ORIGINAL ARTICLE

## Trehalose improves PPR vaccine virus stability in diluent

N. Mohanto[1], A. Khatun[2], J. A. Begum[1], M. M. Parvin[1], M. S. I. Siddiqui[3], S. Begum[4], R. Parvin[1], M. R. Islam[1] and E. H. Chowdhury[1]*

[1]Department of Pathology, Faculty of Veterinary Science, Bangladesh Agricultural University, Mymensingh
[2]Department of Veterinary and Animal Sciences, Faculty of Agriculture, Rajshahi University, Rajshahi
[3]Department of Anatomy and Histology, Faculty of Veterinary, Animal and Biomedical Sciences, Sylhet Agricultural University, Sylhet
[4]Department of Physiology, Faculty of Veterinary, Animal and Biomedical Sciences, Sylhet Agricultural University, Sylhet

**Abstract**

**Background:** Specialized freeze-drying process is being used in the field for different thermostable vaccine preparation worldwide. The thermostability remains only in undiluted conditions. If dilution is made at the morning and used for the whole day, the vaccine efficacy is compromised at high ambient temperature. In this study, trehalose based specialized vaccine diluent was used to improve the stability of *Peste des petits ruminants* (PPR) vaccine in diluted condition.

**Methods:** The available PPR vaccine was reconstituted with conventional diluent and with trehalose based test diluent. The diluted vaccine was kept at ambient temperature without maintaining any cool chain. Stability of diluted vaccine virus was further assessed *in vivo* and *in vitro* at different temperatures. Goats were vaccinated and Vero cells were infected with reconstituted vaccines and were assessed at 0, 3, 6, 9 and 24 hours post dilution. Antibody titer was measured and virus infectivity titer was determined in both cultured cell lysate and supernatant. The presence of the virus particles in Vero cell was confirmed by standard RT-PCR targeting Fusion (F) gene of PPR virus.

**Results:** *In vivo* results revealed that the number of goats possessed antibodies to PPR virus was higher in trehalose based vaccine formulation than the conventional PBS based diluent. Reconstituted vaccine virus (using PBS and trehalose diluent) infected Vero cells produced 70-80% cytopathic effect (CPE) in 5th days of post infection. Both diluents produced and maintained infectivity titer from $\log_{10} TCID_{50}$ 5.5 to $\log_{10} TCID_{50}$ 3.6, until the use of vaccines incubated for 9 hours after dilution. On the other hand, at 24 hours of post dilution only trehalose formulated vaccine produced $\log_{10} TCID_{50}$ 2.5 whereas no infectivity titer was observed at the same time using conventional one.

**Conclusion:** The present study suggests that trehalose preserves the quality of reconstituted vaccine in terms of infectivity titers. Trehalose can be a diluent of choice for reconstitution of PPR vaccine in field.

