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RESEARCH ARTICLE

Effect of Stabilizers on Infectivity Titer of Freeze Dried Peste Des Petits Ruminants Virus Vaccine

Muhammad Zubair Latif[1], Khushi Muhammad[1], Riaz Hussain[2]*, Faisal Siddique[1], Imran Altaf[1], Muhammad Anees[3], Muhammad Imran[4], Muddassar Hameed[1] and Muhammad Farooq[1]

[1]Department of Microbiology, University of Veterinary and Animal Sciences (UVAS), Lahore, Pakistan; [2]University College of Veterinary and Animal Science, The Islamia University of Bahawalpur, Pakistan; [3]Veterinary Research Institute, Lahore, Pakistan; [4]Institute of Biochemistry and Biotechnology, UVAS, Lahore, Pakistan
*Corresponding author: driazhussain@yahoo.com

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ABSTRACT

Peste des Petits Ruminants (PPR) is an acute and highly contagious viral disease of small ruminants caused by Morbilivirus. PPR induces high morbidity and mortality in infected animals and is responsible for huge economic losses to livestock holders and dairy industry. Live attenuated freeze-dried vaccines are commonly used to control the infectious diseases. There is loss of virus titer during freeze drying and post reconstitution of the vaccine resulting in the decrease efficacy of vaccine. Stabilizers are therefore added to protect the virus from freeze drying stress and heat shock during storage and transportation. Therefore, in present study different stabilizers (Weybridge medium-WBM, Lactalbumin hydrolysate sucrose-LS, Lactalbumin hydrolysate sorbitol-LSbG, TrisTrehalose-TT, Tris sucrose-TS and Goat skimmed milk-GSM) were evaluated to protect the infectivity titer of the virus. The stabilizers having carbohydrates (sucrose, sorbitol and trehalose), salts (sodium glutamate) and hydrolyzed proteins (Lactalbumin hydrolysate) are effective to make compact mass of PPR virus vaccine. Casein or other proteins (not hydrolyzed) in stabilizers may act as allergens and could cause anaphylactic shock in the vaccinated animals. However, TS did not make compact mass (cake) of vaccine but all the contents of the virus suspension evaporated while WBM, LS, GSM and LSbG maintained infectivity titer of the vaccine virus (if reconstituted with PBS and stored at 4°C) for 12 hours. However, Tris Trehalose-TT is able to protect infectivity titer of the vaccine virus during freeze drying and even during its storage after rehydration with PBS at 4°C for 24 hours.

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INTRODUCTION

Livestock sector has a major role in the economy of Pakistan because it contributes about 11.8% to the national GDP and more than 56.3% to the agriculture. About 50 to 80 million Pakistani populations is residing in villages and most of the people (8 million) of Pakistan are directly involve in rearing of different animals such as sheep, goats, cattle and buffaloes for their livelihood (Hussain *et al.*, 2013). In Pakistan, the population of small ruminants particularly sheep and goats is increasing at a rate of more than 3% per annum which provide good quality meat, milk and leather (Hussain *et al.*, 2012). At international level, the livestock sector contributes 40% to the gross agriculture domestic product (Rehman *et al.*, 2017).

PPR is a contagious, viral disease of sheep and goats. The disease is listed as a transboundary disease and is characterized by high morbidity and mortality, erosions in the oral cavity, conjunctivitis, catarrhal inflammation in nasal and ocular mucosa, pneumonia and gastroenteritis (Munir *et al.*, 2013; Abubakar and Munir, 2014; Khan *et al.*, 2018). Severity of the disease is more in goats than in the sheep but some studies show equal susceptibility of both the species (Wang *et al.*, 2009; Khan *et al.*, 2018). The disease is incriminated to be cause of economic losses worth 342.15 million US dollars annually worldwide. PPR is endemic in some regions of Asia and African

