CLINICAL AND EXPERIMENTAL VACCINE RESEARCH

Clin Exp Vaccine Res 2020;9:119-125
https://doi.org/10.7774/cevr.2020.9.2.119
pISSN 2287-3651 • eISSN 2287-366X

Preparation and efficacy of freeze-dried inactivated vaccine against bovine viral diarrhea virus genotypes 1 and 2, bovine herpes virus type 1.1, bovine parainfluenza-3 virus, and bovine respiratory syncytial virus

Maha Raafat Abd El Fadeel[1], Ashraf Taha El-Dakhly[2], Ahmad Mohammad Allam[3], Tarek Korany Farag[3], Alaa Abdel-Moneim El-kholy[4]

[1]Department of Rinder Pest like Diseases, Veterinary Serum and Vaccine Research Institute, Agriculture Research Center, Cairo; [2]Department of Lyophilization. Veterinary Serum and Vaccine Research Institute, Agriculture Research Center, Cairo; [3]Department of Parasitology and Animal Diseases, Veterinary Research Division, National Research Centre, Giza; [4]Veterinary Serum and Vaccine Research Institute, Agriculture Research Center, Cairo, Egypt

Received: July 24, 2020
Accepted: July 28, 2020

Corresponding author: Ahmad Mohammad Allam
Department of Parasitology and Animal Diseases, Veterinary Research Division, National Research Centre, EL Buhouth St., 12622 Giza, Egypt
Tel: +20-233371615, Fax: +20-233370931
E-mail: ahmdallam@gmail.com

No potential conflict of interest relevant to this article was reported.

The research protocol was fully funded by the Veterinary Serum and Vaccine Research Institute (VSVRI), Agricultural Research Center, Cairo, Egypt.

Purpose: Bovine respiratory disease is a worldwide health concern in the feedlot cattle causing morbidity and mortality in young with major economic losses to the producer. Programs of vaccination are integral parts of preventive health programs. We aim to prepare and evaluate lyophilized combined inactivated viruses (bovine viral diarrhea virus [BVDV] genotypes 1 and 2, bovine herpes virus type 1.1 [BoHV-1.1], bovine parainfluenza-3 virus [BPI-3V], and bovine respiratory syncytial virus [BRSV]) vaccine using saponin as a solvent and adjuvant in cattle.
Materials and Methods: Lyophilized Pneumo-5 vaccine was formulated to include the inactivated BVDV genotypes 1 and 2, BoHV-1.1, BPI-3V, and BRSV. The saponin solution was used as an adjuvant and solvent. The prepared vaccines were adjusted to contain 1- and 1.5-mg saponin/dose. It was evaluated for its sterility, safety, and potency in mice and calves. The antibody titers in vaccinated calves were measured by virus neutralization test and enzyme-linked immunosorbent assay (ELISA).
Results: The Pneumo-5 vaccine was found to be free from any contaminants and safe in mice. Meanwhile, the vaccine showed safety in calves which inoculated intramuscularly with the double dose of the vaccines. The overall immune response reached its peak in the 2nd-month post-vaccination. The vaccine contained saponin 1.5 mg/dose reached its antibodies peak in the 4th-week post-vaccination. All groups of vaccinated calves with both concentrations of the saponin did not show statistical significance in antibody titers measured by serum neutralization test and/or ELISA.
Conclusion: The prepared vaccine, namely Pneumo-5, and adjuvanted with either 1 or 1.5 mg/dose saponin was proved safe and potent for effectual protection of calves against BVDV genotypes 1 and 2, BoHV-1.1, BPI-3V, and BRSV.

Keywords: Bovine respiratory disease, Viral vaccines, Inactivated vaccines, Freeze drying, Saponins

Introduction

Bovine respiratory disease (BRD) is a worldwide health concern in the feedlot cattle causing morbidity and mortality in young with major economic losses to the producer [1]. BRD occurs most often within 4 weeks after weaning where it is considered a stressful time for calves [2]. It is a multi-factorial syndrome where the viral infection com-

bines with the bacterial agents, and intensified by stress [3]. The viruses involved with BRD include bovine viral diarrhea virus (BVDV), bovine herpes virus (BoHV), bovine parainfluenza-3 virus (BPI-3V), and bovine respiratory syncytial virus (BRSV) [4].