**Key words:** PPR vaccine, Trehalose diluent, Antibody titre, cELISA kit, Vero cell, cytopathic effect

*Correspondence: emdad001@yahoo.com

## Introduction

*Peste des petits ruminants* (PPR) is a highly fatal contagious and economically important disease affecting goats, sheep and wild ruminants (Singh *et al.*, 2014; Banyard *et al.*, 2014). It causes high morbidity and mortality as 100% and 90%, respectively (Couacy Hymann *et al.*, 2002). The disease is caused by *Peste des petits ruminants* virus (PPRV) belongs to genus *Morbillivirus* under the family *Paramyxoviridae* (Gibbs *et al.*, 1979; Bailey *et al.*, 2005), subfamily *Paramyxovirinae* and order *Mononegavirales* (Murphy *et al.*, 1999). The disease is characterized by high fever (106°F-107°F), erosive stomatitis, catarrhal orinasal discharges and necrosis, ulceration of the mucous membrane and inflammation of gastrointestinal tract leading to severe diarrhea (Begum *et al.*, 2018). Administration of live attenuated vaccine along with isolation and disinfection of the contaminated environment is the important method of PPR disease control (Sen *et al.*, 2010). Use of the vaccine in ordinary clinical practice and its effectiveness refers to the reduction of disease, which ultimately benefits the farmer economically (Peter, 2001). In subtropical and tropical countries like Bangladesh, maintenance of cool chain for live attenuated vaccine is challenging due to poor communication framework in these regions and lack of well skilled vaccinators. Commonly vaccinators diluted the vaccine in morning and kept in environmental temperature over a period of the day; even until the next day. Therefore, the later vaccinated dose gradually become ineffective leading to waste of money and time. Furthermore, due to lack of efficient manpower vaccinators cover a vast area under a vaccination program in a day. In tropical and subtropical countries, a thermostable live-attenuated vaccine is a way to avoid cool chain-associated problems. Trehalose is a naturally occurring osmolyte and exceptional stabilizer of proteins especially in thermostability (Koushik and Bhat, 2003) and helps to stimulate a strong immune response (Jamil *et al.*, 2014). The thermostability and potency of lyophilized vaccines remains only in undiluted conditions. Hence, selection of a suitable diluent is in indigence. Thus, we prepared specialized vaccine diluent to increase the stability of PPR vaccine in diluted condition. Here, we study the effectiveness of trehalose-based formulation on the stability of PPR vaccine virus *in vivo* and *in vitro* at selected time intervals, and compared with conventional diluent, phosphate buffered saline.

## Materials and Methods

### Vaccine viruses and cell line

PPR vaccine virus produced by Livestock Research Institute (LRI), Bangladesh which is being currently used to control PPR in the country was used in this study.

Vero cells (CLS, Germany) were maintained in Minimum Essential Medium (MEM) supplemented with 10% Fetal Bovine Serum (FBS), glutamine and gentamicin at 37°C in a humidified incubator provided with 5% $CO_2$.

### Preparation of diluents and vaccine virus

PPR vaccine virus was diluted with conventional diluent phosphate buffered saline (PBS) and a test diluent prepared by using Tris with 20 mM tris-HCl (pH 7.4), 2mM EDTA and 1 M trehalose with required amount of double distilled water. The formulation was stored at 4°C for further use. Two vaccine vials were reconstituted using 5 ml of PBS (Group A) and 5 ml of prepared trehalose test diluents (Group B) respectively. Then both of the vaccine vials were gently mixed and kept at environmental temperature (24 hrs) without maintaining cool chain to vaccinate experimental animals as stated in Table 1.

### Evaluation of efficacy of vaccine diluents *in vivo*

A total of 90 Black Bengal goats of Rajshahi region were selected, grouped (Group A, n=45 and Group B, n=45) and vaccinated (**Table 1**). Blood was collected at day 0 as well as day 81 and 210 of post vaccination and serum was prepared from collected blood. Antibody titers for each group of serum sample were detected by cELISA (IDvet, France) and then immune response were analyzed.

**Table 1. Experimental design to evaluate the efficacy of vaccine diluents *in vivo***

| Time from vaccine dilution to use | Time of vaccination | Group A (Conventional diluent, n=45) | Group B (Trehalose diluent; n=45) | Detection of PPRV antibody |
|---|---|---|---|---|
| 0 hr | 8.00 am | A1; n=10 | B1; n=10 | At day 0 and 81 days |
| 3 hr | 11.00 am | A2; n=10 | B2; n=10 | and 210 days post |
| 6 hr | 2.00 pm | A3; n=10 | B3; n=10 | vaccination |
| 9 hr | 5.00 pm | A4; n=10 | B4; n=10 | |
| 24 hr | 8.00 am | A5; n=05 | B5; n=05 | |

*Virus titration*

Vaccine virus was reconstituted with both conventional (Group A) and prepared trehalose test diluent (Group B). The confluent monolayer of Vero cells grown in 96 well microtiter plates were infected with both reconstituted vaccines at 3, 6, 9 and 24 hrs post dilution (**Table 2**) using 10-fold dilution and 10 replicates of each dilution. Columns 11 and 12 of each plate were left without infection, which served as negative control. The microtiter plates were incubated in the presence of 5% $CO_2$ at 37° C for 6 days with a change of maintenance media (MEM with 5% calf serum) at every alternative day. Cells were observed twice daily for the appearance of cytopathic effect (CEP) under microscope. Virus infectivity titer was quantified by estimating the 50% tissue culture infectivity dose ($TCID_{50}$). $TCID_{50}$/ml of virus suspensions was calculated by observing end point (EP) at 4 to 6 days using Reed and Muench mathematical techniques (1938). The EP was determined when the cells appeared exactly the same, without any minor changes, and 2 separate readings were taken at least 24 hrs apart.

