



Efficacy of Carbomer as a Stabilizer for Lyophilized attenuated Peste des Petits Ruminants (PPR) vaccine

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ABSTRACT

Peste des petits ruminants (PPR) is a category of acute fulminating infectious viral disease, affecting seriously sheep and goats. It is caused by PPRV that classified within a Morbillivirus; family Paramixoviridae. Vaccination is the cornerstone to protect sheep and goats against such diseases. The present work aims to provide a highly potent attenuated lyophilized PPR vaccine where three formulae were prepared including formula (1) stabilized with 1% carbomer and 2% peptone; formula (2) stabilized with 0.5% carbomer and formula (3) stabilized with 2% peptone; 10% sucrose and 0.27% dihydrogen orthophosphate. The three lyophilized PPR vaccine formulae had compact similar cacks without different shape appearances. Also, the three formulae were found to be free from foreign contaminants (aerobic and anaerobic bacteria; fungi and mycoplasma) having no significant difference in the virus titer pre- and post-lyophilization. Reduction in the virus titer post lyophilization was 0.25, 0.5 and 0.5 log₁₀ TCID₅₀/ml in relation to formula 1; 2 and 3 respectively. Although formula (2) induced the peak PPR serum neutralizing antibody titer earlier (128 by the 3rd week) than the other 2 formulae (128 by the 4th week); the potency test revealed that all vaccine formulae were potently inducing high protective PPR immune levels in vaccinated sheep up to 6 months post-vaccination. So, it could be suggested that carbomer accelerate the time to reach the peak of PPR antibody titers in vaccinated sheep.

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INTRODUCTION

Peste des Petits Ruminants (PPR), which is known as the small ruminant plague is a highly contagious disease caused by *Morbillivirus*, family *Paramyxoviridae*. It can infect up to 90% of an animal herd and kills anywhere up to 70% of infected animals. The PPR fulminates contingency because of its rapid outbreak and high mortality rate just which usually develops clinical signs in 3–5 days, followed by the culmination of pyrexia. Symptoms later develop into mucosal hyperemia, mucoid nasal discharges, anorexia, and diarrhea. Susceptible ruminants usually develop acute pulmonary congestion and oedema and give up to death in a week (Olaleye *et al.*, 1989).

Vaccination is the utmost effective controlling manner to PPR without any notable immunosuppressive effect on the animal. Recently, the PPRV strain type (Nigeria 75/1) is adapted in

Vero cells and used as vaccine frequently in African countries (Sarkar *et al.*, 2003; Singh *et al.*, 2010; Singh, 2011). A PPR vaccine dose containing 10³ TCID₅₀ of Vero cell-weakened PPRV is thought to make available protective immunity in small ruminants for 4 years (Singh *et al.*, 2009). Vaccinated animals manufacture many neutralizing antibodies in contrast to the H, F, and N proteins, analogous to those recovered from a natural infection (Diallo *et al.*, 1987 and Sinnathamby, 2001). Commercially available PPR vaccines, such as Nigeria75 and Sungr196, are thought to make available protective immunity against all genetically defined lineages suggesting no serological consequence of lineage classification.

Lyophilization, or freeze-drying, is a complex process that can be used to stabilize vaccines. Stabilizing the vaccine is a vital factor to reserve vaccine more potency and efficiency without

decaying its potency over time and changing in temperature because of its effectiveness against temperature, light, humidity, or acidity during storage. Therefore, choosing the stabilizers of viral vaccine form is contingent chiefly on the type of vaccine, the active pharmaceutical ingredient or viral antigen(s) properties, and the structure of the vaccine (Razieh et al., 2017).

