Assessment of two stabilizers used for lyophilized live attenuated peste des petits ruminants (ppr) vaccine.

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A B S T R A C T

The effect of using Weybridge medium (WBM) and polyvinyl pyrrolidone-NZamine (PPNZ) as Stabilizers during freeze-drying process and under varying storage temperature conditions on peste des petits ruminants (PPR) vaccine was investigated. It was found that both stabilizers gave good freeze dried preparation of PPR virus Nigeria75/1vaccines. During the process of freeze-drying, there was a total decrease of 0.6 log10 TCID50/ml for vaccine with stabilizing medium WBM and an average loss of 0.4 log10 TCID50/ml for vaccine with stabilizing medium PPNZ. The results favored the choice of PPNZ Stabilizers on viability of 75/1 PPR virus during freeze-drying process and preservation at -20◦C and -70◦C. Vaccine with stabilizing medium WBM and PPNZ stored at 4◦C for 12 months maintained sufficient viable PPR virus to protect goat. At room temperature of 25C, the two types of vaccine sufficient viable virus up to 9 months and loss their titers by 19th day of storage at 37◦C. Higher mean neutralizing PPR antibody titers induced by vaccine with stabilizing medium PPNZ than that produced by vaccine with stabilizing medium WBM in goats. In conclusion PPNZ is very effective in the stabilization of PPRV and it is superior to WBM in inducing protective immune response after vaccination of goats with no significant difference between them.

Keywords: PPR, Stabilizers, WBM, PPNZ

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1. INTRODUCTION

Peste des petits ruminant (PPR) is a highly contagious viral disease of sheep and goats characterized by high morbidity and mortality (Khan et al., 2007). The causative agent, PPR virus (PPRV), is a member of the genus Morbillivirus within the Paramyxoviridae family and is antigenically closely related to the Rinderpest (RP) virus (Gibbs et al., 1979). PPR disease is prevalent in Asian countries, sub-Saharan Africa, and in Egypt (Roeder and Obi 1999, Abd El-Rahim et al., 2010). PPR live attenuated vaccine is widely recognized as one of the most effective tools in controlling of such diseases but like other morbillivirus vaccines; has poor thermo stability (OIE, 1998). Vaccine stabilizers are well known as a chemical compounds added to a vaccine to improve its stability during storage, lyophilization, transportation and after dissolving during animal vaccination in the ambient temperature (Moran and Bucklon, 2007). Different stabilizers i.e., lactalbumin hydrolysate sucrose (LS), Weybridge medium (WBM), buffered gelatin-sorbitol (BUGS) and trehalose dihydrate (TD) are 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 this study was planned to improve the keeping quality of the produced live attenuated PPR vaccine by

using Weybridge medium (WBM) and polyvinyl pyrrolidone-NZamine (PPNZ) as stabilizer in order to maximize the vaccine thermo stability during storage, transportation and animal vaccination in hot weather.

2. MATERIAL AND METHODS

2.1.Virus and cell line

The PPR vaccine strain used was PPR virus Nigeria 75/1 obtained from Centre de Cooperation Internationale en Recherche Agronomique pour le Developpement (CIRAD) France, was used as seed virus to prepare the live attenuated PPR cell culture vaccine at Veterinary Serum and Vaccine Research Institute, Abbassia, Cairo, Egypt. African green monkey kidney (Vero) cells were maintained in Minimal Essential medium (MEM) supplemented with nystatin, penicillin and streptomycin sulphate and 10% foetal calf serum.

2.2. *Vaccine and stabilizers*

A batch of live attenuated PPR cell culture vaccine was experimentally prepared (Diallo, 2004) for research purpose. Two different stabilizers i.e., Weybridge medium (WBM) and polyvinyl pyrrolidone-NZamine (PPNZ) were used in the study. WBM stabilizer consisted of 2.5% lactalbumin hydrolysate, 5% sucrose and 1% sodium glutamate, while PPNZ consisted of 2 % polyvinyl pyrrolidone, 4 % NZamineB, 8.2 % sucrose and 1 % sodium glutamate. Equal volumes of the virus suspension and stabilizers were mixed. The vaccine vials containing different stabilizers were then lyophilized simultaneously under identical conditions.

