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Improvement of the Stability of a Live Attenuated Peste Des Petits Ruminants (PPR) Vaccine

Adel M. Khadr[1], Tharwat M. Elshemey[1], Afaf A. Abdelwahab[2], Asmaa G.Abdel-Samad[2]

[1]Department of Medicine and Infectious Diseases, Faculty of Veterinary Medicine, Alexandria University.
[2] Veterinary Serum and Vaccine Research Institute, Abbasia, Cairo.

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Correspondence to: asmaa gamal abdel-samad (asmaagamal223@yahoo.com

ABSTRACT

The study was conducted to find out a way that might lead to potentiation of both cryostability and thermostability criteria of the live attenuated Peste des petits ruminants virus Nigerian75/1 vaccine. This vaccine as with other Morbillivirus vaccines is thermolabile; i.e. thermal degradation occurs on exposure to inconvenient temperatures, even though if kept in a lyophilized state. This draw-back poses difficulties when launching vaccination campaigns especially in arid and semi –arid areas if a cold chain necessity is logistically unavailable to maintain good vaccine titers until delivery of the product to targeted animals. A recently advocated stabilizer formulation (Tris/ Trehalose) was tested for its cryoprotectant as well as thermo- resistant potentialities on being thoroughly incorporated into the PPRV-suspension just prior to lyophilization process and under varying storage temperature conditions, in comparison to the conventionally used Weybridge medium (WBM). It was found that the Tris/ Tre formula was far superior to the WBM one since the loss in virus titre was 0.1 and 0.4 $\log_{10}$ $TCID_{50}$ /ml for Tris/ Tre and WBM stabilized vaccines respectively during the lyophilization process. The results preferred the choice of Tris/Tre Stabilizer formula on viability of 75/1 PPR virus during lyophilization process and preservation at -20◦C and -70◦C. Tris/Tre stabilized vaccines stored at 4◦C and -4◦C for 9,12 months respectively maintained sufficient viable PPR virus to protect goats. Even at a drastic high temperatures (37°C and 45°C), the Tris/Tre formula could conserve satisfactory vaccine titre after 5 and 2 days of exposure, corresponding to only 1.5 and 1 day with the WBM formula, respectively. In conclusion, Tris/Tre stabilized PPRV- attenuated vaccine, enhanced to a reasonable extent both cryoprotectant and thermotolerence potentialities as compared to the WBM one and it is superior to WBM in inducing protective immune response after vaccination in tested goats.

1. INTRODUCTION:

Peste des petits ruminants (PPR) is an acute highly contagious viral disease of sheep and goats and wild small ruminants that was included in the OIE list of notifiable terrestrial animal diseases (OIE, 2004). it is considered one of the highly fatal and economically important viral diseases due to its high morbidity and mortality rates reaching up to 100% and 90%, respectively, depending upon the endemicity in the area (Banyard et al., 2010). It is caused by PPR virus (PPRV), which is a member of the genus Morbillivirus, family Paramyxoviridae and is antigenically closely related to the Rinderpest (RP) virus (Gibbs et al., 1979). PPR disease is widespread throughout Asian countries, sub-Saharan Africa, and in Egypt (Roeder and Obi 1999, Abd El-Rahim et al., 2010).

PPR homologous live attenuated vaccine is well-known as one of the most effective tools in controlling of such disease, but like other morbillivirus vaccines; has poor thermostability (OIE, 1998). Vaccine stabilizers are well recognized as chemical

compounds added to a vaccine to improve its stability during storage, lyophilization, transportation and after reconstitution during animal vaccination in the ambient temperature (Moran and Bucklon, 2007).

lactalbumin hydrolysate sucrose (LS), Weybridge medium (WBM), buffered gelatin-sorbitol (BUGS) and trehalose dihydrate (TD) are different stabilizers, used to prepare the lyophilized vaccines. However, LS and TD are more stable than rest of the stabilizers to lyophilize PPR vaccine (Sarkar et al., 2003).

As PPR living vaccine is used in areas with hot weather and is affected by high temperature, so the present study was aiming at improving the keeping quality of the produced attenuated PPR vaccine through using Weybridge medium (WBM) and Tris/Trehalose (Tris/Tre) as stabilizers to optimize the vaccine thermostability during storage, transportation and animal vaccination in hot weather.