countries. In Pakistan, PPR has been documented since 1991; however, the disease outbreaks in Punjab province were confirmed in 1994. Afterwards, PPR epidemics have been reported from time to time in several districts of the Punjab (Jalees *et al.,* 2013; Ullah *et al.,* 2015). It causes immunosuppression as shown by lymphopenia in the infected goats (Rajak *et al.,* 2005). The disease is caused by PPR virus (member of genus Morbillivirus of Paramyxoviridae). It is pleomorphic, with negative sense ssRNA that encodes two non-structural and six structural proteins. There is only one serotype of PPR virus but its virulence varies among strains in different geographic locations of world. Nucleotide sequence of each virus strain and genetic makeup of the host species are presumably important factors for virulence of the virus (Couacy-Hymann *et al.,* 2007). Virus is subdivided into four lineages on the basis of the partial sequence homology of fusion and haemagglutinin genes (Couacy-Hymann *et al.,* 2007). The live attenuated PPR virus vaccine is used to immunize the sheep and goats. Mass vaccination is the most effective preventive strategy for the disease control. In Pakistan, the virus vaccine is produced using 75/I strain of the virus (Abbas *et al.,* 2012). The vaccine is thermolabile and requires maintenance of cold chain supply to preserve the virus infectivity which is vital for such vaccine to be effective. During freeze drying and after dilution of freeze dried vaccine there is loss of virus titer resulting to poor efficacy of vaccine. Stabilizers are therefore added to protect from freeze drying stress and heat shock during storage and transportation. Various proteins (lactalbumin-hydrolysate, gelatin etc.), carbohydrates (sucrose, trehalose, sorbitol etc.), salts (sodium glutamate, potassium phosphates etc.) and amino acids (histidine, alanine etc.) are used as stabilizers for lyophilization of various microbial vaccines (Kang *et al.,* 2010). If freeze drying of Japanese encephalitis vaccine virus is done without addition of any stabilizers, then there is loss of 90% of the antigenicity. The present study was therefore designed to evaluate ability of different stabilizers to protect the infectivity titer of the virus during freeze drying and post dilution storage.

MATERIALS AND METHODS

Maintenance and propagation of Vero cell: The Vero cell line (cryovial) obtained from Veterinary Research Institute, Lahore, was processed for thawing and preparation of monolayer in T-25 cm^2 (Orange scientific, Braine-l'Alleud, Belgium) containing Dulbecco Modified Essential Medium (Caisson Laboratories Inc., North Logan, USA). Ultimately one flask of the cell line monolayer was split into five cell culture flasks (Ammerman *et al.*, 2008).

Cultivation of PPR virus: Live attenuated PPRV Nigeria 75/1, strain obtained from VRI Lahore was used to give infection each of the five T-25 cm^2 cell culture flasks. Each of the flasks was incubated in the humidified incubator at 37°C temperature and 5% concentration of CO_2 for 5 days and examined under inverted microscope daily for development of cytopathic effect (CPE). The virus thus harvested was stored at a -70°C temperature until required for further processing (Anees *et al.,* 2014).

Vaccine preparation: The virus infectivity titer was calculated as mean TCID50 (Reed and Muench, 1938) and processed for development of vaccines with different stabilizers. For development of vaccine 0.5ml virus suspension (106.17 units of TCID50/ml) was mixed with 0.5ml of either of the sterile stabilizers (Weybridge medium-WBM; 2.5% Lactalbumin hydrolysate, 5% sucrose, 1% monosodium glutamate, LS; 5% Lactalbumin hydrolysate, 10% sucrose, LSbG; 2.5% Lactalbumin hydrolysate, 5% sorbitol, 1% monosodium glutamate, TS; 20 mM Tris HCl, 2 mM EDTA, 0.02% (w/v) Tween 80, 1 M sucrose, TT; 20 mM Tris HCl, 2 mM EDTA, 0.02% (w/v) Tween 80, 1M Trehalose and GSM; Goat skimmed milk). Each of the vaccine virus suspension was processed for 2 hours for pre-freeze drying and for 48 hours for freeze drying (Silva *et al.,* 2011).