BRD in Egyptian cattle is caused by a variety of viral agents which include BVDV genotypes 1 and 2 [5], BoHV type 1 (BoHV-1) [6], BPI-3V, and BRSV [7].

Programs of vaccination for breeding herds are integral parts of preventive health programs which are performed to decrease the effects of infectious respiratory diseases and considered the most cost-effective method in controlling disease particularly viral infectious diseases [8]. Selecting the adjuvant is one of the keys for the success of the vaccine. There are two main technologies for veterinary adjuvant in the field can fit with multiple expectations as safety and efficacy in various species such as aluminum salts and emulsion [9].

In Egypt, prevention and control of the syndrome are considered successful and economically attainable than treatment. One of the ideal protocols includes vaccination of the herd with safe, potent and effective inactivated vaccine (Pneumo-5) which contains the five viral antigens adjuvanted with aluminum hydroxide and/or oil [10]. Locally prepared inactivated gel Pneumo-3 vaccine containing BVDV-1, BoHV-1, and BPI-3V was the first edition to face the syndrome in Egypt. Then, Pneumo-4 vaccine was produced by adding BRSV to Pneumo-3 [11].

The saponin substance (*Quillaja Sapinaria* Molina) is extracted from bark of the tree Soapbark [12]. The saponin can be used as solvent and adjuvant [13]. The motivation of immune-cell proliferation and increasing the titer of all major immunoglobulin G (IgG) subclasses *in vivo* is one of main function of the saponin substance [14-16]. In the meantime, the strong immune adjuvant activity reported for the crude extract of the plant bark, and its derivatives, makes them ideal adjuvant for the vaccine development [17].

Aim of the work, it was carried out to prepare and evaluate polyvalent inactivated viruses (BVDV genotypes 1 and 2, BoHV-1.1, BPI-3V, and BRSV) vaccine using saponin (*Quillaja Saponaria* Molina extract) as solvent and adjuvant to the lyophilized vaccine.

Materials and Methods

Ethical approval

Care and use of laboratory animals in this study were approved by the Medical and Veterinary Research Ethics Committee at the National Research Centre in Egypt (No., 20/053).

Viruses and cell line

The local Egyptian strains of BVDV genotype-1 (BVDV-1) "Iman strain" with a titer of $10^{6.5}$ tissue culture infectious dose 50% ($TCID_{50}$)/mL; BVDV genotype-2 (BVDV-2) cytopathic strain "strain 125" with a titer of $10^{6.5}$ $TCID_{50}$/mL; BoHV type 1.1 (BoHV-1.1) "Abou Hammad strain" with a titer of $10^{7.5}$ $TCID_{50}$/mL; BPI-3V "strain 45" with a titer of 10^{8} $TCID_{50}$/mL; and BRSV "strain 375L" with a titer of 10^{8} $TCID_{50}$/mL were kindly supplied by the Veterinary Serum and Vaccine Research Institute (VSVRI), Cairo, Egypt. All viruses were propagated and titrated on Madin-Darby bovine kidney (MDBK) cell line which has been proved free of any extraneous contamination. The cell lines were developed at 37°C, in minimum essential medium with Earle's salts (Minimum Essential Medium Eagle) supplemented with heat-inactivated 10% fetal calf serum, 100 U/mL penicillin, 100 μg/mL streptomycin, and 25 IU/mL mycostatin. Prior to experimental work, these viruses were tested for their identity by virus neutralization test using the respective specific reference antisera which were supplied by VSVRI, Cairo, Egypt.

Experimental animals

Mice

Fifteen Albino Swiss mice were obtained from Laboratory Animal Breeding Farm at VSVRI and were used to study the safety of the prepared vaccines divided into three groups (five mice/group).

Calves

Eighteen clinically apparent healthy mixed bred calves aging 6–8 months, in a private farm in Beni Seuf Governorate, were used to study the safety and potency of the vaccines (nine calves for each experiment).

Before vaccination, all calves and mice were proved seronegative to the viral strains incorporated in the prepared vaccine and examined for internal and external parasites. Mice were kept in the hygienic animal facilities at the VSVRI.