Vaccination with live or inactivated vaccines necessitates adjuvants preparations acting as immunostimulants such as synthetic carbomer and acrylic acid having the properties of polymers and high molecular weight. Carbomer is using as adjuvants in veterinary vaccines since 1970s by enabling vaccine conveyance and promoting cellular immune response with longtime, such as: modified-live vaccine for porcine reproductive and respiratory syndrome (PRRS1), live-attenuated Newcastle disease virus (NDV) vaccine, swine parvovirus vaccine; circovirus type 2 vaccine; freeze-dried inactivated bovine viral respiratory combined vaccine, *Staphylococcus aureus* vaccine for sheep, inactivated equine herpes virus-1 vaccine, and inactivated rabies vaccine and freeze-dried bovine ephemeral fever virus vaccine (Mair et al., 2015). Previous studies had evidenced that the utilizing of carbomer is safe in vaccine preparation and has more robust immune responses than those by orthodox vaccines. Carbopol® boosts cellular immunity by polarizing a strong type-1 T-cell (Th1) and producing interferon-gamma (IFN γ) as well as boosts antigen capture by macrophages, especially the dendritic cells (Naglaa et al., 2020).

This study attempted to evaluate the use of Carbomer as a stabilizer to the locally produced PPR live attenuated vaccine and to enhance its immunogenicity.

MATERIALS AND METHODS

Ethical approval

This research was accepted via the Animal Ethics Committee of the Veterinary Serum and Vaccine Research Institute (VSVRI), Agricultural Research Center (ARC), Egypt and coordinated with the VSVRI guidelines for animal research.

1. Sheep

Twelve susceptible native breed sheep, aged 6-8 months and screened by serum neutralization test (SNT) for free from PPR virus-neutralizing antibodies, were allocated into four clusters, three sheep each. All sheep housed under hygienic measures received a balanced ration and adequate water with clinical observation for two weeks of the experimentation period. The prepared PPR vaccine formulae were then applied to evaluate their potency on those sheep.

2. Cell Culture

African green monkey Kidney (Vero) cells supplied by Veterinary Serum and Vaccine Research Institute (VSVRI) were cell-cultured in the Minimum Essential Medium with Earle's salts (MEM, Sigma Chemical Company, UK). This media was supplemented by heat-inactivated 10% Newborn calf serum, 100 IU/mL penicillin, 100 μ g/mL streptomycin sulphate, and 25 IU/L myostatin (Gibco Laboratories, New Zealand). The temperature was adjusted at 37°C.

3. Peste des Petits Ruminant Virus

The live attenuated PPR virus, with a titer of 10^{5.5} TCID₅₀/mL, was attenuated from the Department of Rinderpest Vaccine Research (DRV), VSVRI, and titrated on the Vero cell line. The virus had verified free of any inessential contamination. Both were then prepared for vaccine and serum neutralization test (SNT).

4. Virus Titration

The prepared live PPR vaccine formulae via carbomer and peptone/sucrose were titrated on the Vero cell line pre- and post-lyophilization using the microtiter technique (Rossiter and Jessette, 1982). Then, virus titer was stated as log₁₀ TCID₅₀/ml according to Reed and Meunch (1938).

5. Carbomer

The used carbomer in the PPR vaccine is Carbopol® 940 NF (Lubrizol®) polymer. It was diluted to 0.25%, 0.5%, and 1% by the hot ddH₂O. Solutions were separately autoclaved at 121°C/20min and then stored at 4°C. All solutions were calibrated to pH 7.3 before use.

6. Preparation of lyophilized attenuated PPR vaccine formulae

PPR virus with titer 10^{5.5} TCID₅₀/mL was inoculated into the Vero cell line and collected when the full cytopathic effect was obtained. After three freeze-thaw cycles, centrifugation was processed at 2000rpm for 10 minutes in a cool centrifuge and the supernatant was collected and subjected to virus titration. Then three freeze-dried PPR vaccine formulae were prepared via many stabilizers as the following:

Formula-1 stabilized with 1% carbomer and 2% peptone.

Formula-2 stabilized with 0.5% carbomer only.

Formula-3 stabilized with 10% sucrose; 2% peptone and 0.2% dihydrogen orthophosphate.