Effect of stabilizers on biological titre of PPRV: Each of the vaccine (n=3 vials) was diluted with the PBS (7.2 pH) and stored at 4°C, 25°C and 37°C. Infectivity titer of the virus in each of the vaccines was determined on Vero cell lines at zero, 12, 24 and 36 hours post storage using MTT assay. The optical density (OD) value of all wells were recorded by using a multi-well ELISA reader (Multiskan EX, Electron Corporation, Finland) at 570 nm wavelength (Twentyman and Luscombe, 1987). Following formula was used for the calculation of cell survival percentage (CSP).

CSP = (Mean OD of sample − Mean OD of blank) / Mean OD of cell control – Mean OD of blank × 100

Cell inhibition or CPE percentage = 100 - CSP
Then PD 50 was calculated and finally mean TCID50 of virus of vaccine was calculated.

Statistical analysis: The results of the experiments were in form of infectivity titer/tissue culture infectious dose 50 $TCID_{50}$. Mean values of the infectivity titer of each of the vaccine formulation was analyzed with statistical analysis by one-way ANOVA at level of significance 0.05 with the help of SPSS (Statistical Package for the Social Sciences) software 22.0 version.

RESULTS

In the present research**,** stabilizer like goat skimmed milk (GSM) produced maximum infectivity titer of PPR vaccine followed by Tristrehalose-TT, lactalbumin sucrose-LS, WyeBridge medium WBM and lactalbumin sorbitol-LSbG (Fig. 1). Results indicated that at different post storage freeze dried vaccine temperature including 4°C, 25°C and 37°C there were no statistically significant difference in reduction of infectivity titer of the virus either used of the GSM, TT, LS and WBM stabilizers. Results revealed that among different stabilizers, LS showed significant reduction in infectivity titer of the virus if the vaccine was used after 12 and 24 h post storage at all different temperatures (Fig. 1) when compared to other stabilizers. Results showed that the infectivity titer of vaccine with sorbitol-LSbG stabilizer was significantly decreased after 24 h of post storage freeze dried at 4°C and 25°C, while decreased infectivity

titer of vaccine with sorbitol- WBM and LSbG stabilizers after post storage freeze dried at 37°C. After 36 h post storage, the infectivity titer of vaccine with LS and LSbG stabilizers significantly decreased as compared to all other stabilizers at 4°C while at 24°C and 37°C, the infectivity titer of vaccine with all stabilizers except TT. Results showed that each of the stabilizers such as TT, LS, WBM, GSM, and LSbG made compact mass (cake) of the vaccine in the respective vials so proved as an effective preservative for the virus during freeze drying process except Tris sucrose (TS) that did not make any compact mass in the vial, the solute was evaporated and proper cake of the vaccine was not made. Each of the stabilizers (WBM, LS and LSbG) showed minimum reduction (less than $\log_{10}$ 0.32) of infectivity titer of the freeze dried PPRV vaccine (when reconstituted with PBS-pH 7.2 and stored at 4°C) for 12 h while there was significantly high reduction of infectivity titer of the PPR virus vaccine (if reconstituted with PBS and stored at different temperature for more than 12 h (Fig.1). Tris-Trehalose did not induce any significant reduction of infectivity of the freeze dried PPR vaccine virus (when reconstituted the PBS and stored at 4°C) up to 24 hours. However, there was significant reduction in infectivity titer of the hydrated PPR vaccine virus during its storage at 4°C, 25°C and 37°C temperature.