Vaccine preparation

The lyophilized Pneumo-5 vaccine was formulated to include the five inactivated antigens BVDV-1 and 2, BoHV-1, BPI-3V, and BRSV. The saponin solution (*Quillaja Saponaria* Molina extract) was used as adjuvant and for reconstitution. Briefly,

 https://doi.org/10.7774/cevr.2020.9.2.119

confluent monolayers of MDBK cells grown in Roux bottles were inoculated with the five agents separately at multiplicity of infection 2 to 1 (virus to cell) and incubated at 37°C in the presence of 1 mg/mL of trypsin. After 70%–80% of the infected cells showed cytopathic effect, the culture fluid was harvested, clarified, and titrated.

Viruses were inactivated individually by stirring with 0.01 M binary ethyleneimine (BEI 10% volume/volume [v/v]) at 37°C overnight. Sodium thiosulphate (20%) was then added with a final concentration of 2% to stop the action of BEI [18, 19]. Then we mixed equal amounts from the five inactivated viruses fluid. Thereafter, we add the stabilizer (sucrose and lactalbumen) v/v and then aliquot and lyophilized. The lyophilization process was carried out using a freeze-dryer apparatus (2014-033; Shanghai Tofflon Science & Technology Co. Ltd., Shanghai, China). On final vaccine formulation, the vaccine dose should contain least titer of 10^6 $TCID_{50}$ of each virus [20].

Quality control of the vaccine

Sterility test

Different steps of vaccine preparation were subjected to in-process testing for sterility to prove that the prepared vaccine was free from any contaminants [20].

Safety of vaccines

Safety in mice: Fifteen adult albino mice were used to study the safety of the vaccine. The mice were divided into three groups each of five mice. All mice were inoculated intraperitoneal with 0.2 mL/mice of the prepared vaccines as follows; the first two groups were inoculated with the lyophilized vaccine reconstituted in saponin 1 and 1.5 mg/dose, respectively. The 3rd group was inoculated with physiological saline as control. All mice were kept under observation for 2 weeks for the detection of any clinical abnormalities [20].

Safety in calves: Nine male calves were divided into three groups (3 calves/group) used to evaluate the vaccine safety. The first two groups were inoculated intramuscular with double dose of the lyophilized vaccines which reconstituted in saponin 1 and 1.5 mg/dose, respectively [21]. The 3rd group was inoculated with the same dose and route by physiological saline solution. All animals were observed up to 21 days afterwards for development of any clinical abnormalities.

Potency of the vaccines in calves

Nine male calves were divided into three groups (3 calves/group) where the first two groups were inoculated with 1 mL of the lyophilized vaccine which is reconstituted in 1 and 1.5 mg saponin/dose, respectively. The 3rd group was kept as non-vaccinated control calves. Poster dose was given to all calves 2 weeks post-vaccination [20].

Serum samples were collected from all calves on the first day of vaccination (0 day), 2nd week, 4th week, and every month up to 6 months post-vaccination. 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 vaccine.

Serological investigations

Virus neutralization test

The test carried out [22] and the antibody titer calculated [23]. The antibody titer expressed as $\log_{10}$ using logarithmic tables.

Indirect ELISA

Seroconversion of vaccinated calves and mice against viral components of the prepared vaccine were estimated by indirect in-house enzyme-linked immunosorbent assay (ELISA) with modifications [24]. The results were recorded using a computer-assisted micro plate reader (ELx808TM Absorbance Micro Plate Reader; BioTek Instruments Inc., Winooski, VT, USA).

Statistical methodology

Statistical analysis was performed with the aid of Microsoft Excel 2010 (Microsoft Corp., Redmond, WA, USA). The data were expressed as mean±standard deviations. Data were compared using the unpaired Student t-test. A p-value <0.05 was considered to indicate statistical significance.

Results

The freeze-dried inactivated vaccines prepared against BVDV genotype 1 and 2, BoHV-1.1, BPI-3V, and BRSV (Pneumo-5) were found to be free from any contaminants. Besides, it was safe in mice and calves inoculated with both concentrations (1 and 1.5 mg saponin/dose) of saponin. All animals did not show abnormal clinical signs and remained survive until the end of experiment.