**Table 2. Experimental design to evaluate the efficacy of vaccine diluents *in vitro***

| Time from vaccine dilution to use | Time of infection into Vero cell | Group A (Conventional diluent) | Group B (Test diluent) | Group C (Control group) |
|---|---|---|---|---|
| 0 hr | 8.00 am | Plate 1 | plate 6 | Column 11 |
| 3 hr | 11.00 am | plate 2 | plate 7 | and 12 were |
| 6 hr | 2.00 pm | plate 3 | plate 8 | used as |
| 9 hr | 5.00 pm | plate 4 | plate 9 | control in |
| 24 hr | 8.00 am | plate 5 | plate 10 | each plate |

**Identification of the virus by RT- PCR**

Viral RNA was isolated from the culture supernatant (1st dilution i.e. $10^{-1}$) infected at selected time (0 hr and 24 hrs) point using the PureLink™ Viral RNA/DNA Mini Kit (Invitrogen, USA). RNA was subjected to amplify fusion (F) gene by RT-PCR (Forsyth and Barrett, 1995) using Verso 1-Step RT-PCR ReddyMix Kit (ThermoFisher Scientific, USA). The PCR products were analyzed by gel electrophoresis.

**Results**

Blood was collected from both groups on day 0, 81 and 210 days post vaccination (dpv). The sera were subjected to cELISA for the detection of PPR virus antibody. Since at day 0, 5 animals of 3 subgroups (A1, A2 and A4) of group A and 1 animal of Group B (subgroup B4) were found positive, they were discarded from the calculations. Again farmers sold two animals from B5 subgroup and thus discarded from the calculations. In group A (PBS diluted vaccine), 60% animals seroconverted after using the freshly (0 hr) diluted vaccine and the seroconversion rate were gradually decreased up to 45% at 81 and 120 dpv when diluted vaccine was incubated for 9 hrs at ambient temperature (Table 3). All animals from group A showed no seroconversion at 24 hours post dilution. On the other hand, in group B (trehalose diluted vaccine), 70% animals seroconverted at 81 and 120 dpv using freshly diluted vaccine (Table 3) and the sero-conversion rate were slightly decreased over time (3 hpd to 24 hpd) up to 56% at 81 dpv and remained same at 210 dpv (**Table 3**).

**Table 3. Antibody titer of vaccinated goats at 81 and 210 days post vaccination**

| Hour of vaccine dilution to vaccination | Group A (Conventional diluent) | | | Group B (Trehalose diluent) | | |
|---|---|---|---|---|---|---|
| | SG | No. of +ve at 81 dpv | No. of +ve at 210 dpv | SG | No. of +ve at 81 dpv | No. of +ve at 210 dpv |
| 0 hpd | A1 (n=7) | 4 (60%) | 4 (60%) | B1 (n=10) | 7 (70%) | 7 (70%) |
| 3 hpd | A2 (n=9) | 4 (45%) | 4 (45%) | B2 (n=10) | 6 (60%) | 6 (60%) |
| 6 hpd | A3 (n=10) | 4 (40%) | 4 (40%) | B3 (n=10) | 6 (60%) | 6 (60%) |
| 9 hpd | A4 (n=9) | 4 (45%) | 5 (56%) | B4 (n=9) | 5 (56%) | 5 (56%) |
| 24 hpd | A5 (n=5) | 0 | 0 | B5 (n=5) | 3 (60%) | 3 (60%) |

N.B: G=Group, SG= Subgroup, +ve= Positive, -ve=Negative, hpd: hour post dilution, dpv= Day post vaccination