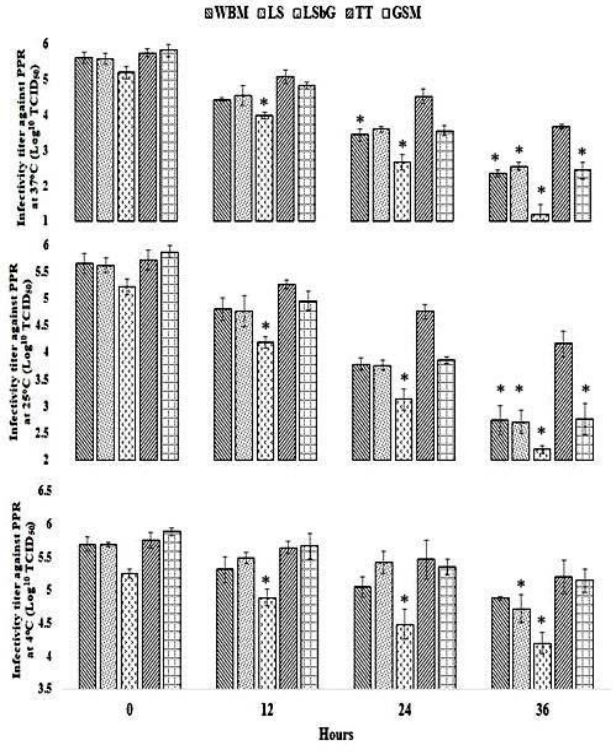

Fig. 1: Effect of different stabilizers i.e. Weybridge medium (WBM), Lactalbuminhydrolysate sucrose (LS), Lactalbuminhydrolysate sorbitol (LSbG), Tristrehalose (TT) and Goat skimmed milk (GSM) on infectivity titer against PPR vaccine at various temperature and time periods.

DISCUSSION

Peste des Petits Ruminants Virus (PPRV) vaccine contains live attenuated virus that is thermo-sensitive (Kumar *et al.*, 2014). Freeze drying of such virus vaccines along with some suitable stabilizers is common practice to maintain biological titer of the virus. Dehydration and freezing of vaccines without stabilizer cause drastic conformational changes in the virus. Such changes in the virus are not revived upon reconstitution of the vaccine but can be mitigated by addition of stabilizers. Sugars make hydrogen bonding with polar residues of the bio-molecules and lower the nucleation temperature of the water molecules on surface of the virus capsid and prevent large ice crystal formation between the virus and the external medium. A mixture of sugar and protein is denser as compared to sugar or protein when used alone. Various proteins, carbohydrates, salts and amino acids are used as stabilizers for lyophilization of various microbial vaccines. It is established that without addition of stabilizers, the antigenicity of vaccine is reduced up to 90% during freeze drying conditions (Kang *et al.*, 2010).

In live attenuated virus vaccine, the vaccine virus is attenuated and can multiply in cells of the organs that are not essential for life such as epithelial cells of intestine which are desquamated and are replaced rapidly with newly developing underline cells. In this way the vaccine virus continues to propagate and maintain its retention in the body like Newcastle disease virus, Rotaviruses, Calci viruses, Influenza virus and Respiratory syncytial virus, Simian immunodeficiency virus, Bovine viral diarrhea virus types 1 and 2, Infectious bovine rhinotracheitis virus, Bovine parainfluenza-3 virus, and Bovine respiratory syncytial virus and HIV/AIDS (Xue *et al.*, 2010). The retention of virus in the body is essential to induce long lasting immunity. Longer the virus retains in the host, higher will be the titer of the specific antibody (Anees *et al.*, 2014). Inactivated virus alone does not multiply in the vaccinated animals so is degraded and excreted out from the body without inducing any immunity. In such vaccines, the microorganisms are made water insoluble by adding some chemicals such as oil that encapsulates the immunogen, gel that adsorbs the microbes and alum that precipitates the vaccinal microbes (Awate *et al.*, 2013).

The TS stabilizer disqualified its ability to maintain the viability/stability of the virus. Skimmed milk at concentration of 2.5% in microbial suspension is the optimum level resulting in minimal percent reduction in Salmonella cell viability (18.9%) after exposure to the defined heat stress. Moreover, skimmed milk gives best thermal stabilization of live attenuated *Salmonella enteritidis* vaccine.

