ($CCID_{50}$/dose), whereas for SPV and GPV vaccines, the shelf-lives were calculated considering the minimum required titre of 3 $\log_{10}$ $CCID_{50}$/dose (Indian Pharmacopoeia, 2018) [5]. The shelf-lives of all the three vaccine batches were calculated from linear regression analysis assuming a 100-dose pack size (Riyesh *et al.*, 2011) [13].

3. Results

3.1 Evaluation of pellet profile of lyophilized vaccines

Cake appearance, which may or may not be critical with respect to product quality, is however an important attribute during the design and development of a lyophilized product (Patel *et al.*, 2017) [12]. A circular and uniform cake was observed throughout the vaccine batches (Fig. 1). No fragmented detachment of the lyophilized cake was observed when vials were subjected to hand agitation. The dried pellets led to fine granular powder at lower forces. The lyophilized pellet appeared pale yellow to yellow depending on the stabilizer used for lyophilization and was consistent throughout all the batches. The lyophilized pellet was completely solubilized within 10±5 seconds of reconstitution without leaving any visible particulate.

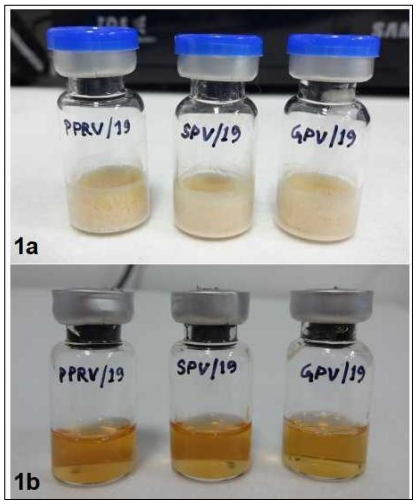

Fig 1(a): Lyophilized PPRV, SPV and GPV vaccine vials showing a circular uniform cake texture (b) Reconstituted PPRV, SPV and GPV vaccine vials completely solubilized within 10±5 sec on reconstitution

3.2 Vaccine quality

The vaccine viruses PPRV and SPV lyophilized with stabilizer A and GPV lyophilized with stabilizer B respectively, were assessed for their quality after lyophilization. The titres of respective vaccine batches and loss on lyophilization were summarized in Table 1. lyophilization of PPR and SPV vaccine using Stabilizer A resulted in loss of 0.25 $\log_{10}$ $CCID_{50}$/vial and 0.75 $\log_{10}$ $CCID_{50}$/vial respectively, whereas GPV vaccine virus stabilized with Stabilizer B induced a lyophilization loss of 0.5 $\log_{10}$ $CCID_{50}$/vial. All the lyophilized vaccine batches were found to have vacuum packaging when tested with a vacuum spark tester. The residual moisture levels in the lyophilized vaccine vials ranged from 1.8-2.2 %, which was within the acceptable limit of 3% as per Indian Pharmacopoeia standards (Indian Pharmacopoeia, 2018) [5].

Table 1: Vaccine quality of lyophilized PPRV, SPV and GPV vaccines

Vaccine batches	Stabilizer	Titre ($\log_{10}$ $CCID_{50}$/vial)		Loss on lyophilization ($\log_{10}$ $CCID_{50}$/vial)	Moisture content (%)
		Before lyophilization	After lyophilization		
PPRV	A	5.75	5.5	0.25	1.8
SPV	A	6	5.25	0.75	2.2
GPV	B	6	5.5	0.5	2.0

3.3 Real time and accelerated stability of lyophilized vaccine viruses

Evaluation of real-time stability was done by storing the lyophilized vaccines at 5±3℃ for 6 months. Based on the experimental data generated from regression analysis it was observed that there was no loss of infectivity titres of PPRV and SPV under real time conditions up to 6 months and hence the shelf-lives could not be estimated. GPV vaccine, which however was stable up to 4 months, showed a subsequent degradation at month 5 providing a shelf-life of 23.82 months. The degradation curves and shelf-lives were represented in Fig. 2 and Table 2 respectively.