The Vero cells cultured in 96 well plates were infected with reconstituted vaccine virus considering selected time intervals (0, 3, 6, 9 and 24 hrs) from the time of dilution to its use (**Table 2**). The infectivity titer of the virus suspension between conventional diluents (group A) and trehalose diluents (group B) was compared on the basis of cytopathic effect (CPE) in Vero cells. CPE was defined as initial cell rounding, ballooning and aggregations of cells, syncytial development, detachment and floating of rounded cell. The infected Vero cells with reconstituted vaccine virus (both PBS and trehalose) started to produce CPE at 72 hours post infection (**Figure 1B, 1C**) and 70-80% CPE was observed in 5th days of post infection (**Figure 1D**) and complete CPE were observed on 6th day post infection (**Figure 1E**).
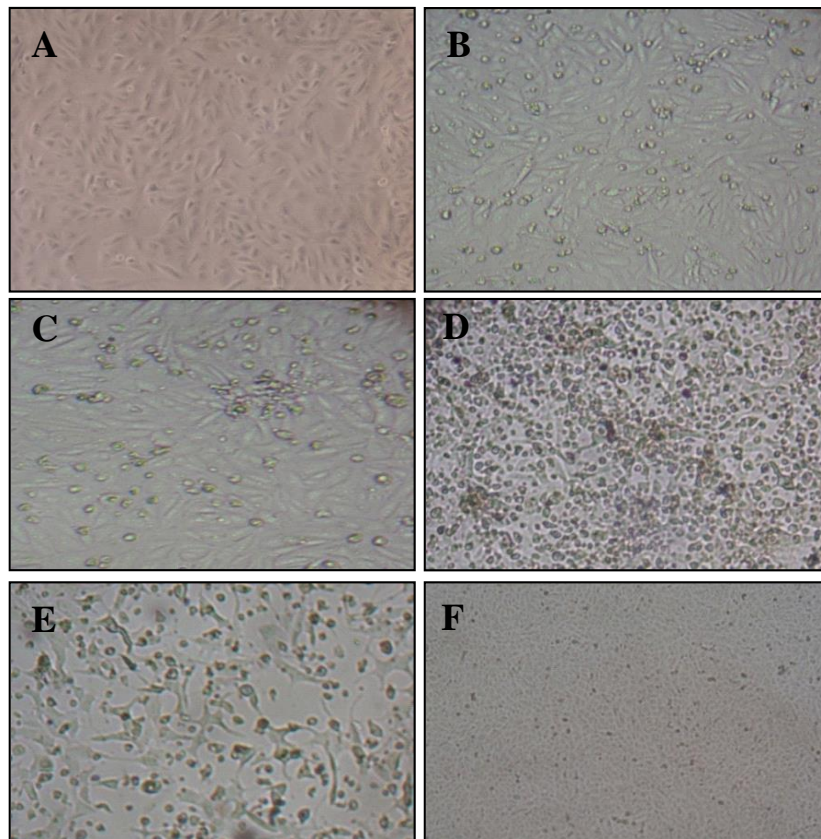


**Figure 1:** Cytopathic effects (CPE) in Vero cells produced by test diluent (trehalose). (**A**): Normal Vero cell (90-100% confluent); (**B**): Individual cell rounding at 3rd day post infection, 10x; (**C**): Aggregation of rounded cell at 4th day post infection, 10x; (**D**): Aggregation of rounded cell at 5th day post infection, 10x; (**E**): Detachment of cell at 6th day post infection, 10x; (**F**): Control at 6th day post infection; 10x.