Regarding the serological examination explained in Tables 1 and 2 and Figs. 1 and 2, generally speaking, there was gradual increase in the values of virus neutralizing antibody titers and indirect ELISA values from the 2nd-week post-vaccina-

Table 1. Mean virus neutralizing antibody titers of calve vaccinated with Pneumo-5 vaccine reconstituted in saponin (1 and 1.5 mg/dose) expressed as log_{10}

Period post-vaccination	Detected serum neutralizing antibody titers (log_{10}) in calves receiving									
	1 mg saponin per dose					1.5 mg saponin per dose				
	BVDV-1	BVDV-2	BoHV-1	BPI-3V	BRSV	BVDV-1	BVDV-2	BoHV-1	BPI-3V	BRSV
0 Day	0.2±0.0	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0	0.2±0.0	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0
2nd WPV	0.45±0.01	0.45±0.01	0.65±0.04	0.61±0.05	0.43±0.05	0.6±0.01	0.6±0.01	0.7±0.02	0.69±0.33	0.6±0.01
4th WPV	1.64±0.2	1.7±0.25	1.85±0.03	1.85±0.05	1.63±0.2	2.1±0.56	2.1±0.2	2.3±0.25	2.3±0.2	2.16±0.25
2nd MPV	1.8±0.2	1.8±0.4	2.05±0.55	2.0±0.2	1.82±0.05	1.9±0.05	1.91±0.2	2.06±0.25	2.0±0.25	1.9±0.4
3rd MPV	1.6±0.4	1.65±0.25	1.8±0.02	1.8±0.4	1.6±0.2	1.69±0.33	1.78±0.4	1.9±0.05	1.9±0.2	1.75±0.33
4th MPV	1.45±0.25	1.5±0.2	1.5±0.04	1.6±0.33	1.4±0.04	1.5±0.2	1.56±0.33	1.72±0.2	1.7±0.4	1.6±0.4
5th MPV	1.2±0.2	1.2±0.25	1.2±0.14	1.4±0.33	1.15±0.4	1.3±0.05	1.35±0.33	1.5±0.4	1.5±0.2	1.42±0.2
6th MPV	0.9±0.01	0.94±0.05	0.9±0.05	1.2±0.05	0.9±0.01	1.09±0.33	1.19±0.33	1.32±0.6	1.3±0.03	1.2±0.2

Values are presented as mean±standard deviation.
BVDV, bovine viral diarrhea virus; BoHV, bovine herpes virus; BPI-3V, bovine parainfluenza-3 virus; BRSV, bovine respiratory syncytial virus; WPV, week post-vaccination; MPV, month post-vaccination.

Table 2. Mean enzyme-linked immunosorbent assay value in sera of calf vaccinated with Pneumo-5 reconstituted in saponin (1 and 1.5 mg/dose)

Period post-vaccination	1 mg saponin per dose					1.5 mg saponin per dose				
	BVDV-1	BVDV-2	BoHV-1	BPI-3	BRSV	BVDV-1	BVDV-2	BoHV-1	BPI-3	BRSV
0 Day	0.2±0.0	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0
2nd WPV	1.1±0.03	1.1±0.035	1.4±0.3	1.2±0.04	0.92±0.01	0.83±0.01	0.85±0.01	0.9±0.01	0.88±0.02	0.85±0.02
4th WPV	1.95±0.35	1.95±0.36	2.1±0.03	2.05±0.2	1.9±0.2	2.3±0.25	2.3±0.36	2.54±0.3	2.5±0.6	2.48±0.36
2nd MPV	2.0±0.36	2.0±0.04	2.3±0.03	2.25±0.2	2.01±0.36	2.1±0.25	2.1±0.36	2.32±0.25	2.3±0.54	2.22±0.36
3rd MPV	1.75±0.4	1.75±0.05	1.9±0.2	1.9±0.4	1.76±0.33	1.9±0.35	1.9±0.2	2.16±0.25	2.1±0.36	2.02±0.25
4th MPV	1.55±0.4	1.55±0.2	1.7±0.36	1.7±0.6	1.5±0.2	1.72±0.2	1.72±0.24	1.91±0.2	1.9±0.2	1.8±0.2
5th MPV	1.3±0.2	1.3±0.06	1.5±0.4	1.5±0.4	1.3±0.4	1.54±0.4	1.54±0.36	1.73±0.08	1.7±0.4	1.55±0.4