GSM contains proteins (lactalbumin, lactglobulins, casein, lactoferrin), lactose and minerals. The albumin and globulins of GSM may be recognized as self-antigen in the goat so do not induce any untoward reaction while source of milk casein is not liver, but is milk acini (Khokha and Werb, 2011), so it may act as sequestered antigen in the animal vaccinated 1st time. The primed goat when boosted with the same vaccine may show signs of anaphylactic shock. Addition of GSM is therefore not suggested to be used as stabilizer in the freeze-dried vaccines. Skimmed milk of any source can be added in the vaccines as stabilizers which are administered orally or through spray method. Skim milk is therefore routinely added as stabilizer in freeze dried orally administered poultry vaccines. Proteins if essential to be added in the vaccine for freeze drying, may be hydrolyzed so as to

make it non-antigenic such as hydrolyzed lactalbumin is an essential component of WBM, LS and LSbG (Ibrahim *et al.,* 2004).

Tris-Trehalose as stabilizer maintained infectivity titer of the virus with minimum loss during freeze drying process. Trehalose is a naturally occurring disaccharide sugar that protects living cells from drying. It forms gel between cells and environment so protects their normal structure and functioning. It is used effectively for freeze drying of thermo labile viruses as trehalose form a foamy glass which can protect virus in dehydrated state up to 14 days at 45°C. It is an effective stabilizer for mumps vaccine (Jamil *et al.,* 2014), influenza virus vaccine (Murugappan *et al.,* 2013) and PPRV vaccine (Silva *et al.,* 2011). Trehalose (TT), sucrose (LS, WBM) and sorbitol (LSbG) are carbohydrates while Tween 80-(TT) is lipid molecule. These molecules are T cell independent antigens and are not presented on surface of antigen presenting cells (APCs) such as macrophages, B cells, dendritic cells etc., along with their MHC-II antigen (Iyer *et al.,* 2012). Hence, specific Th2 cells can't recognize such antigens (allergens), unable to undergo process of blast formation, proliferation and differentiation into effector cells for production of cytokines including IL-4 that are cellular and molecular basis for induction of hypersensitivity type 1 reactions in the vaccinated hosts (Chaplin, 2010). Sodium glutamate (WBM/LSbG) and EDTA and Tris buffer (TT) are salts which are non-antigenic molecule so is safe to use as stabilizers in freeze dried injectable vaccines without any risk of hypersensitivity reactions.

PPR virus is reconstituted in phosphate buffered saline solution (pH 7.2), normal saline, magnesium sulphate, magnesium chloride or distilled water before injection to the sheep/goat (Riyesh *et al.,* 2011; Anees *et al.,* 2014). Normal saline, distilled water, glucose-saline or other pharmaceutical injectable saline preparations usually have low pH (some time below 5.0). The low pH might be due to incorporation of carbon dioxide from environment and formation of carbonic acid in the injectable. The pharmaceutical preparations are not toxic for human or animals because of buffering capacity of the blood (Turner *et al.,* 2011) but may be detrimental for the freeze-dried vaccine virus sensitive to extreme pH. The freeze-dried vaccine containing PPR virus is sensitive to pH so was hydrated with phosphate buffered saline (pH 7.2). However, other diluents such as distilled water or normal saline may be utilized in the cases when the vaccine viruses show resistance to wide range of pH. The PPR virus is thermo-sensitive (Kumar*et al.,* 2014) so more the temperature of the virus storage after hydration, higher is the reduction of the infectivity titer of the virus. The freeze-dried vaccine when rehydrated in drinking water for oral administration, infectivity titer of the virus in the vaccine starts declining. Reconstitution of freeze dried vaccine in tap water causes 90% loss in the infectivity titer of the NDV. The addition of 0.25% skimmed milk in drinking water (tap water) and distilled water minimize the rate of virus inactivation at 37°C.

