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Research Article

Protective Value of Freeze-Dried Inactivated Bovine Viral Respiratory Combined Vaccine Stabilized by Carbomer and Adjuvanted with Saponin

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Abstract | Adjuvants have been used in veterinary vaccines. Carbomer is a synthetic polymer which has a potential vast of applications in the pharmaceuticals. This work aimed to examine the immunologic effect of the carbomer as a stabilizer and adjuvanted with saponin in the lyophilized combined inactivated vaccine (Pneumo-4) instead of regular stabilizers. Freeze-dried vaccine named Pneumo-4 contained the inactivated bovine viral diarrhea virus (BVDV) genotypes, bovine herpesvirus type 1 (BoHV-1), bovine parainfluenza virus type 3 (BPI3V), and bovine respiratory syncytial virus (BRSV). The carbomer 0.3% and 0.5% were used as a stabilizer. The novel stabilizer was compared with 5% lactalbumin hydrolysate with 2.5% sucrose. The saponin solution 1mg saponin/dose was used as an adjuvant and solvent. Twelve local breed calves were utilized in the study. The calves were divided into four groups where each group contained three calves. The first two groups were vaccinated with bovine viral respiratory combined vaccine stabilized by carbomer 0.3% and 05%, respectively. The third group vaccinated with the vaccine stabilized by 5% lactalbumin hydrolysate with 2.5% sucrose. The fourth group was kept without vaccination as a test control. The poster dose was given to the calves of the first three groups 2 weeks post-vaccination. The antibody titer in vaccinated calves was evaluated by the virus neutralization test (VNT). The vaccines showed safety in calves. The immune response against the four viruses got its peak in the 4th month post vaccination. The vaccines stabilized by carbomer showed an appropriate level of protective antibody which lasted until the 9th month post vaccination. There was a very high significance ($p \leq 0.05$) between the values of neutralizing antibodies between different groups of the experiment. Finally, the use of carbomer as a stabilizer, in addition to saponin as an adjuvant, in the bovine viral respiratory combined vaccine enhances the antibody production in the immunized animals for a time of approximately 9 months. Consequently, the prepared vaccine has a significant economic impact, as it reduces the numbers of vaccination, and thus reduces the costs involved in terms of labor and tools as well as reducing the stress on animals.

Keywords | Bovine respiratory disease complex, vaccines, Freeze drying, Carbomer, Saponins

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INTRODUCTION

Vaccines need development through using new stabilizer and adjuvant to induce satisfactory immunological response and to attain protection during times of challenge. Adjuvants have been used in veterinary vaccines for almost a century in trying to raise vaccine immunogenicity, chiefly by motivating innate immunity and increasing adaptive

immune responses (Petrovsky, 2015). The development in existing vaccine design is mainly directed to the enhancement of the immune responses of commercial vaccines by means of using novel stabilizer and adjuvants (Levitz and Golenbock, 2012). So, there is a necessity for designing an uncommon and new stabilizer and adjuvant able to increase the efficacy of the immune response (Chauhan et al., 2017). The steadiness of the vaccine can be judged according to the circumambient temperature which affects its expiration. Such studies are important for vaccines used in developing countries where vaccine quality agonizes due to logistics issues such as transportation, storage, power failures and unqualified labor (Sarkar et al., 2003).

Carbapol (carbomer) is a synthetic polymer which has a potential vast of applications in the pharmaceuticals. Some advantages of using aqueous carbomer gels are their compatibility with many constituents, thermos-stability and, smooth flow during various routes of application (Islam et al., 2004). Carbapol is a cross-linked chain of acrylic acid, and has several properties such as high safety, non-toxic and suspending agent (Ahuja et al., 1997). Nowadays, carbapols are gaining considerable interest as adjuvants for veterinary vaccines.

Previous studies used the carbomer as an adjuvant in various vaccine formulations on different animals. Studies proved the high safety margin and superior immune response in comparison to the traditional vaccines (Mair et al., 2015, Stadler et al., 2016, Abd El-Moneam et al., 2020).

Carbapol (carbomer) can enhance and activate cellular and humoral immunity in mammals (Gartlan et al., 2016). The merits of using aquatic carbomer gels include easy flow behavior, compatibility mode with several active ingredients; and good thermal steadiness (Zhang et al., 2018).