The infectivity titers ($TCID_{50/ml}$) of vaccine virus suspension using conventional diluent and trehalose based diluent were calculated (**Figure 2**). Conventionally diluted (group A) vaccine virus produced higher infectivity titer (5.5 $\log_{10}$ $TCID_{50/ml}$) at 0 hour and 3 hour of post dilution (hpd) and infectivity titer gradually decreased to 2.5 $\log_{10}$ $TCID_{50/ml}$ at 9 hpd. No virus yield was recorded at 24 hpd. Whereas, the reconstituted vaccine virus with trehalose-based diluent (group B) showed high infectivity titer (5.5 $\log_{10}$ $TCID_{50/ml}$) at 0 and 3 hpd and infectivity titer of 2.5 $\log_{10}$ $TCID_{50/ml}$ was sustained for the vaccine virus even after 24 hpd indicating that the trehalose-based diluent maintained better infectivity of vaccine virus for longer time than the conventional diluent.
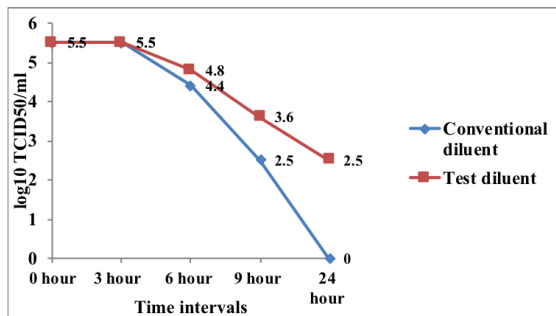


**Figure 2:** Comparative study of infectivity titer of PPR vaccine virus using two vaccine diluents at selected time intervals.

Furthermore, we confirmed the presence of F gene of PPR virus from cultured supernatant (1st dilution i.e. $10^{-1}$) of studied time points at 0 and 24 hpd for both diluents. Out of four samples, three samples (0 hour diluted samples of both diluents and 24 hour diluted sample of test diluent) which produced CPE and infectivity titer in Vero cell, amplified 448 bp of F gene fragment (**Figure 3**).



**Figure 3:** Amplification of a 448 bp fragment of F gene by conventional RT-PCR. S1= Test diluent (0 hour of dilution); S2= Test diluent (24 hour of dilution); S3= Conventional diluent (0 hour of dilution); S4= Conventional diluent (24 hour of dilution); PC= Positive control; NC= Negative control; M= 100 bp DNA size marker.

## Discussion

The study was focused on the determination of the thermostability of PPR vaccine using conventional (PBS based) and test (trehalose based) diluents. In Bangladesh, the thermostability of lyophilized vaccine remains only in undiluted conditions. The common practice in field is to make dilution at the morning and the diluted vaccine is being used for the whole day or sometimes even for the next day, which compromises the efficacy of the vaccine. In this study, trehalose based specialized vaccine diluents was prepared to increase the stability of PPR vaccine in diluted condition and their efficacy was tested *in vivo* and *in vitro*. Our study showed comparatively better stability of the vaccine virus in trehalose based diluent.

Goats from all subgroups of group B (vaccine diluted with trehalose based diluent) showed 10-20% better seroconversion when compared with goats of subgroups of group A (vaccine diluted with conventional diluent) (**Table 3**). Similar results were found *in vitro*; the infectivity titer was maintained better with trehalose diluent (**Figure 2**). These indicated that trehalose maintained better stability of the vaccine virus than the conventional diluent. However, the reconstituted vaccine with both diluents showed

presence of virus particle at RT-PCR but that does not exhibit the sustainability of infectivity titer or immune stimulatory properties of virus. Trehalose was used as stabilizer to prepare the PPRV sungri 96 vaccine during freeze drying procedure (Silva *et al.,* 2011) and it produced higher stability of the lyophilized PPRV vaccine. The study by Sarker *et al.* (2003) revealed that trehalose was more stable than other stabilizers at different temperatures such as 4°C, 25°C and 37°C. Trehalose that was used as test diluent is a naturally occurring osmolyte and exceptional stabilizer of proteins especially in the condition for thermostability. It offers better thermal protection to this vaccine virus followed by conventional diluents. When trehalose was used, it forms a protective layer around the protein and other biomolecules under adverse conditions like extreme temperatures and dehydration, and creates a freezing condition in vaccine (Kaushik and Bhat, 2003). For this reason, the stability of the vaccine virus in trehalose based diluent is relatively better when the cool chain is not maintained.