TT stabilizer maintains the infectivity titer of the PPR virus of the PBS diluted freeze-dried vaccine (stored at 37°C) for 4 hours (Silva *et al.,* 2011). The trehalose along with other amino acids maintains the stability of PPR vaccine virus in reconstituted form with 0.85% sodium chloride for 48 h both at 4°C and 25°C and for 24-30 h at 37°C (Riyesh *et al.,* 2011). The freeze dried PPR virus vaccine with trehalose as stabilizer is an effective vaccine for the control of the disease in the countries with minimum failure of immunoprophylaxis on account of breakdown in cold-chain maintenance. Thermo-tolerance of virus in freeze dried vaccine has an additive effect on its shelf life even after reconstitution with PBS. Trehalose maintains infectivity titer of camel pox virus in rehydrated vaccine more than the minimum effective dose ($log10^3$.0 units of $TCID_{50}$) with a maximum titer of $log10^6$.53 $TCID_{50}$ for 60 h at 37°C and 45°C (Prabhu *et al.,* 2014). Trehalose dihydrate can maintain virus titer at 4°C for 45 days, at 25°C for 15-19 days and at 37°C for 1-2 days. Tris-firoin formulation of pH 8.0 has been proved better for adenoviral vectors for storage upto 1 year (Cruz *et al.,* 2006). When different microbes were freeze-dried with 100mM trehalose as stabilizer, 57% of the *B. thuringiensis* and 70% of the *E. coli* survival occurred as compared to sucrose where 44 and 56%, survived respectively (Leslie *et al.,* 1995).

Conclusions: Our study concluded that stabilizers having carbohydrates (sucrose, sorbitol, trehalose), salts (sodium) and hydrolyzed proteins (lactalbumin hydrolysates) are effective to make compact mass (freeze drying) of PPR virus vaccine. WBM, LS and LSbG maintain infectivity of the PPR virus vaccine (if reconstitution with PBS and stored at 4°C) for 12 hours. However, TT is able to protect infectivity titer of the PPR virus vaccine during freeze drying and even during its storage after hydration with PBS at 4°C for 24 hours.

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Authors contribution: KM, MZL and FS planned and conducted the research; RH, MA and MF analyzed the data; RH, FS, MH and MF finalized the article.

REFERENCES

Abbas F, Ullah A, Awan MA, *et al.,* 2012. Production of tissue culture based peste des petits ruminants (PPR) vaccine at CASVAB, Quetta, Pakistan. Pak J Life Soc Sci 10:80-3.

Abubakar M and Munir M, 2014. Peste des petits ruminants virus: An emerging threat to goat farming in Pakistan. Transbound Emerg Dis 61:7-10.

Ammerman NC, Beier SM and Azad AF, 2008. Growth and maintenance of Vero cell lines. Curr Protoc Microbiol 4:1-7.

Anees M, Muhammad K, Rabbani M, *et al.,* 2014. In process quality control factors effecting potency of peste des petits ruminants virus vaccines in goats. J Anim Plant Sci 24:749-54.

Awate S, Babiuk LAB and Mutwiri G, 2013. Mechanisms of action of adjuvants. Front Immunol 4:114-20.

Chaplin DD, 2010. Overview of the immune response. J Allergy Clin Immunol 125:3-23.

Couacy-Hymann E, Bodjo S, Danho T, *et al.,* 2007. Early detection of viral excretion from experimentally infected goats with peste-des-petits ruminant's virus. Prev Vet Med 78:85-8.

Cruz PE, Silva AC, Roldão A, *et al.,* 2006. Screening of novel excipients for improving the stability of retroviral and adenoviral vectors. Biotechnol Progr 22:568-76.

Hussain R, Auon M, Khan A, *et al.,* 2012. Contagious caprine pleuro-pneumonia in Beetal goats. Trop Anim Health Prod 44:477-81.

Hussain R, Javed MT, Khan A, *et al.,* 2013. Risks factors associated with subclinical mastitis in water buffaloes in Pakistan. Trop Anim Health Prod 45:1723-9.

Ibrahim HR, Taniyama N and Aoki T, 2004. Distinct dimerization between α-lactalbumin and lysozyme exhibiting novel antimicrobial activity against Gram-positive and Gram-negative bacteria. Letters Drug Design Disc 1:101-9.