2. MATERIALS AND METHODS:

2.1. Virus and Cell line:

The PPR vaccine strain used was PPR virus Nigerian 75/1, obtained from the reference laboratory PANVAC, Debrezeit, Ethiopia; from which a primary and a secondary seed lots were produced on vero cells at Rinderpest department, VSVRI, Abbasia, Cairo, Egypt. Vero cells were preserved in Minimal Essential medium (MEM) supplemented with nystatin, penicillin and streptomycin sulphate and 10% foetal calf serum.

2.2. Vaccine and Stabilizers:

Two different compositions of vaccine stabilizers i.e., Weybridge medium (WBM) and Tris/Trehalose (Tris/Tre) were used in this study. WBM stabilizer consisted of 2.5% lactalbumin hydrolysate, 5% sucrose and 1% sodium glutamate, while (Tris/Tre) consisted of 20 mM Tris HCL PH 7.4, 2 mM EDTA, and 0.02 % (w/v) Tween 80 and 1 M Trehalose. Equal volumes of the virus suspension and stabilizers were mixed. The vaccine vials containing different stabilizers were then lyophilized simultaneously under identical conditions. A batch of live attenuated PPR cell culture vaccine was experimentally prepared (Diallo, 2004) for research purpose.

2.3. Lyophilization:

The lyophilization cycle was undertaken according to the lyophilization protocol used at CIRAD, the reference laboratory for PPR (Diallo, 2004). Briefly, [1] Freezing,−40 ◦C for 2 h; [2] Primary drying,−40 ◦C for 14 h at 0.060 mBar; [3] Secondary drying,−35 ◦Cfor 2 h;−30 ◦C for 2 h;−25 ◦C for 2 h;−20 ◦C for 2 h;−15 ◦C for 14 h;−10 ◦C for 2 h;−5 ◦C for 2 h; 0 ◦C for 2 h; 10 ◦C for 2 h; 20 ◦C for 2 h; all at 0.060 mBar; and [4] Final drying, 35 ◦C for 2 h at 0.060 mBar. One milliliter of the formulated PPRV was dispensed in sterile 5 ml capacity glass vials and partially sealed with vented rubber stoppers. At the end of the lyophilization process, the caps were closed and the samples were stored at different temperatures until reconstitution for titration. Some samples were reconstituted with 1 ml of normal saline solution and titrated immediately after lyophilization to evaluate the effect of the process on virus stability.

2.4. Cryo and Thermostability of lyophilized Vaccines:

Adequate numbers of lyophilized vaccine vials for each stabilizer were stored at 4 ◦C, -4 ◦C, -20◦C, -70◦C, (up to 12 months), at 37 ◦C (up to 9 days) and at 45 ◦C (up to 5 days). Samples were taken along time and all removed vials were stored at - 70°C. All stored vaccinal vials were subjected for the virus titration of the samples at the same time.

2.5. Virus Titration:

After the specific incubation or exposure time, the re-hydrated freeze-dried vaccines were subjected to virus titration (OIE, 2004). Serial ten-fold dilutions of exposed virus suspension were made immediately in maintenance medium and the viruses were titrated in monolayers of Vero cells grown in 96-well microtiter plates using four replicates as per dilution (100µl/well). The plates were incubated in the presence of 5% CO2 for 6 days with a change of maintenance media at every alternative day and cells were observed for cytopathic effects (CPE) regularly under microscope. Virus infectivity was quantified by estimating the 50% tissue culture infectivity doses ($TCID_{50}$) and end points were calculated as per Reed and Muench (1938).

2.6. Animals and Experimental Design:

Eight local breed male goats of 9-12 months of age were used. These goats were apparently healthy and free from antibodies against PPR virus as proved by using serum neutralization test**.** The goats were used to compare the potency (Efficacy) of the different

stabilized vaccine by dividing into three groups as follow:

Group I: Each of three goats was vaccinated subcutaneously with 1ml of 2 $\log_{10}$ $TCID_{50}$ live attenuated PPR virus vaccines with stabilizing medium WBM.

Group II: Each of three goats was vaccinated subcutaneously with 1ml of 2 $\log_{10}$ $TCID_{50}$ live attenuated PPR virus vaccines with stabilizing medium Tris/Tre.

Group III: two Goats were left as non-vaccinated controls. Each of these Goat was subcutaneously injected with an equal volume physiological saline and was left as control.

Goats were housed in mosquito proof isolated stable and daily observed besides body temperature was recorded.