Values are presented as mean±standard deviation.
BVDV, bovine viral diarrhea virus; BoHV, bovine herpes virus; BPI-3V, bovine parainfluenza-3 virus; BRSV, bovine respiratory syncytial virus; WPV, week post-vaccination; MPV, month post-vaccination.

tion against all antigens in vaccinated calves. There was ascending increment reaching the peak on the 2nd month in vaccinated calves with the Pneumo-5 vaccine reconstituted in saponin (1 mg/dose) while in calves' vaccinated Pneumo-5 vaccine reconstituted in saponin (1.5 mg/dose) showed increased antibody titers reaching their peak in the 4th-week post-vaccination. Controls had any reactions. There was no statistical significance in antibodies titers measured by serum neutralization test (SNT) and/or ELISA among all vaccinated calve groups.

Discussion

BRD syndrome usually causes major economic losses mainly due to decreased performance, high morbidity, mortality, the expenses of medication, and labor to treat the sick animals [1]. It is still challenging due to the multi-factorial nature and complexity of the disease. Thus, availability of reliable combined vaccines has been considered the appropriate alternative for combating the syndrome through traditional approaches [25]. Vaccination against the presumed causal organisms is a commonly used tactic to assist in the prevention of BRD. With significant concern for prudent antibiotic use in the beef industry, it is vital that decision making with regards BRD management be based on understanding of the efficacy of vaccination programs and management factors that might modify the efficacy of the preventive management practice [26].

In the present study, the Pneumo-5 vaccine performance was assessed against BVDV genotypes 1 and 2, BoHV-1.1, BPI-3V, and BRSV. The saponin extracted from *Quillaja Sapinaria* Molina was used as adjuvant to stimulate and activate the humeral immune responses [27].

 https://doi.org/10.7774/cevr.2020.9.2.119

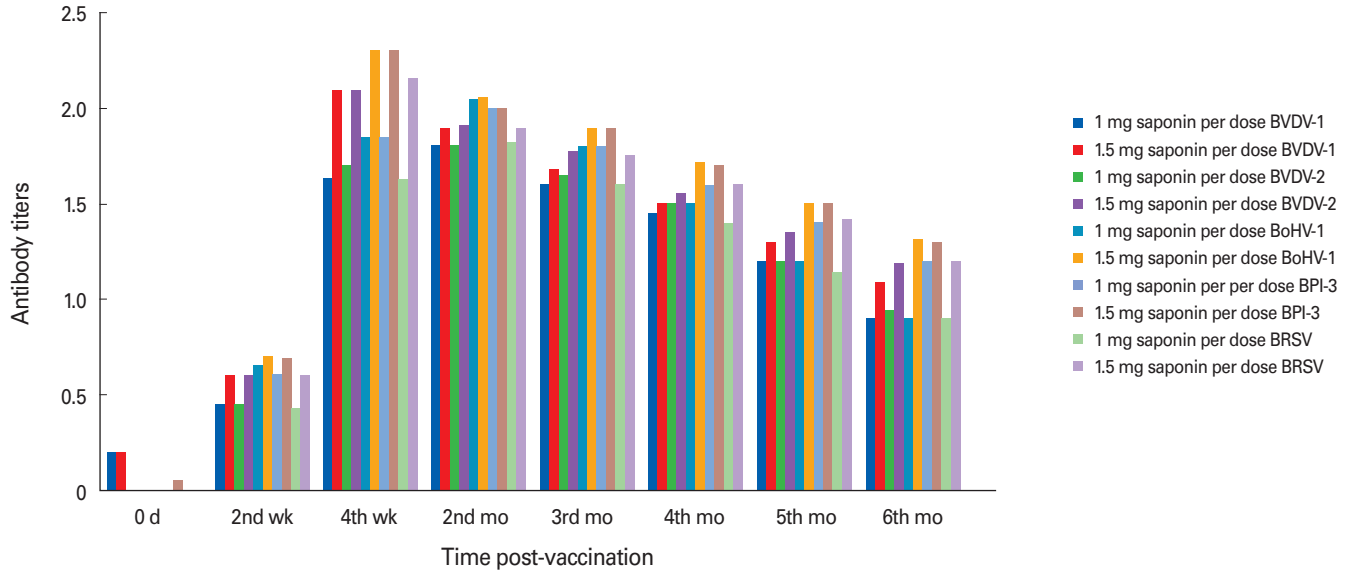

Fig. 1. Mean log_{10} virus neutralizing antibody titers of calves vaccinated with Pneumo-5 vaccine reconstituted in saponin (1 and 1.5 mg/dose). BVDV, bovine viral diarrhea virus; BoHV, bovine herpes virus; BPI-3V, bovine parainfluenza-3 virus; BRSV, bovine respiratory syncytial virus.