Saponins, extracted from *Quillaja saponaria* Molina, have been used to a large degree as adjuvants for decades in various animal vaccines. However, despite its recognized adjuvant potential, its use still debatable due to unwanted side effects, such as local reactions, hemolytic activity and systemic toxicity (Sun et al., 2009).

For the control of bovine respiratory disease (BRD), the usual practice was to use an inactivated combined vaccine (O'Connor et al., 2019). Recently, an inactivated vaccine has been developed at Veterinary Serum and Vaccine Research Institute (VSVRI), Egypt named Pneumo-4. It is formulated from inactivated Bovine viral diarrhea virus (BVDV), Bovine herpesvirus type 1 (BoHV-1), Bovine Respiratory Syncytial Virus (BRSV), and Bovine Parainfluenza Type-3 virus (BPI3V) and adjuvanted with alum gel and/or oil (EL-Hawary and Mostafa, 2017).

This work aims to study the immunologic effect of the Carbapol as stabilizer and adjuvanted with saponin in the lyophilized combined inactivated vaccine (Pneumo-4) instead of regular stabilizer lactalbumin hydrolysate with sucrose.

MATERIAL AND METHODS

Ethical approval

The use of the animals and its care were granted by the Medical and Veterinary Research Ethics Committee at the National Research Centre in Egypt (No., 20/053).

Viruses and cell line

The native Egyptian strains were used in the preparations of these study vaccines. The viruses were bovine respiratory syncytial virus (BRSV), bovine parainfluenza-3 virus (BPI3V), bovine viral diarrhea virus genotype-1 (BVDV-1), and bovine herpesvirus type 1 (BoHV-1) with titers of $10^{8}TCID_{50}$/mL (tissue culture infectious dose 50%), $10^{6.5}TCID_{50}$/mL, $10^{6.5}TCID_{50}$/mL and $10^{7.5}TCID_{50}$/mL, respectively. The viruses were provided from VSVRI, Cairo, Egypt. These viruses were propagated and titrated on the Madin-Darby bovine kidney (MDBK) cell line which was free from any irrelevant contaminant. The identity of these viruses was confirmed by the VNT using specific reference antisera.

Stabilizers

In this study, three freeze-dried vaccines against the mentioned above viruses were prepared using different stabilizers. The first vaccine was stabilized by the common stabilizer which is 5% lactalbumin hydrolysate (Sigma-Aldrich GmbH) with 2.5% sucrose according to Riyesh et al. (2011). The other two vaccines were stabilized by 0.3% and 0.5% carbomer (Lubrizol, USA) according to Abd El-Moneam et al. (2020).

Adjuvant

The saponin solution 1 mg/dose (*Quillaja Saponaria* Molina) (Sigma-Aldrich GmbH) extract was used as adjuvant during reconstitution of lyophilized vaccine according to Abd El Fadeel et al. (2020).

Vaccine preparation

The bovine viral respiratory combined vaccine was articulated to comprise the four inactivated antigens of BVDV-1, BoHV-1, BPI3V, and BRSV. Viruses were inactivated separately by 0.01M binary ethyleneimine (BEI) 10% volume/volume [v/v]. Then, Sodium thiosulphate (20%) was used to stop the effect of BEI after 24 h from the start of inactivation (Bahnemann, 1990). Meanwhile, equal amounts from the four inactivated viruses' fluid were blended. Thereafter, the stabilizers v/v were added and the

vaccines were aliquoted and lyophilized. The lyophilization process was conceded using a lyophilize apparatus (2014-033; Shanghai Tofflon Science & Technology Co. Ltd., Shanghai, China).

Experimental animals

Calves: Twelve healthful local breed calves with ages between 4-8 months were utilized in the evaluation of the safety and protective values of the prepared vaccines. Calves were free from external parasite and seronegative to the viral strains of the bovine viral respiratory combined vaccine. Calves were divided into four groups where each group contained three calves. The first group was vaccinated (3ml each animal) with bovine viral respiratory combined vaccine stabilized by carbomer 0.3%. The second group was vaccinated with the vaccine (3ml each animal) stabilized by carbomer 0.5%. The third group vaccinated with the vaccine (3ml each animal) stabilized by 5% lactalbumin hydrolysate with 2.5% sucrose. The fourth group was kept without vaccination as a test control. The poster dose was given to the calves of the first three groups 2 weeks post-vaccination (WHO, 2019).