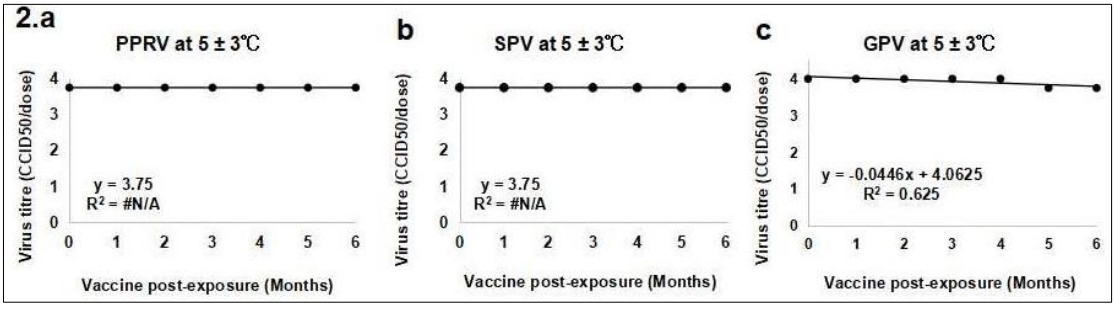

Fig 2: Degradation curves (Regression lines) showing the infectivity titres of lyophilized (a) PPRV (b) SPV and (c) GPV vaccines at different time points under real time storage conditions (5±3℃)

Accelerated stability studies at 25±1℃ and 36±1℃ were performed to assess the degree of degradation obtained at other arbitrarily selected storage conditions (30 days at 25±1℃ and 15 days at 36±1℃) and verify the simulated predictions. Lyophilized PPR vaccine showed a linear decrease in infectivity titres with respect to the exposed time points (Fig. 3a) and maintained a shelf-life of 26.51 days. At similar conditions of storage, SPV and GPV vaccines induces lower degradation with respect to infectivity titres and retained its minimum infectious titre up to 35.65 days and 28.1 days respectively. The degradation curves and shelf-lives were represented in Fig. 3 and Table 2 respectively.

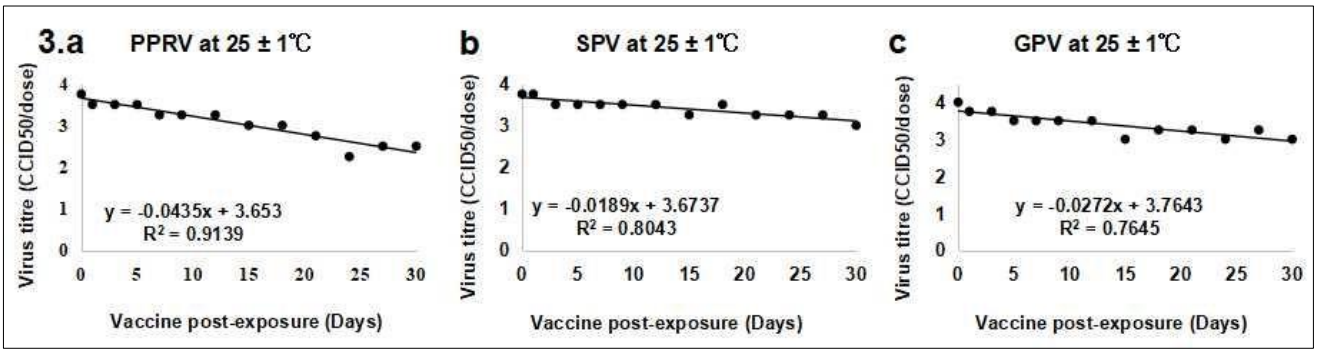

Fig 3: Degradation curves (Regression lines) showing the infectivity titres of lyophilized (a) PPRV (b) SPV and (c) GPV vaccines at different time points at 25±1℃.

At 36 ±1℃, gradual decrease of infectivity titres were observed for all the vaccine viruses. PPRV induced a rapid initial decrease followed by a more gradual loss of infectivity (Fig. 4a). Lyophilized SPV and GPV vaccine maintained an infectivity titre of 3.25 and 3 $\log_{10}$ $CCID_{50}$ /dose at the end of the exposure period at 36 ±1℃. The estimated shelf-lives at 36±1℃ for PPRV, SPV and GPV vaccines were 15.82, 21.55 and 13.31 days respectively. The degradation curves and shelf-lives were represented in Fig. 4 and Table 2 respectively.