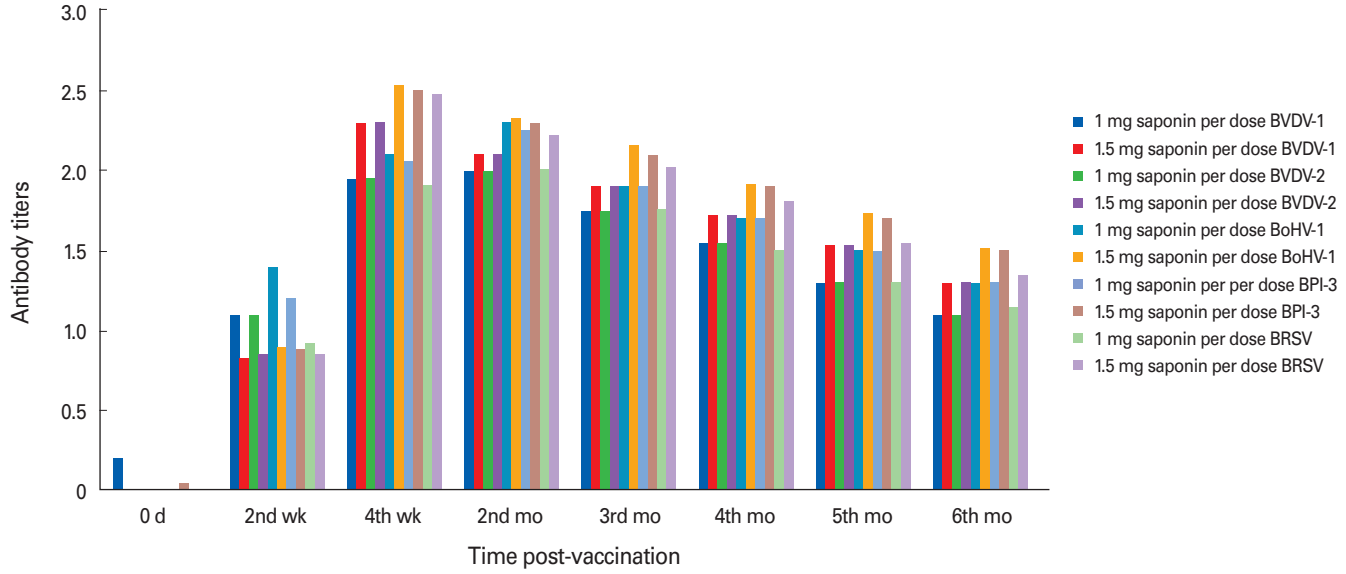

Fig. 2. Mean enzyme-linked immunosorbent assay value in sera of calf vaccinated with Pneumo-5 reconstituted in saponin (1 and 1.5 mg/dose). BVDV, bovine viral diarrhea virus; BoHV, bovine herpes virus; BPI-3V, bovine parainfluenza-3 virus; BRSV, bovine respiratory syncytial virus.

Results of sterility testing of the prepared vaccine indicated its freedom from any bacterial, fungal, or mycoplasma contamination on inoculated media. It was also found safe in mice and claves with even the double field dose. These findings meet the safety requirements and recommendations of the World Organization for Animal Health manual [20].

Vigor assessment of the prepared freeze-dried inactivated vaccine against BVDV genotypes 1 and 2, BoHV-1.1, BPI-3V, and BRSV which reconstituted in 1 and 1.5 mg saponin/dose was evaluated by the SNT and indirect ELISA. The results revealed that all vaccinated calves have robust neutralizing antibodies against all viral components of the vaccine starting from the 2nd-week post-vaccination in agreement with [28,29]. The titer of antibodies increased gradually and the duration of immunity lasted up to 6th month of vaccination in accordance with the results of [30,31]. The minimum protective log_{10} antibody titers were achieved for all viral agents where that of BVDV are 0.9 and for BoHV, BPI-3V, and BRSV is 0.6 [11].