2.3.Lyophilization

The lyophilization protocol was adapted to the lyophilizer from the 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 distilled water and titrated immediately after lyophilization to evaluate the effect of the process on virus stability.

2.4.Thermo-stability of lyophilized vaccines

Sufficient numbers of lyophilized vaccine vials for each stabilizer were stored at -20 ◦ C, -70 ◦ C, and 4 ◦ C (up to 12 months), and at 37 ◦ C (up to19 days). Samples were taken along time and the lyophilized samples were reconstituted with 1 ml of distilled water. The samples stored at 4 ◦ C were immediately titrated; those samples kept at 37 ◦ C were stored at − 80 ◦ C until titration in order to perform the 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 (TCID50) and end points were calculated as per Reed and Muench(1938).

2.6.Goats and experimental design

Twelve local breed female goats of nine to twelve months old 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 of the different stabilized vaccine by dividing into three groups as follow: Group I: Each of five goats was vaccinated subcutaneously with 1ml of 2 log10 TCID50 live attenuated PPR virus vaccines with stabilizing medium WBM. Group II: Each of five goats was vaccinated subcutaneously with 1ml of 2 log10 TCID50 live attenuated PPR virus vaccines with stabilizing medium PPNZ. Group III: two Goats were left as non vaccinated controls. Each of these Goats was subcutaneously injected with physiological saline and was left as control. Goats were housed in mosquito proof isolated stable and daily observed as well as body temperature was recorded

2.7.*Serum samples*

All sera were collected from groups I, II, III 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 (SNT)*

It was used to evaluate the immune response of the vaccinated goat as the collected sera were titrated against a constant titer of PPR virus (100 TCID50) according to OIE (2004).

3. RESULTS

3.1.PPR virus titers of wet vaccines and immediately after lypohilization

The titer of PPR virus in wet vaccines with stabilizer WBM or PPNZ was 6.6 log10 TCID50/ml and the titer immediately after lypohilization was 6.0 log10 TCID50/ml and 6.2 log10 TCID50/ml for the vaccine with stabilizer WBM and PPNZ respectively. The total virus losses during lypohilization process were 0.6 log10 TCID50/ml and 0.4 log10 TCID50/ml WBM and PPNZ stabilized vaccines respectively (table1).

Table (1) PPR virus titer in vaccine with stabilizers WBM and PPNZ pre and post lypohilization

Type of stabilizer	Virus titer expressed by log 10 $TCID_{50}$/ml		
	Pre-lypohilization	Post-lypohilization	Loss in virus titer
WBM	6.6	6.0	0.6
PPNZ	6.6	6.2	0.4

3.2.Stability of live attenuated PPR virus vaccines stored at -20°C and -70°C

Among WBM and PPNZ stabilizers, PPR vaccines with stabilizing medium PPNZ stabilized the initial titer (6.2 log10 TCID50/ml) of the vaccine for < 5 months at -20°C and up to 12 months at -70°C, while vaccines with stabilizing medium WBM stabilized the initial titer (6.0 log10 TCID50/ml) of the vaccine for < 3 months at -20°C and < 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 0.4 log10 TCID50/ml for vaccine with stabilizing medium WBM and 0.2 log10 TCID50/ml for vaccine with stabilizing medium PPNZ (table2).