Quality control of the vaccine

Sterility test: According to WHO (2019), all stages of vaccine manufacture were succumbed to in-process testing for sterility to confirm free from any contaminants.

Safety in calves: Twelve male calves were divided into four groups (3calves/group) and used to evaluate the safety of the prepared vaccines. The first three groups were injected intramuscularly (I/M) with a double dose (6ml each animal) of the lyophilized vaccines stabilized by carbomer 0.3%, 0.5% and lactalbumin hydrolysate 5% with sucrose 2.5%, respectively. While the calves in the 4th group were injected I/M with the same dose by physiological saline instead (Code of Federal Regulations, 2015). All calves were kept under observation for 21 days to record the appearance of any clinical abnormalities.

Serological tests to evaluate the potency of the vaccines in calves

Serum samples were collected on the first day of vaccination (0 days), 2nd week, 4th week, and every month until reaching the lowest unprotective levels of neutralizing antibodies. All serum samples were inactivated at 56°C for 30 minutes and stored at -20°C until used in virus neutralization test against all viral components of the prepared vaccines. The virus neutralization test was done according to Robson et al (1960) and the antibody titer calculated according to Reed and Muench (1938). The antibody titer was expressed as $\log_{10}$.

Statistical analysis

The statistical analysis was done by Microsoft Excel 2010 (Microsoft Corp., Redmond, WA, USA). The records data were expressed as mean ± standard deviations. Data between groups in the same virus were compared using the one way analysis of variance (ANOVA) test. A p-value ≤ 0.05 was considered statistical significance.

RESULTS

The analyses of the data showed that the prepared vaccines were sterile and free from any contaminant and safe to all calves. There were no clinical abnormalities that appeared during experiment time.

The physical examination of the discs produced after freeze-drying of the vaccines stabilized by carbomer 0.3%, 0.5% and lactalbumin hydrolysate 5% with sucrose 2.5% showed that the use of carbomer (0.3% or 0.5%) gave white and coherent disc which was more acceptable than the disc produced from the lyophilized vaccine stabilized by lactalbumin hydrolysate 5% with sucrose 2.5%.

The virus neutralization test was used to monitor the mean serum neutralizing antibody titers expressed in $\log_{10}$ in the serum sample against BVDV-1, BoHV-1, BPI3V, and BRSV. All vaccinated calves demonstrated high as well as protective levels of antibody titers.

The mean virus neutralizing antibody titers of BVDV-1 in lyophilized vaccine stabilized by carbomer 0.3% and 0.5% reached to 2.3±0.2, 2.1±0.56 in the 4th weeks post vaccination (WPV), respectively while it was 1.8±0.4 in the 2nd month post vaccination (MPV) in case of vaccine stabilized by lactalbumin hydrolysate 5% with sucrose 2.5%. Then, the antibody titer was decreased gradually to 0.91±0.05 and 1.08±0.03 in 8th MPV against the BVDV in the vaccine stabilized by carbomer 0.3% and 0.5%, respectively. The vaccine stabilized by lactalbumin hydrolysate 5% with sucrose 2.5% reached the unprotective level 0.75±0.05 on the 8th MPV (Table 1).

BoHV-1 antibodies titer of the vaccinated calves reached the peak protective level at the 4th WPV 2.5±0.03, 2.3±0.25 for the vaccine stabilized by carbomer 0.3% and 0.5%, respectively. While the vaccine stabilized by lactalbumin hydrolysate 5% with sucrose 2.5% reached the peak level of antibodies 1.86±0.02 at the 4th MPV. The vaccines stabilized by lactalbumin hydrolysate 5% with sucrose 2.5% lost its protective level of antibodies at the 8th MPV 0.60±0.00 while the other two vaccines maintained the appropriate immune level (Table 2).

The serological response of the vaccinated calves against

Table 1: Mean virus neutralizing antibody titers expressed in log_{10} against BVDV-1 in vaccinated calves with Pneumo-4 vaccine stabilized by 0.3% and 0.5% carbomer and 5% lactalbumin hydrolysate with 2.5% sucrose.