## Conclusion

Taken together, the stability of reconstituted vaccine virus diluted with conventional and trehalose based diluent was assessed at selected time intervals (0, 3, 6, 9 and 24 hrs) after dilution. Although the virus titer was gradually decreased with time for both of the diluents, trehalose reconstituted PPR vaccine developed antibody against PPR virus and the infectivity titer was also sustained after 24 hours of dilution. Therefore, trehalose based formulation was found superior to stabilize the PPR vaccine and enhanced the utility of vaccine without maintenance of cool chain.

## Acknowledgment

## Competing Interest

The authors declare that they have no competing interests.

## References

1. Bailey D, Banyard A, Dash P, Ozkul A, Barrett T. Full genome sequence of *Peste des petits ruminants* virus, a member of the morbillivirus genus. Virus Research 2005; 110: 119–124.
2. Banyard AC, Wang Z, Parida S. *Peste des petits ruminants* virus, Eastern Asia. Emerging Infectious Diseases. 2014; 20: 2176–2178.
3. Begum S, Nooruzzaman M, Parvin M, Mohanto N, Parvin R, Islam MR *et al.* Peste des petits Ruminants virus infection of Black Bengal goats showed altered haematological and serum biochemical profiles. Onderstepoort Journal of Veterinary Research. 2018; 85(1): a1595.
4. Couacy-Hymann E, Roger F, Hurard C, Guillou JP, Libeau G, Diallo A. Rapid and sensitive detection of *Peste des petits ruminants* virus by a polymerase chain reaction assay. Journal of Virological Methods. 2002; 100: 17–25.
5. Forsyth MA and Barrett T. Evaluation of polymerase chain reaction for the detection and characterisation of rinderpest and peste des petits ruminants viruses for epidemiological studies. Virus Research. 1995; 39: 151-163.
6. Jai K. KaushikGibbs EP, Taylor WP, Lawman MJ, Bryant J. Classification of *Peste des petits ruminants* virus as the fourth member of the genus morbillivirus. Intervirology. 1979; 11: 268–274.
7. Jamil RK, Taqavian M, Sadigh ZA, Shahkarami MK, Esna-Ashari F, Hamkar R, *et al..* Evaluation of the thermal stability of a novel strain of live-attenuated mumps vaccine (RS-12 strain) lyophilized in different stabilizers. Journal of Virological Methods. 2014; 199: 35-8.
8. Koushik JK, Bhat R. Why is trehalose an exceptional protein stabilizer? An analysis of the thermal stability of proteins in the presence of the compatible osmolyte trehalose. The Journal of Biological Chemistry. 2003; 278 (29):26458–26465.
9. Murphy FA, Gibbs EPJ, Horzinek MC, Studdert MJ. Classification and nomenclature of viruses. In: Veterinary Virology, 3rd edn. Academic press, NewYork. 413; 1999.
10. Peter B. Vaccination. Van Roitt, Jonathan brostoff and David male. Immunology 6th edn.; 277–87, 2001.

11. Reed LJ and Muench H. Simple method of estimating 50% end points. American Journal of Hygiene. 1938; 27: 493–497.

12. Sarkar J, Sreenivasa BP, Singh RP, Dhar P, Bandyopadhyay SK. Comparative efficacy of various chemical stabilizers on the thermostability of a live-attenuated *Peste des petits ruminants* (PPR) vaccine. US National Library of Medicine, National Institue of Health (Pub Med.gov). 2003; 21(32): 4728–35.

13. Sen A, Saravanan P, Balamurugan V, Rajak KK, Sudhakar SB, Bhanuprakash V, Parida S, Singh RK. Vaccines against *Peste des petits ruminants* virus. US National Library of Medicine, National Institue of Health (Pub Med.gov). 2010; 9(7): 785–96.

14. Silva AC, Carrondo MJT, Alves PM. Strategies for improved stability of *Peste des petits ruminants* vaccine. Vaccine. 2011; 29: 4983–4991.

15. Singh B, Bardhan D, Verma MR, Shiv P, Sinha DK, Prasad S . Estimation of economic losses due to *Peste des petits ruminants*in small ruminants in India. Veterinary World. 2014; 7: 194–199.