2.7. Serum Samples:

All sera were collected from vaccinated and unvaccinated goats through the jugular vein blood of each animal on the day of vaccination (zero day), then weekly till 28th day post vaccination. The sera were stored at -20°C and inactivated at 56°C for 30 minutes before being examined by the Serum Neutralization Test (SNT).

2.8. Serum Neutralization Test:

Both qualitative and quantitive estimations were performed on the sera of goats using the microtitre technique as described by Rossiter and Jessett (1982) in flat bottom tissue culture micro titer plates containing Vero cells . The SNT was applied on goat's sera before and at one week intervals after vaccination with PPR vaccine for one month. The end point neutralizing antibody titer was expressed as the reciprocal of the final dilution of serum inhibiting the CPE of 100 $TCID_{50}$ of PPR virus on Vero cells.

3. RESULTS:

3.1. PPR Virus Titers of Stabilized Vaccines Just Pre and Post Lyophilization:

The PPR virus titer in wet vaccines with stabilizer WBM or Tris/Tre was 6.5 $\log_{10}$ $TCID_{50}$/ml and 6.8 $\log_{10}$ $TCID_{50}$/ml and the titer immediately after lyophilization was 6.1 $\log_{10}$ $TCID_{50}$/ml and 6.7 $\log_{10}$ $TCID_{50}$/ml for both vaccines, respectively. The total virus losses during lyophilization process were 0.4 $\log_{10}$ $TCID_{50}$/ml and 0.1 $\log_{10}$ $TCID_{50}$/ml for both stabilized vaccines respectively (Table1).

3.2. Cryostability of Live Attenuated PPR Virus Vaccines Stored at 4°C and - 4°C:

Exposure of PPR vaccines with stabilizing medium WBM and Tris/Tre to 4°C for 12 months resulted in a loss of 2.5 and 2.3 $\log_{10}$ $TCID_{50}$/ml, respectively. While, Exposure to - 4°C for 12 months resulted in a loss of 1.7 and 1.0 $\log_{10}$ $TCID_{50}$/ml for both stabilized vaccines, respectively. Results of these tests are given in (Table 2).

Table (1): PPR virus titer in vaccine with WBM and Tris/Tre stabilizers pre and post lypohilization.

Type of stabilizer	Virus titer expressed as log $_{10}$ $TCID_{50}$/ml		
	Pre- lypohilization	Post- lypohilization	Loss in virus titer
WBM	6.5	6.1	0.4
Tris/Tre	6.8	6.7	0.1

Table (2): PPR virus titer in lyophilized vaccine with WBM and Tris/Tre stabilizers stored at 4°C and - 4°C.

Months	Virus titer expressed as log $_{10}$ $TCID_{50}$/ml			
	4°C		-4°C	
	PPR virus vaccine with WBM	PPR virus vaccine with Tris/Tre	PPR virus vaccine with WBM	PPR virus vaccine with Tris/Tre
1	5.8	6.6	5.9	6.7
3	5.3	6.5	5.6	6.7
5	5.0	6.1	5.5	6.6
7	4.4	5.7	5.2	6.5
9	4.1	5.3	4.9	6.0

12	3.6	4.4	4.4	5.7

Table (3) PPR virus titer in lyophilized vaccine with WBM and Tris/Tre stabilizers stored at -20°C and -70°C.

Months	Virus titer expressed as log $_{10}$ $TCID_{50}$/ml			
	-20°C		-70°C	
	PPR virus vaccine with WBM	PPR virus vaccine with Tris/Tre	PPR virus vaccine with WBM	PPR virus vaccine with Tris/Tre
1	6.0	6.7	6.1	6.7
3	6.0	6.6	6.1	6.7
5	5.9	6.5	6.1	6.7
7	5.8	6.5	6.1	6.7
9	5.7	6.4	6.1	6.7
12	5.6	6.4	6.1	6.7

Table (4) PPR virus titer in lyophilized vaccine with WBM and Tris/Tre stabilizers stored at 37°C.

Days	Virus titer expressed as log $_{10}$ $TCID_{50}$/ml	
	PPR virus vaccine with WBM	PPR virus vaccine with Tris/Tre
1	5.2	5.9
3	4.1	5.3
5	3.0	4.8
7	2.4	4.2
9	1.5	3.4

3.3. Cryostability of live Attenuated PPR Virus Vaccines Stored at -20°C and -70°C:

PPR vaccines with stabilizing medium Tris/Tre stabilized the initial titer of the vaccine for < 3 months at -20°C and up to 12 months at -70°C, while vaccines with stabilizing medium WBM stabilized the initial titer of the vaccine for 1 month at -20°C and up to 12 months at - 70°C. The little amount of virus total loss during 12 months of storage period were observed at -20°C where it was 0.5 log_{10} $TCID_{50}$/ml for vaccine with stabilizing medium WBM and 0.3 log_{10} $TCID_{50}$/ml for vaccine with stabilizing medium Tris/Tre (Table3).