Time post vaccination	Carbomer 0.3% (mean± SD)	Carbomer 0.5% (mean± SD)	Lactalbumin hydrolysate 5% with sucrose 2.5% (mean± SD)
0 Day	0.05±0.0	0.2±0.0	0.02±0.0
2nd WPV	0.12±0.01[a]	0.6±0.01[b]	0.45±0.01[c]
4th WPV	2.3±0.2[a]	2.1±0.56[b]	1.7±0.25[c]
2nd MPV	2.15±0.2[a]	1.9±0.05[b]	1.8±0.4[c]
3rd MPV	2.0±0.4[a]	1.9±0.02[b]	1.75±0.05[c]
4th MPV	1.86±0.25[a]	1.5±0.2[b]	1.5±0.2[c]
5th MPV	1.7±0.2[a]	1.3±0.05[b]	1.2±0.25[c]
6th MPV	1.54±0.01[a]	1.32±0.04[b]	0.94±0.05[c]
7th MPV	1.4±0.4[a]	1.17±0.2[b]	0.10±0.04[c]
8th MPV	0.91±0.05[a]	1.08±0.03[b]	0.75±0.05[c]
9th MPV	0.22±0.04[a]	0.25±0.04[b]	0.60±0.04[c]

BVDV-1: Bovine viral diarrhea virus type 1
WPV: week post vaccination
MPV: month post vaccination
SD: Standard deviation
Means with different superscripts in the same row are significantly different at P≤0.05.

Table 2: Mean virus neutralizing antibody titers expressed in log_{10} against BoHV-1 in vaccinated calves with Pneumo-4 vaccine stabilized by 0.3% and 0.5% carbomer and 5% lactalbumin hydrolysate with 2.5% sucrose.

Time post vaccination	Carbomer 0.3% (mean± SD)	Carbomer 0.5% (mean± SD)	Lactalbumin hydrolysate 5% with sucrose 2.5% (mean± SD)
0 Day	0.03±0.0	0.02±0.0	0.30±0.00
2nd WPV	1.55±0.04[a]	0.7±0.02[b]	1.01±0.02[a]
4th WPV	2.5±0.03[a]	2.3±0.25[b]	1.13±0.02[a]
2nd MPV	2.35±0.55[a]	2.06±0.25[b]	1.47±0.01[a]
3rd MPV	2.28±0.02[a]	1.9±0.05[b]	1.52±0.01[a]
4th MPV	2.14±0.04[a]	1.72±0.2[b]	1.86±0.02[a]
5th MPV	2.0±0.14[a]	1.5±0.4[b]	1.53±0.03[a]
6th MPV	1.68±0.05[a]	1.32±0.6[b]	1.2±0.01[a]
7th MPV	1.7±0.25[a]	0.86±0.05[b]	1.02±0.03[a]
8th MPV	1.51±0.4[a]	0.75±0.02[b]	0.60±0.00[a]
9th MPV	1.3±0.05[a]	0.20±0.04[b]	0.42±0.03[a]

BoHV-1: Bovine herpes virus type 1
WPV: week post vaccination
MPV: month post vaccination
SD: Standard deviation
Means with different superscripts in the same row are significantly different at P≤0.05.

BPI3V increased gradually and reached 2.51±0.05, 2.3±0.2 in the 4th WPV and 2.00±0.02 in the 4th MPV in case of using carbomer 0.3%, 0.5% and lactalbumin hydrolysate 5% with sucrose 2.5%, respectively. Then, the antibody level was declined gradually to reach low levels in vaccine stabilized by lactalbumin hydrolysate 5% with sucrose 2% at the 8th MPV. The other two vaccines showed an appropriate level of protective antibody 1.32±0.04 and 0.88±0.04 at the 9th MPV, respectively (Table 3).

The mean antibody titer of BRSV in vaccinated calves reached the highest level 2.28±0.2 and 2.16±0.25 in the 4th WPV in the case of using carbomer 0.3% and 0.5%. While in the case of the vaccine stabilized by lactalbumin hydrolysate 5% with 2.5% sucrose was a little pit late, at the 4th MPV, to show adequate protective levels 2.05±0.01. The antibody level decremented gradually to reach 0.85±0.05, 0.85±0.03 and 0.60±0.00 at the 9th MPV in case of using carbomer 0.3%, 0.5% and lactalbumin hydrolysate 5% with sucrose 2.5% as a stabilizer, respectively (Table 4).