Table (2) PPR virus titer in lyophilized vaccine with stabilizers WBM and PPNZ stored at -20C and -70C

Months	Virus titer expressed by log 10 $TCID_{50}$/ml			
	-20C		-70C	
	PPR virus vaccine with WBM	PPR virus vaccine with PNZ	PPR virus vaccine with WBM	PPR virus vaccine with PPNZ
1	6.0	6.2	6.0	6.2
3	5.9	6.2	6.0	6.2
5	5.9	6.1	6.0	6.2
7	5.8	6.1	6.0	6.2
9	5.8	6.1	6.0	6.2
12	5.6	6.0	5.9	6.2

3.3.Stability of live attenuated PPR virus vaccines stored at 4°C

The vaccine with stabilizing medium WBM fell from 5.7 to 3.9 log10 TCID50/ml after 12 months of storage at 4C, while vaccine with stabilizing medium PPNZ fell from 6 to 4.3 log10 TCID50/ml for the same period (table3). The total loss of virus during the storage period was 1.8 log10 TCID50/ml for vaccine with stabilizing medium WBM and 1.7 log10 TCID50/ml for vaccine with stabilizing medium PPNZ.

Table (3) PPR virus titer in lyophilized vaccine with stabilizers WBM and PPNZ stored at 4C

Months	Virus titer expressed by log 10 $TCID_{50}$/ml	
	PPR virus vaccine with WBM	PPR virus vaccine with PPNZ
1	5.7	6.0
3	5.4	5.8
5	5.0	5.6
7	4.8	5.1
9	4.4	4.9
12	3.9	4.3

3.4.Stability of live attenuated PPR virus vaccines stored at 25°C

Titer of vaccine with stabilizing medium WBM fell from 5.2 to 1.9-log10 TCID50/ml after 9 months of storage at 25C, while titer of vaccine with stabilizing medium PNZ fell from 5.7 to 2.4 log10 TCID50/ml for the same period, while both vaccines fell to zero at 12th month of storage (table4). The total loss of virus during 9 months of storage period was 3.3 log10 TCID50/ml for both types of stabilized vaccines.

Table (4) PPR virus titer in lyophilized vaccine with stabilizers WBM and PPNZ stored at 25C

Months	Virus titer expressed by log 10 $TCID_{50}$/ml	
	PPR virus vaccine with WBM	PPR virus vaccine with PPNZ
1	5.2	5.7
3	4.4	5.0
5	3.9	4.3
7	2.9	3.4
9	1.9	2.4
12	0	0

3.5.Stability of live attenuated PPR virus vaccines stored at 37°C

The vaccine with stabilizing medium WBM dropped from 5.5 to 1.2 log10 TCID50/ml after 17 days of storage at 37C, while vaccine with stabilizing medium PPNZ dropped from 5.9 to 1.5 log10 TCID50/ml for the same period, while both vaccines dropped to zero by 19th day of storage (table5). The total loss of virus during the storage period was 4.3 log10 $TCID_{50}$/ml for vaccine with stabilizing medium WBM and 4.4 log10 TCID50/ml for vaccine with stabilizing medium PPNZ.

3.6.Efficacy of live attenuated PPR virus vaccines with WBM and PPNZ stabilizer

Both live attenuated PPR virus vaccines with stabilizing medium WBM or PPNZ induce neutralizing antibodies by the 7th day post vaccination (DPV) till 28th DPV. Higher mean neutralizing PPR antibody titers induced by vaccine with stabilizing medium PPNZ than that produced by vaccine with stabilizing medium WBM (table6).

Table (5) PPR virus titer in lyophilized vaccine with stabilizers WBM and PPNZ stored at 37C

Days	Virus titer expressed by log 10 $TCID_{50}$/ml	
	PPR virus vaccine with WBM	PPR virus vaccine with PPNZ
1	5.5	5.9
3	5.0	5.4
5	4.9	5.1
7	4.2	4.7
9	3.7	4.1
11	3.0	3.6
13	2.5	3.0
15	1.9	2.4
17	1.2	1.5
19	0	0

Table (6) mean neutralizing PPR antibody titer in sera of goat vaccinated with WBM and PPNZ stabilized live attenuated vaccine.

Days post vaccination	Mean neutralizing antibody titer*		
	PPR virus vaccine with WBM	PPR virus vaccine with PPNZ	Non vaccinated group
0	0	0	0
7	0.8**	3.2	0
14	5.2	14.4	0
21	17.6	28.8	0
28	19.2	28.8	0

* The titer expressed as the reciprocal of the least serum dilution that inhibit the appearance of CPE produced by 100 TCID50/0.1 ml of PPRV on vero cells, **the mean of five goats each vaccinated S/C with 1ml of 2 log10 TCID50 PPR.