3.4. Thermostability of Live Attenuated PPR Virus Vaccines Stored at 37°C and 45°C:

Exposure of PPR vaccines with stabilizing medium WBM and Tris/Tre to 37°C for 9 days resulted in a loss of 4.6 and 3.3 log_{10} $TCID_{50}$/ml, respectively. Results of these tests are given in (Table 4). While, Exposure for 5 days at 45°C resulted in complete loss of potency of vaccine with stabilizing medium WBM and a loss of 5.7 log_{10} $TCID_{50}$/ml for vaccine with stabilizing medium Tris/Tre. Results of these tests are given in (Table 5).

3.5. Efficacy Testing of Live Attenuated PPR Virus Vaccines With WBM and Tris/Tre Stabilizers:

Both live attenuated PPR virus vaccines with stabilizing medium WBM or Tris/Tre induced neutralizing antibodies by the 7th day post vaccination (DPV) and increased gradually and reached the peak on the 28th DPV. Higher mean neutralizing PPR antibody titers prompted by vaccine with stabilizing medium Tris/Tre than that produced by vaccine with stabilizing medium WBM (Table 6).

4. DISCUSSION:

Along with all members of the Paramyxoviridae family, PPRV is thermo labile; this is a serious problem for the efficient use of the live attenuated vaccine in the endemic areas, which have hot climatic environments. In addition these regions usually have poor infrastructures, being difficult to sustain a cold chain so, the distribution, storage and use of vaccines therefore present challenges that could be reduced by enhanced thermo-stability, with resulting improvements in vaccine effectiveness (kristensen et al., 2011).

Table (5) PPR virus titer in lyophilized vaccine with WBM and Tris/Tre stabilizers stored at 45°C.

Days	Virus titer expressed as log $_{10}$ $TCID_{50}$/ml	
	PPR virus vaccine with WBM	PPR virus vaccine with Tris/Tre
1	5.0	5.5
2	3.6	4.5
3	2.8	3.8
4	1.4	2.7

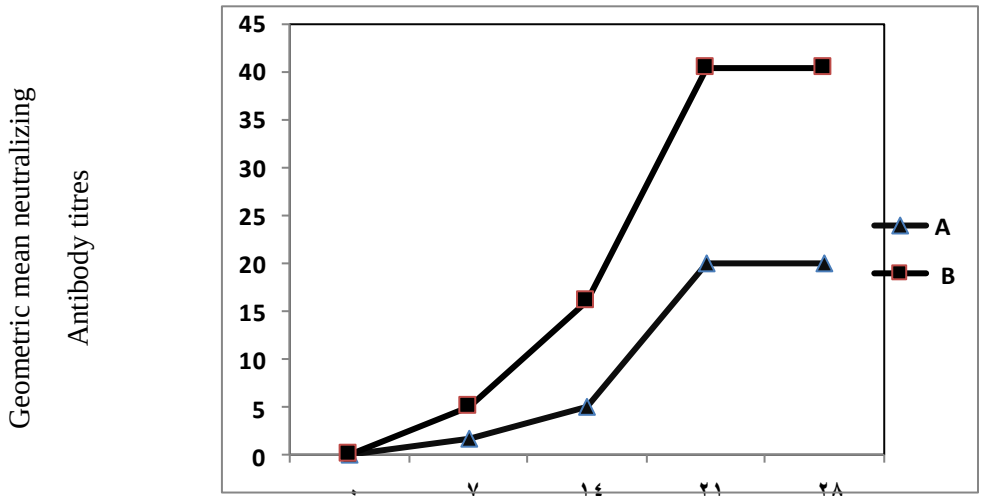
5	0.0	1.0

Table (6) Mean neutralizing PPR antibody titers in sera of goats vaccinated with WBM and Tris/Tre stabilized live attenuated vaccine.