These stabilizers were added to PPR virus suspension in a proportion of 1:1 (V/V), then dispensed as 2.5ml / glass vial and exposed to lyophilization on Teflon lyophilize apparatus. The freeze-drying system was made full into 2.5ml sterilized glass vials, inner diameter 1.9cm, covered

with a semipermeable rubber plug. Vials were then quickly frozen on the freeze-dryer that precooled to -60°C (Wang and Zhang, 2007), for 2 hours. The primary drying began. The shelf temperature was set at -32°C , and the vacuum was controlled under 10 Pa (Zhou *et al.*, 2007). The primary drying process continued for 16h. The shelf was then warmed up to 20°C at a rate of $0.2^{\circ}\text{C}/\text{min}$ and kept for 6h. After then, vials were plugged in and held for 2h at room temperature (Shao-zhi *et al.*, 2010). They were then stored at -20°C until used.

7. Quality control testing of the prepared PPR vaccine formulae

7.1. Physical appearance

All lyophilized PPR vaccine formulations including Carbopol® and peptone/sucrose stabilizer undertook physical examination.

7.2. Sterility

Random samples from all prepared PPR vaccine formulae were verified for any contaminations, aerobic bacteria, fungi and mycoplasma on thioglycolate medium, Sabouraud agar, nutrient agar and mycoplasma medium according to WHO (2009)

7.3. Safety Test

Three susceptible sheep were exposed to 10X Carbopol® stabilized PPR vaccine field dose inoculated S/C in the neck side and subjected to daily clinical examination up to 15 days post vaccination.

7.4. Potency Test

Twelve sheep were separated into four clusters (three sheep per cluster), where all clusters were vaccinated with the prepared PPR vaccine formulae 1, 2, and 3, respectively except the fourth one was held as control.

The used vaccine dose was the recommended field dose ($10^{2.5}\text{TCID}_{50}/\text{animal}$) inoculated S/C in the neck side according to Khodeir and Mouaz (1998). Serum samples were attained from all sheep clusters for monitoring of PPR antibodies in their sera pre and on week intervals four times post vaccination; then on monthly intervals up to 6 months post vaccination.

8. Serum neutralization test (SNT)

SNT was carried out on serum samples gained from all sheep groups via the microtiter technique according to Ferreira (1976). The end point of neutralizing antibody titers was expressed as the reciprocal of the final dilution of serum inhibiting the CPE according to Sing *et al.*, (1967).

9. Indirect Enzyme Linked Immune Sorbent Assay (ELISA)

It was carried out on sheep sera according to Voller, *et al.*, (1976) and Hubschle *et al.*, (1981).

RESULTS

The physical appearance of the three prepared lyophilized PPR vaccine formulae revealed compact cack (fig.1) without detectable apparent difference. Also, all vaccine preparations were free from any contaminations like fungi and mycoplasma contaminants and aerobic and anaerobic bacteria. Virus titration lyophilization, as shown in Table (1), revealed that the loss of virus titer was $0.25\log_{10}\text{TCID}_{50}/\text{ml}$ using 1% carbomer and $0.5\log_{10}\text{TCID}_{50}/\text{m}$ using 2% peptone with 0.5% carbomer and using 2% peptone; 10% sucrose and 0.2% dihydrogen orthophosphate.

Table 1: PPR virus titers in the prepared lyophilized vaccine formulae

Vaccine formulae	Virus titer $\log_{10}\text{TCID}_{50}/\text{ml}$	
	Before lyophilization	After lyophilization
PPRV + carbomer 0.5% only	↑ 6 ↓	5.75
PPRV + carbomer 1% with 2% peptone		5.50
PPRV with peptone/sucrose.		5.50

Final freeze-dried vials PPRV vaccine; Vial-1: Stabilized vaccine with 2% peptone; 10% sucrose and 0.27% dihydrogen orthophosphate Vial-2: Stabilized vaccine with 1% carbomer+ 2% peptone; Vial-3: Stabilized vaccine with 0.5% carbomer as showed in Fig (1).