4. DISCUSSION

Most vaccines are susceptible to damage by elevated temperatures and many are also damaged by freezing. 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). The development of a stable live attenuated PPR virus vaccine resisting heat stress during lyophilization, transportation, storage or during vaccination in tropical countries is highly recommended. Previous studies with the lyophilized PPRV Nigeria 75/1 vaccine with lactalbumin hydrolysate-sucrose (LS), Weybridge medium (WBM) and lactalbumin hydrolysate-manitol (LM) have shown that the WBM formulation could maintain the virus titer for longer (Asim et al 2008). Different stabilizers, i.e. LS, WBM, buffered gelatin-sorbitol (BUGS) and trehalose dihydrate (TD) were also used to prepare the PPRV Sungri 96 vaccine. The results showed that LS and TD allowed for higher stability of the lyophilized PPRV vaccine (Sarkar et al. 2003). This study focused on the stability evaluation of PPR virus vaccine during storage and production beside their efficacy in vaccinated goats by using two different stabilizers WBM and PPNZ. The vaccines were freeze-dried in batches with WBM and PPNZ stabilizers. Under these freeze-drying conditions, there was a loss of virus titer to a very low degree that was within the acceptable limit. The total virus losses during lypohilization process were 0.6 log10 TCID50/ml and 0.4 log10 TCID50/ml WBM and PPNZ stabilized vaccines respectively (table1). This indicated that PPNZ is one of the best cryoprotectants like Trehalose dihydrate (TD), a natural cryoprotectants in a dehydrating environment and has been used for dehydration of PPR vaccine without the need for freezing during lyophilization or for desiccation under vacuum conditions (Worrall et al 2000). PPR vaccines with stabilizing medium PPNZ stabilized the initial titer of the vaccine for < 5 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 < 3 months at -20°C and < 12 months at -70°C (table2). This demonstrated the effect of low temperature on virion stability beside presence of stabilizer (Silva et al, 2011). At 4 ◦C, both lyophilized formulations (PPNZ and WBM) showed similar, higher half-lives of 12 months (Table 3). These results are superior to the 30 days reported by Sarkar et al. for the WBM under the same conditions, for the PPRV Sungri 96 vaccine (Sarkar et al. 2003) and in agreement with (Silva et al, 2011) At 25◦C, stabilizer WBM and PPNZ saved the titer till 9 months of storage (Table 4), but temperatures of 37□ are normally encountered in many parts of the tropical regions of the country during summer months, the lyophilized WBM and PPNZ stabilized titer till 17th day of storage (Table 5). Un-similar to the 10 h reported by Sarkar et al. (2003) for the same formulation used in the PPRV vaccine of the Asian origin. While Riyesh et al. (2011) reported that the lyophilized PPR vaccine showed an expiry period of 7-8 days at 37°C. This favored the OIE (2004), which concludes that WBM medium is a stabilizer of choice for freeze-drying of PPR cell culture vaccine to save the vaccinal titer and we add PPNZ. PPR neutralizing antibodies in goats were induced by the 7th DPV with live attenuated PPR virus vaccines stabilized with WBM or PPNZ and higher mean neutralizing PPR antibody titers induced by vaccine with stabilizing medium PPNZ (Table 6). This may attributed to the long standing stabilization effect of PPNZ beside may have a role in immunogenicity due to presence of amine in addition to disaccharide sucrose (Crowe, et.al. 1987; Pitaksuteepong 2005)

From the obtained results it could be concluded that PPNZ is very effective in the stabilization of PPRV and it is superior to WBM in inducing protective immune response after vaccination with no significant difference between them. So the use of PPNZ could be recommended for protection of PPRV vaccine from heat inactivation during lyophilization, storage and transportation in tropical and subtropical areas as Egypt.

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