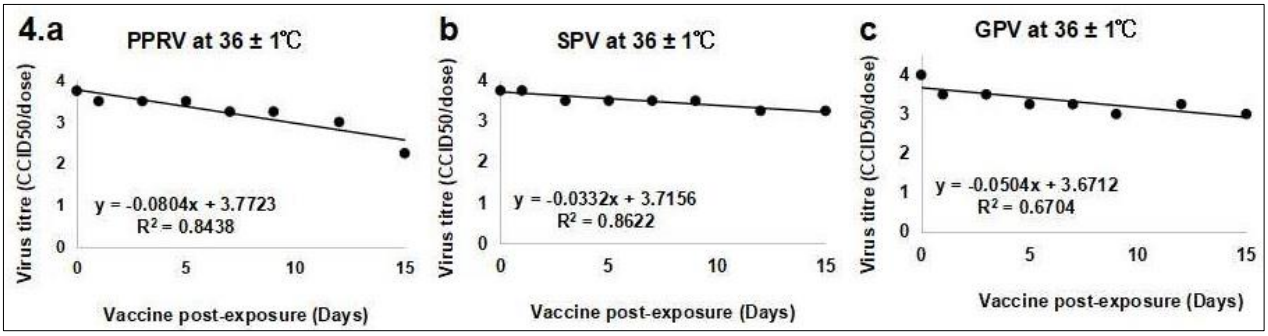

Fig 4: Degradation curves (Regression lines) showing the infectivity titres of lyophilized (a) PPRV (b) SPV and (c) GPV vaccines at different time points at 36±1℃.

Table 2: Shelf-lives of lyophilized PPRV, SPV and GPV vaccines after reconstitution with 100 ml of diluent and storage at temperatures 5 ± 3℃, 25 ±1℃ and 36 ±1℃

Vaccine batches	Temperatur (℃)	Sample size	Regression equation	R^2 value	Shelf-lives (months/days)
PPRV	5 ± 3	7	0x + 3.75	NA	NS
	25 ±1	13	-0.0435x + 3.653	0.9139	26.51 days
	36 ±1	8	-0.0804x + 3.7723	0.8438	15.82 days
SPV	5 ± 3	7	0x + 3.75	NA	NS
	25 ±1	13	-0.0189x + 3.6737	0.8043	35.65 days
	36 ±1	8	-0.0332x + 3.7156	0.8622	21.55 days
GPV	5 ± 3	7	-0.0446x + 4.0625	0.625	23.82 months
	25 ±1	13	-0.0272x + 3.7643	0.7645	28.1 days
	36 ±1	8	-0.0504x + 3.6712	0.6704	13.32 days

NA= not applicable; NS= non-significant

4. Discussion

Live attenuated vaccines are more heat sensitive to potency loss during storage and distribution and is one of the major constraint in control of viral diseases (Riyesh *et al*., 2011) [13]. For stability perspectives, these vaccines necessitate presence of improved excipient formulations to provide sufficient stability during long term storage (Kumru *et al*., 2014) [8]. The present study provided an insight in thermo-stability of lyophilized PPRV, SPV and GPV vaccines under real time and accelerated temperature conditions. Herein, we evaluated the importance of product formulation and lyophilization process for consistent pellet profile, vaccine quality and stability.

The lyophilized vaccines were evaluated for their pellet profile which includes cake appearance, texture, dissolution time and presence of particulate matter in the reconstituted cake or pellet. In the present study, an uniform and consistent lyophilized cake was observed throughout all the vaccine

batches which was readily dissolved without leaving any undissolved matter. A non-ideal cake appearance is a visual indicator of a poor formulation, a process that is not under proper control or a poor drug product presentation (Patel *et al*., 2017) [12]. The dry and easily reconstituted cake thus can be correlated with the selection of an improved stabilizer formulation and an optimum lyophilization condition.