Days post vaccination	*Mean neutralizing antibody titers		
	PPR virus vaccine with WBM	PPR virus vaccine with Tris/Tre	Non vaccinated group
0	0	0	0
7	**1.6	5.0	0
14	5.0	16.0	0
21	20.0	40.3	0
28	20.0	40.3	0

* The titer expressed as the reciprocal of the last serum dilution that inhibit the appearance of CPE produced by 100 $TCID_{50}$/0.1 ml of PPRV on Vero cells.
**The mean of three goats each was vaccinated S/C with 1ml of 2 $\log_{10}$ $TCID_{50}$ / PPRV vaccine.

Fig. (1): The level of neutralizing PPRV antibody titers in sera of goats vaccinated with live attenuated PPR virus vaccines with stabilizing medium WBM (A) or Tris/Tre (B) in an Efficacy test.



Trials to formulate stabilizer compositions aiming at potentiating thermotolerence to obtain a stable PPR virus live attenuated vaccine resisting heat stress during lyophilization, transportation, storage or during vaccination in tropical countries are abundant. (Sarkar et al., 2003) used LS, WBM, buffered gelatin-sorbitol (BUGS) and trehalose dihydrate (TD) to prepare the PPRV Sungri 96 vaccine. The results showed that LS and TD allowed for higher stability of the lyophilized PPRV vaccine.

In this study a Tris/Trehalose stabilizer formulation was evaluated in the thermal stability studies during storage and production beside their efficacy in vaccinated goats in comparison to the current used formulation of the live attenuated PPR vaccine, the Weybridge medium.

The vaccines were lyophilized in batches with WBM and Tris/Tre stabilizers. Under these lyophilization conditions, there was a loss of virus titer to a very low degree that was within the acceptable limit. The Tris/Tre formula presented a negligible loss of titre (0.1 $\log_{10}$ $TCID_{50}$ /ml), where as a discernible loss of titre (0.4 $\log_{10}TCID_{50}$ /ml) was detected with the WBM formula (Table 1). Such an advantage could be attributable to inherent properties characteristic of the components of the Tris/Tre formulation.

Exposure of the lyophilized vaccine to 4°C, showed that after 9 months ,the needed 2.5-3 $\log_{10}$ $TCID_{50}$ /dose/head is reachable if the vial vaccine is manufactured to vaccinate 100 animals (OIE, 2013) for the Tris/Tre formula compared to 5 months for WBM one. While Exposure of the lyophilized vaccine

to -4 ◦C indicated that after 12 months , the needed field dose is saved for the Tris/Tre formula compared to 9 months for WBM one. However, exposure of the vaccine to -20°C assured a period of 12 months at least through which the required vaccination field dose (2.5-3 $\log_{10}$ $TCID_{50}$) is kept. It is well known that -20°C can keep the vaccine quite valid for at least 24 months (OIE, 2013). Furthermore, exposure of the vaccine to -70°C gave approximate results to those of -20°C (Table 2, 3).

At 37°C, the Tris/Tre formula could maintain the supposed vaccine titre required for vaccination of 100 animals (4.8 - 5.0 $\log_{10}$ $TCID_{50}$) after 5 days of exposure to 37 °C, corresponding to only 1 to 1.5 days with the WBM formula (Table 4). These results are approximately similar to 6 days obtained by (Silva et al., 2011) and disagree with 10 h reported by (Sarkar et al., 2003).

Even at a drastic high temperature (45°C), the Tris/Tre formula could conserve the supposed vaccine titre required for vaccination of 100 animals (4.5 $\log_{10}$ $TCID_{50}$) after 2 days of exposure to 45°C, corresponding to only 1 day with the WBM formula (Table 5). These results agree with 49 hrs obtained by (Silva et al., 2011) and unsimilar to 3 hrs reported by (Sarkar et al., 2003).

PPR neutralizing antibodies in test goats were induced by the 7^{th} DPV with live attenuated PPR virus vaccines stabilized with WBM or Tris/Tre and higher mean neutralizing PPR antibody titers prompted by vaccine with stabilizing medium Tris/Tre. Potentiation of such a response with the use of Tris/Tre composition might be attributable to long standing stabilization effect of Tris/Tre or to inherent stimulants in this composition and / or to individual animal immune functional variations (Table 6).

From the results presented herein, it could be concluded that Tris/Tre stabilized PPRV- attenuated vaccine, enhanced to a reasonable extent both cryoprotectant and thermotolerence potentialities as compared to the WBM one and it is superior to WBM in inducing protective immune response after vaccination in tested goats. So, it is highly recommended to use the Tris/Tre formulation for protection of PPRV vaccine from heat inactivation during lyophilization, storage and transportation in tropical and subtropical areas as Egypt.

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