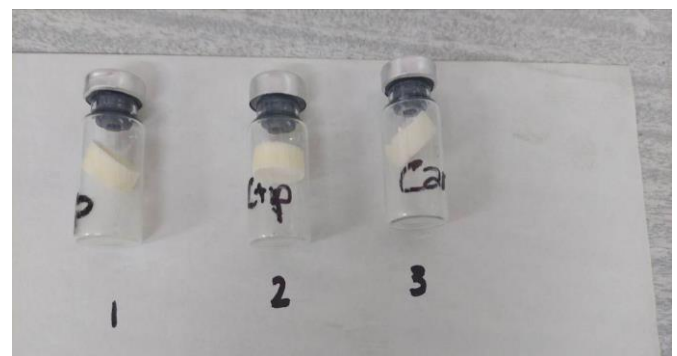


Fig.1: Final freeze-dried vials PPRV vaccine

Testing the safety of the prepared PPR vaccine formula stabilized with carbomer showed that it did not promote any abnormal systemic or local post inoculation reaction with 1% concentration in the vaccine formula indicating its safety (the safety of 0.5% carbomer did not tested as it is the lower concentration and that of peptone and sucrose was previously determined).

The results of SNT in table (2) and ELISA in table (3) showed that, the specific PPR antibodies in vaccinated sheep since the first week post-vaccination but the formula stabilized with 1% carbomer induced earlier antibody peak titer (128 by the 3rd week) than in case of the use of the other 2 formulae (128 by the 4th week) although all induced immune levels remains with their highest titer up to 6 months post vaccination.

Table 2: PPR serum neutralizing antibody titers in different vaccinated sheep groups

Sheep Groups	PPR serum neutralizing antibody titers*									
	Animal number	1WPV**	2WPV	3WPV	4WPV	2MPV***	3MPV	4MPV	5MPV	6MPV
Group (1)	1	4	16	64	128	128	128	128	128	128
	2	4	16	64	128	128	128	128	128	128
	3	2	16	64	64	128	128	128	128	128
Geometric mean		3.33	16	64	106.6	←128→				
Group (2)	4	2	32	128	128	128	128	128	128	128
	5	2	32	128	128	128	128	128	128	128
	6	4	32	128	128	128	128	128	128	128
Geometric mean		2.6	32	128	128	←128→				
Group (3)	7	4	16	32	64	128	128	128	128	128
	8	4	16	32	64	128	128	128	128	128
	9	4	16	32	64	128	128	128	128	128
Geometric mean		4	16	32	64	←128→				
Group (4)	10	All sheep in this group remain seronegative all over the experimental period								
	11									
	12									
Geometric mean										

*PPR serum neutralizing antibody titer = the reciprocal of the final serum dilution which neutralized and inhibited the CPE of 100 TCID₅₀ of PPR virus

**WPV= week post vaccination

***MPV= month post vaccination

Group (1): vaccinated with PPRV with 1% carbomer and 2% peptone

Group (2): vaccinated with PPRV with 0.5% carbomer only.

Group (3): vaccinated with PPRV with 2% peptone; 10% sucrose and 0.27% dihydrogen orthophosphate only.

Group (4): unvaccinated control.

Table 3: ELISA Delta Optical density (Δ OD) for PPR antibodies in vaccinated sheep

Sheep groups	PPR antibody ELISA Δ OD									
	Animal number	1WPV*	2 WPV	3 WPV	4 WPV	2 MPV**	3 MPV	4 MPV	5 MPV	6 MPV
Group (1)	1	0.082	0.281	0.551	0.671	0.825	1.853	1.654	1.755	1.366
	2	0.089	0.271	0.571	0.692	0.865	1.862	1.655	1.565	1.480
	3	0.092	0.321	0.507	0.683	0.915	1.852	1.665	1.585	1.385
Mean		0.088	0.291	0.543	0.682	0.868	1.856	1.658	1.635	1.410
Group (2)	1	0.079	0.287	0.562	0.685	0.866	1.838	1.685	1.590	1.390
	2	0.087	0.261	0.560	0.643	0.885	1.819	1.690	1.520	1.550
	3	0.083	0.323	0.585	0.717	0.860	1.817	1.670	1.555	1.460
Mean		0.083	0.290	0.569	0.682	0.870	1.825	1.682	1.555	1.467
Group (3)	1	0.075	0.162	0.565	0.681	0.620	1.817	1.660	1.560	1.525
	2	0.065	0.293	0.555	0.635	0.655	1.855	1.650	1.565	1.530
	3	0.080	0.255	0.545	0.625	0.665	1.885	1.635	1.885	1.540
Mean		0.073	0.237	0.555	0.647	0.647	1.852	1.648	1.534	1.532
Group (4)	1	0.084	0.081	0.087	0.085	0.082	0.083	0.086	0.084	0.087
	2	0.081	0.079	0.085	0.083	0.081	0.085	0.087	0.081	0.083
	3	0.073	0.078	0.074	0.081	0.076	0.084	0.079	0.085	0.080
Mean		0.079	0.079	0.082	0.083	0.079	0.084	0.084	0.083	0.083