Jalees MM, Hussain I, Arshad M, *et al.,* 2013. Occurrence of peste des petitis ruminants in five districts of Punjab, Pakistan. Pak Vet J 33:165-9.

Jamil RK, Taqavian M, Sadigh ZA, *et al.,* 2014. Evaluation of the thermal stability of a novel strain of live-attenuated mumps vaccine (RS-12 strain) lyophilized in different stabilizers. J Virol Methods 199:35-8.

Kang M, Jang H, Kim M, *et al.,* 2010. Development of a stabilizer for lyophilization of an attenuated duck viral hepatitis vaccine. Poult Sci 89:1167-70.

Khan A, Saleemi MK, Ali F, *et al.,* 2018. Pathophysiology of peste des petits ruminants in sheep (Dorper & Kajli) and goats (Boer & Beetal). Microb Pathogen 117:139-47.

Khokha R and Werb Z, 2011. Mammary gland reprogramming: metalloproteinases couple form with function. Cold Spring Harb Perspect Biol 3:4333.

Kumar N, Maherchandani S, Kashyap SK, *et al.,* 2014. Peste des petits ruminant's virus infection of small ruminants: a comprehensive review. Viruses 6:2287-327.

Leslie SB, Israeli E, Lighthart B, *et al.,* 1995. Trehalose and sucrose protect both membranes and proteins in intact bacteria during drying. Appl Environ Microb 61:3592-7.

Munir M, Zohari S and Berg M, 2013. Immunology and immunopathogenesis of peste des petits ruminants virus. In: Molecular Biology and Pathogenesis of Peste des Petits Ruminants Virus. Springer Briefs in Animal Sciences. Springer, Berlin, Heidelberg pp:49-68.

Murugappan S, Patil HP, Kanojia G, *et al.,* 2013. Physical and immunogenic stability of spray freeze-dried influenza vaccine powder for pulmonary delivery: comparison of inulin, dextran, or a mixture of dextran and trehalose as protectants. Eur J Pharm Biopharm 85:716-25.

Prabhu M, Bhanuprakash V, Venkatesan G, *et al.,* 2014. Evaluation of stability of live attenuated camel pox vaccine stabilized with different stabilizers and reconstituted with various diluents. Biologicals 42:169-75.

Rajak K, Sreenivasa B, Hosamani M, *et al.,* 2005. Experimental studies on immunosuppressive effects of peste des petits ruminants (PPR) virus in goats. Comp Immunol Microb 28:287-96.

RehmanA, Jingdong L and Chandio AA, 2017. Livestock production and population census in Pakistan: Determining their relationship with agricultural GDP using econometric analysis. Inform Process Agri 4:168-77.

Riyesh T, Balamurugan V, Sen A, *et al.,* 2011. Evaluation of efficacy of stabilizers on the thermos stability of live attenuated thermo-adapted peste des petits ruminant's vaccines. Virol Sin 26:324-37.

Silva AC, Carrondo MJ and Alves PM, 2011. Strategies for improved stability of peste des petits ruminant's vaccine. Vaccine 29:4983-91.

Turner PV, Pekow C, Vasbinder MA, *et al.,* 2011. Administration of substances to laboratory animals: equipment considerations, vehicle selection, and solute preparation. J Am Assoc Lab Anim Sci 50:614-27.

Ullah RW, Zahur BA, Asma L, *et al.,* 2015. Mild form of peste des petits ruminants virus (PPRV) in Pakistan. Pak J Zool 47:276-9.

Wang Z, Bao J, Wu X, *et al.,* 2009. Peste des petits ruminant's virus in Tibet. China Emerg Infect Dis 15:299-307.

Xue W, Ellis J, Mattick D, *et al.,* 2010. Immunogenicity of a modified-live virus vaccine against bovine viral diarrhea virus types 1 and 2, infectious bovine rhino tracheitis virus, bovine parainfluenza-3 virus, and bovine respiratory syncytial virus when administered intranasally in young calves. Vaccine 28:3784-92.