Table 3: Mean virus neutralizing antibody titers expressed in log10 against BPI3V in vaccinated calves with Pneumo-4 vaccine stabilized by 0.3% and 0.5% carbomer and 5% lactalbumin hydrolysate with 2.5% sucrose

Time post vaccination	Carbomer 0.3% (mean± SD)	Carbomer 0.5% (mean± SD)	Lactalbumin hydrolysate 5% with sucrose 2.5% (mean± SD)
0 Day	0.01±0.0	0.01±0.0	0.30±0.00
2nd WPV	1.5±0.05[a]	0.69±0.33[b]	1.2±0.03[c]
4th WPV	2.51±0.05[a]	2.3±0.2[b]	1.9±0.02[c]
2nd MPV	2.39±0.2[a]	2.0±0.25[b]	1.70±0.01[c]
3rd MPV	2.25±0.4[a]	1.9±0.2[b]	1.80±0.01[c]
4th MPV	2.14±0.33[a]	1.7±0.4[b]	2.00±0.02[c]
5th MPV	2.01±0.33[a]	1.5±0.2[b]	1.92±0.01[c]
6th MPV	1.58±0.05[a]	1.3±0.03[b]	1.74±0.02[c]
7th MPV	1.69±0.05[a]	1.1±0.2[b]	0.96±0.03[c]
8th MPV	1.5±0.05[a]	1.07±0.3[b]	0.72±0.03[c]
9th MPV	1.32±0.04[a]	0.88±0.04[b]	0.60±0.00[c]

BPI3V: Bovine Para influenza type 3 virus
WPV: week post vaccination
MPV: month post vaccination
SD: Standard deviation
Means with different superscripts in the same row are significantly different at P≤0.05.

Table 4: Mean virus neutralizing antibody titers expressed in log10 against BRSV in vaccinated calves with Pneumo-4 vaccine stabilized by 0.3% and 0.5% carbomer and 5% lactalbumin hydrolysate with 2.5% sucrose.

Time post vaccination	Carbomer 0.3% (mean± SD)	Carbomer 0.5% (mean± SD)	Lactalbumin hydrolysate 5% with sucrose 2.5% (mean± SD)
0 Day	0.01±0.0	0.01±0.0	0.30±0.00
2nd WPV	1.19±0.05[a]	0.6±0.01[b]	0.93±0.01[c]
4th WPV	2.28±0.2[a]	2.16±0.25[b]	1.03±0.01[c]
2nd MPV	2.12±0.05[a]	1.9±0.4[b]	1.40±0.02[c]
3rd MPV	1.98±0.2[a]	1.75±0.33[b]	1.60±0.02[c]
4th MPV	1.82±0.04[a]	1.6±0.4[b]	2.05±0.01[c]
5th MPV	1.68±0.4[a]	1.42±0.2[b]	1.92±0.01[c]
6th MPV	1.5±0.01[a]	1.2±0.2[b]	1.3±0.03[c]
7th MPV	1.38±0.4[a]	1.1±0.02[b]	1.04±0.02[c]
8th MPV	1.0±0.06[a]	1.0±0.04[b]	0.72±0.03[c]
9th MPV	0.85±0.05[a]	0.85±0.03[b]	0.60±0.00[c]

BRSV: Bovine respiratory syncytial virus
WPV: week post vaccination
MPV: month post vaccination
SD: Standard deviation
Means with different superscripts in the same row are significantly different at P≤0.05.

There was a very high significance (p≤ 0.05) between the values of neutralizing antibodies between different groups of the experiment. The titer of the included viruses in the prepared vaccines was compared between the first two groups (0.3% & 0.5%) and showed very high significance in BVDV (p=0.0000001), BoHV-1 (p=0.005), BPI3V (p=0.0006) and BRSV (p=0.000005). In general, when compared each group against the third one, there was significance (p≤ 0.05) but not such high like the above results.

DISCUSSION

The manufacture of vaccines is a complex method. There were several tryouts to evaluate the quality of vaccines by using different stabilizers and adjuvants. The stabilizer plays an important role in keeping the vaccine efficacy particularly when exposed to harsh conditions such as high temperature. It has important role in prolonging the shelf life of vaccine (Anderson et al., 1980).