*WPV = week post vaccination

**MPV = month post vaccination

Group 1: sheep vaccinated with PPR vaccine stabilized with 1% carbomer and 2% peptone.

Group2: sheep vaccinated with PPR vaccine stabilized with and 0.5% carbomer only.

Group3: sheep vaccinated with PPR vaccine with 2% peptone; 10% sucrose and 0.2% K. dihydrogen orthophosphate

Group4: control non vaccinated, Positive average PPR antibody ELISA O.D. is > 0.500.

DISCUSSION

The prepared lyophilized PPR vaccine appeared as compact cack as shown in (fig.1), and free from any contaminations like fungi and mycoplasma contaminants and aerobic and anaerobic bacteria which in agreement with the recommendations of **OIE (2019)**.

The results of Virus titration lyophilization showed loss of virus titer was $0.25 \log_{10} \text{TCID}_{50}/\text{ml}$ using 1% carbomer and $0.5 \log_{10} \text{TCID}_{50}/\text{m}$ using 2% peptone with 0.5% carbomer and using 2% peptone;

10% sucrose and 0.2% dihydrogen orthophosphate as shown in table (1), these findings indicate that the least virus loss was obtained using 1% carbomer in agreement with those of **Alexander et al., (2015)** who reported the produced water is subjected to a high vacuum (freeze-drying) and frozen during the Lyophilization process. These aspects reflect stress factors that damage and decrease in the viability of viruses. Similar results were attained by **Latif et al., (2018)** and **Ayatollah et al., (2019)** they found that prepared FP and PPR vaccines with different concentrations of skimmed milk after lyophilization showed the same lessening in virus titer (0.25

log₁₀EID₅₀) but the reduction was (0.5 log₁₀EID₅₀) by using Lactalbumin sucrose stabilize.

The safety test of the prepared PPR vaccine formula stabilized with carbomer showed safe post inoculation reaction, even with 1% concentration in the vaccine formula indicating its safety, but the safety of 0.5% carbomer did not tested as it is the lower concentration and that of peptone and sucrose was previously determined, as these finding determined also by **Mair et al., (2015)** and **Naglaa et al., (2020)**.

Regarding the effectiveness of the prepared PPR vaccine formulae; it was found that SNT (table-2) and indirect ELISA (table-3) revealed that all formulae were able to induce detectable specific PPR antibodies in vaccinated sheep since the first week post-vaccination but the formula stabilized with 1% carbomer induced earlier antibody peak titer (128 by the 3rd week) than in case of the use of the other 2 formulae (128 by the 4th week) although all induced immune levels remains with their highest titer up to 6 months post vaccination. Same results were got by **Abeer (1997)**; **Afaf et al., (1998)**; **Khodeir and Mouaz (1998)** indicated that after sheep vaccination with PPR vaccine, the antibody titer improved the highest value on day 28. Regarding the effect of carbomer on the potency of PPR lyophilized vaccine, it could be noticed that 1% carbomer accelerates the induction of PPR antibodies in vaccinated sheep in agreement with what stated by **Mumford et al., (1994)**; **Zhang et al., (2018)**; **Maha et al., (2019)** and **Naglaa et al., (2020)** determined that carbomer was proved to be pure, fully safe and highly potent.

CONCLUSION

In conclusion, it could be said that the use of 1% carbomer in PPR lyophilized vaccine enhance and accelerate the induction of PPR antibodies in vaccinated sheep.

Conflicts of interest

The authors declare that there is no conflict of interest regarding the research data and tools used with this study.

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