The quality of the vaccine viruses lyophilized with stabilizer A and B were evaluated based on the loss on lyophilization, vacuum packaging and RM content. It was observed that all the vaccine batches lost virus in the range of 0.25-0.75 $\log_{10}$ $CCID_{50}$/vial under similar lyophilization conditions. The lyophilization loss could be due to the impact of sublimation process of the freeze dryer. The previous studies on lyophilization of PPR vaccine virus reported a loss of around 0.15 to 0.3 $\log_{10}$ $CCID_{50}$/vial (Riyesh *et al*., 2011) [13], whereas Sarkar *et al.* reported a loss of 1.04 $\log_{10}$ $CCID_{50}$/vial with lactalbumin hydrolysate-sucrose (LS) stabilizer which is commonly used for lyophilization of PPR vaccines (Sarkar *et al*., 2003) [14]. The change in RM levels during the storage period is an additional parameter which may influence the infectivity titre (May, 2003). According to the Indian Pharmacopoeia (IP 2018) [5], lyophilized vaccine vial should not contain RM content of more than 3%. In the present study, the RM levels in the lyophilized vaccine vials ranged between 1.8-2.2%, which was within the acceptable limit of 3% as per IP standards, indicating sufficient drying using the controlled freeze-drying parameters. In addition, the key to increasing product shelf-life is determining the ideal atmosphere within the packaging for the product being packaged (Patel *et al*., 2017) [12]. All the lyophilized vaccine batches were found to have vacuum packaging indicating the efficiency of the lyophilization process.

To determine the stability of lyophilized PPRV, SPV and GPV vaccines, the vaccine viruses were evaluated based on their shelf-life under real time and at accelerated temperature conditions. Storage of vaccine vials up to 6 months at 5±3°C did not impact the infectivity level of PPRV and SPV retaining the initial titre throughout the storage period. Since the regression coefficient for PPRV and SPV at 5±3°C was positive, the shelf-lives could not be estimated indicating that the infective titre against time period has an increasing trend. GPV vaccines provided a shelf-life of 23.82 months, and thus can be estimated that these vaccines would provide a shelf-life of at-least 2 years under real time conditions. At 25±1°C, all the lyophilized vaccine batches maintained its shelf-life up to at-least 25 days. Our results are in support of previous report on thermo-stability of thermo-adapted PPRV strains PPR Revati/2006 and PPR Jhansi/2003, where both the strains maintained a shelf-life of 22-25 days on storage at 25±1°C. The degradation with the lyophilized vaccine virus looked more pronounced at elevated temperature, except for SPV at 36±1°C. No significant loss of infectivity titre was observed with SPV when compared to PPRV and GPV, making it the more thermo-stable variant by maintaining a shelf-life of 21.55 days. The lower shelf-life (13.32 days) obtained with GPV might be due to initial decrease of titres on exposure to 36±1°C. The lyophilized PPR vaccine stability profile seems to be promising having retention of adequate infective titres even after 10 days of the exposure period at 36±1°C. Previous studies on PPRV containing LS as a stabilizer component reported a shelf-life of not more than 7 days using the conventional freeze-drying technique (Sarkar *et al*., 2003; Riyesh *et al*., 2011) [14, 13]. In our study, all the lyophilized vaccine viruses were found to be stable at higher temperatures which could be related with the use of gelatin hydrolysate as an additional component to the conventional formulation. Gelatin and gelatin derivatives such as hydrolyzed gelatin have been successfully used as stabilizers in a number of vaccine compositions. The ability of gelatin to form a thermo-reversible gel and low immunogenicity makes it a preferred vaccine stabilizer and ensures longer shelf-life of vaccines (Vaccines and porcine gelatin, 2018) [16].

5. Conclusion

To successfully carry out global immunization programs, ensuring the stability of vaccines is crucial. In this context, predicting vaccine stability and preventing product damage due to excessive temperature excursions outside of the recommended storage conditions is crucial. In the present study, lyophilized PPRV, SPV and GPV vaccines are evaluated for their pellet profile and stability at 5±3°C, 25±1°C and 36±1°C. Gelatin hydrolysate as an additive in both the stabilizer formulations (A&B) containing LS as base formulation was found to be effective by evaluation of the visual attributes and vaccine quality of the lyophilized pellets. All the lyophilized vaccine batches tested in the present study were found to be stable without significant loss in infectivity titres under real time storage conditions (5±3°C) and maintained a shelf-life of at-least 10 days at 36±1°C. Therefore, the vaccines tested in the present study can be used effectively during mass immunization programs and would be stable during temperature excursions at hot climatic regions or in areas where the stringency of maintaining the cold chain is limited.

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