It is clear from the results that the using of Pneumo-5 re-

constituted in 1.5 mg saponin/dose gave higher and protective titers in the 4th-week post-vaccination than the other dose 1 mg saponin/dose. These results agree with those of Ojiako et al. [27] who mentioned that the using of saponin with vaccine as adjuvant by certain concentration become well than other adjuvants, where saponin able to stimulate strong antibody production.

Numerous publications proved the suitability of Quillaja extract to be used as adjuvant with diverse of viral vaccines. Saponin was used as an adjuvant in bovine viral diarrhea virus vaccine in mice [32]. These findings revealed its capability of stimulating both cellular and humoral immune responses. They found that the total specific IgG against BVDV were elevated in all groups inoculated with saponin adjuvanted preparations. On the other hand, Quillaja leaves extract was used as an adjuvant in BoHV-5 vaccine and revealed a significant increase in total IgG in vaccinated mice compared with other known adjuvants [33]. Meanwhile, the Quillaja extract saponins used in BoHV-1 vaccines in mice showed significant induction of antibodies against the virus in immunized mice [34].

In conclusion, the prepared vaccine, namely Pneumo-5, and adjuvanted with either 1 or 1.5 mg saponin/dose and the intramuscular route was proved safe and potent for effectual protection of calves against BVDV genotypes 1 and 2, BoHV-1.1, BPI-3V, and BRSV.

ORCID

Maha R. Abd El Fadeel *https://orcid.org/0000-0002-7457-5005*
Ashraf T. El-Dakhly *https://orcid.org/0000-0003-1324-7988*
Ahmad M. Allam *https://orcid.org/0000-0001-9776-7205*
Tarek K. Farag *https://orcid.org/0000-0002-0717-4519*
Alaa A. El-kholy *https://orcid.org/0000-0003-1016-727X*

References

1. Hilton WM. BRD in 2014: where have we been, where are we now, and where do we want to go? Anim Health Res Rev 2014;15:120-2.
2. Boyles SL, Loerch SC, Lowe GD. Effects of weaning management strategies on performance and health of calves during feedlot receiving. Prof Anim Sci 2007;23:637-41.
3. Kasimanickam R. Bovine respiratory disease "shipping fever" in cattle. Pullman, WA: Veterinary Medicine Extension, Washington State University; 2010.
4. O'Connor AM, Hu D, Totton SC, et al. A systematic review and network meta-analysis of bacterial and viral vaccines, administered at or near arrival at the feedlot, for control of bovine respiratory disease in beef cattle. Anim Health Res Rev 2019;20:143-62.
5. Abd El-Fadile MR. Serosurveillance of bovine viral diarrhea genotype in breeding farm animal in Egypt [master's thesis]. Benha: Benha University Faculty of Veterinary Medicine; 2007.
6. Mahmoud MA, Mahmoud NA, Allam AM. Investigations on infectious bovine rhinotracheitis in Egyptian cattle and buffaloes. Glob Vet 2009;3:335-40.
7. Mahmoud MA, Allam AM. Seroprevalence of bovine viral diarrhea virus (BVDV), bovine herpes virus type 1 (BHV-1), parainfluenza type 3 virus (PI-3V) and bovine respiratory syncytial virus (BRSV) among non vaccinated cattle. Glob Vet 2013;10:348-53.
8. Haase CJ. Serological responses to commercial vaccination in Holstein dairy cattle [Internet]. Ann Arbor, MI: ProQuest LLC.; 2019 [cited 2020 May 1]. Available from: https://search.proquest.com/openview/b0df22afe52d236629b05928201f9b94/1?pq-origsite=gscholar&cbl=18750&diss=y.
9. Parker R, Deville S, Dupuis L, Bertrand F, Aucouturier J. Adjuvant formulation for veterinary vaccines: Montanide Gel safety profile. Procedia Vaccinol 2009;1:140-7.
10. EL-Hawary RI, Mostafa HA. Immunological response of locally prepared oil adjuvanted pneumo-5 vaccine in calves. J Vet Med Res 2017;24:41-7.
11. Taha SS, El-Sabbagh MM, Ghaly HM. Preparation of combined inactivated BVD, IBR, PI3 and respiratory syncytial virus (BRSV). J Egypt Vet Med Ass 2001;61:251-63.
12. Fleck JD, Betti AH, da Silva FP, et al. Saponins from Quillaja saponaria and Quillaja brasiliensis: particular chemical characteristics and biological activities. Molecules 2019;24:171.
13. Saleh NS, Ebeid EM, Warda FF, et al. Lyophilized EHV-1 vaccine inactivated and adjuvanted by Quillaja Saponaria Molina extract. J Virol Sci 2017;1:123-34.
14. Kensil CR, Patel U, Lennick M, Marciani D. Separation and characterization of saponins with adjuvant activity from Quillaja saponaria Molina cortex. J Immunol 1991;146:431-7.
15. Plohmann B, Bader G, Hiller K, Franz G. Immunomodulatory and antitumoral effects of triterpenoid saponins. Pharmazie 1997;52:953-7.