Use of the carbomer (0.3% and 0.5%) as a stabilizer was characterized by its nontoxic properties (Zhang and Oyama, 2007; Kaczmarek et al., 2014; Gartlan et al., 2016). Naturally, the carbomer binds to antigens and makes a strict seal that protect the antigen molecule allowing retain of antigen integrity even after extended periods of heat exposure (Mudhivarthi et al., 2012; Varges et al., 2019). Vaccination of animals with pure protein antigens, that's may stimulate the immune system to produce a low level of antibodies but adding an adjuvant to it, enhance the efficacy of the vaccine to produce high levels of antibodies. Nevertheless, the adjuvants in combination with the antigens can help in using low doses of it so that; the vaccines would be of economic value (Steven et al., 2013; Wegmann et al., 2015).

In this study, Carbomer 0.3%, 0.5% and lactalbumin hydrolysate 5% with sucrose 2.5% were used as a stabilizer in the preparation of bovine viral respiratory combined vaccine. Saponin solution 1 mg/dose was used as an adjuvant during reconstitution of lyophilized vaccine according to Abd El Fadeel et al. (2020).

The use of the carbomer 0.3%, and 0.5% as a stabilizer in combination with the saponin solution 1 mg/dose as an adjuvant gave a high protective level of antibodies against BVDV-1, BoHV-1, BPI3V, and BRSV with very high significance between first two groups. The antibody titers lasted till the 9th month post-vaccination in comparison to lactalbumin hydrolysate 5% with sucrose 2.5% which lasted till the 7th month post-vaccination. In a previous study, the use of a regular stabilizer with the saponin 1%/dose as an adjuvant gave a protective level of antibodies against the four viruses only up to 6 months post vaccination (Abd El Fadeel et al., 2020; Abd El-Moneam et al., 2020). This means that the presence of carbomer may augment the efficacy of the used adjuvants pushing the duration of immunity up to 9 months post-vaccination.

The persistence of antibodies up to 9 months post-vaccination in the case of using carbomer 0.3%, and 0.5% as a stabilizer and saponin as an adjuvant was attributed to the polymers nature of the carbomer which help in the slow release of the antigens post vaccination (Shakya and Kumar, 2012). However, the use of lactalbumin hydrolysate 5% with sucrose 2.5% as a stabilizer with saponin solution as an adjuvant has not such an advantage so that it gave a protection up to 6 months only.

Abd El Fadeel et al. (2020) mentioned that the Pneumo-5 vaccine which was stabilized by lactalbumin hydrolysate 5% with sucrose 2.5% and adjuvanted with 1mg saponin/dose gave protection to calves against the contained viruses up to 6 months post-vaccination. On the other hand, Abd El-Moneam et al. (2020) concluded that, the using of carbomer as a stabilizer as an alternative of skimmed milk in preparation of the live-attenuated LaSota vaccines reinforced the enhancement and modulation of critical immune reactions.

When the results were compared between the Pneumo-4 vaccine which was stabilized by 0.3% and 0.5% carbomer and adjuvanted with 1mg saponin/dose with the vaccine stabilized by the regular stabilizer, it gave a high titer of antibodies against BVDV-1, BoHV-1, BPI3V, and BRSV than the other one (Table 1-4). These results impute to carbomer act as stabilizer along with an adjuvant, at the same time; it has an additive immune stimulant effect if compared with lactalbumin hydrolysate 5% with sucrose 2.5% (Shakya and Kumar, 2012, Aly et al., 2020). This combination helped in maintaining the antibody level up to the 9th month post-vaccination.

CONCLUSION

The immuno defensive values of inactivated bovine viral respiratory combined vaccine stabilized by carbomer and adjuvanted with saponin increase and persist for longer period of time than using lactalbumin hydrolysate with sucrose as stabilizer and adjuvanted with saponin/dose.

In the end, the use of carbomer as a stabilizer, in addition to saponin as an adjuvant, in the bovine viral respiratory combined vaccine enhances the immune status of the immunized animals for a time of approximately 9 months. This has a significant economic impact, as it reduces the numbers of vaccination, and thus reduces the costs involved in terms of labor and tools as well as reducing the stress on animals.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

AUTHOR CONTRIBUTIONS

All authors shared the ideas and writing of the manuscript. AMA and TKF inoculated the animals and collected the samples. MRA and ATE formulated the vaccines. NIA formulated different concentrations of the carbomer. MRA run the serology.

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