16. Lacaille-Dubois MA, Hanquet B, Cui ZH, Lou ZC, Wagner H. A new biologically active acylated triterpene saponin from Silene fortunei. J Nat Prod 1999;62:133-6.
17. Rajput ZI, Hu SH, Xiao CW, Arijo AG. Adjuvant effects of saponins on animal immune responses. J Zhejiang Univ Sci B 2007;8:153-61.
18. Bahnemann HG. Inactivation of viral antigens for vaccine preparation with particular reference to the application of binary ethylenimine. Vaccine 1990;8:299-303.
19. Daoud AM, Zeidan SM, Wassel MS, Gerges SM, Effat L. Evaluation and field application of locally prepared combined inactivated Rota/Corona viruses and E. coli K99 (Entero-3) vaccine. Proceedings of the 7th Scientific Congress of Egyptian Society for Cattle Diseases; 2003 Dec 7-9; Assiut, Egypt. [place unknown]: Egyptian Society for Cattle Diseases; 2003. p.257-70.
20. World Organisation for Animal Health. Tests for sterility and freedom from contamination of biological materials intended for veterinary use. In: World Organisation for Animal Health, editor. Manual of diagnostic tests and vaccines for terrestrial animals 2019. Paris: OIE; 2019. p.109-22.
21. Code of Federal Regulations: animals and animal products: parts 1-199. Washington, DC: Office of the Federal Register, National Archives and Record Administration; 2015.
22. Robson DS, Gillespie JH, Baker JA. The neutralization test as an indicator of immunity to virus diarrhea. Cornell Vet 1960;50:503-9.
23. Reed LJ, Muench H. A simple method of estimating fifty percent endpoints. Am J Hyg 1938;27:493-7.
24. Voller A, Bidwell DE, Bartlett A. Enzyme immunoassays in diagnostic medicine. Theory and practice. Bull World Health Organ 1976;53:55-65.
25. Knezevic N, Kosanovic P, Rogan D. Immunoprophylaxis of respiratory diseases of cattle with inactivated vaccines: III. study of the immunogenicity of a bivalent, inactivated oil vaccine against IBR and PI-3. Vet Glas 1990;44:503-12.
26. O'Connor AM, Wang C, Sargeant JM, et al. What is the efficacy of viral and bacterial vaccines in feedlot cattle to reduce bovine respiratory disease (BRD) and subsequent antibiotic use? [Internet]. Ames, IA: Veterinary Diagnostic and Production Animal Medicine at Iowa State University Digital Repository; 2018 [cited 2020 May 20]. Available from: https://lib.dr.iastate.edu/vdpam_reports/11.
27. Ojiako CM, Okoye EI, Oli AN, Ike CJ, Esimone CO, Attama AA. Preliminary studies on the formulation of immune stimulating complexes using saponin from Carica papaya leaves. Heliyon 2019;5:e01962.
28. Allam AM, El-Bayoumy MK, Taha SS, Elsabbag MM. Use of rabbits as a laboratory model for evaluation of the combined inactivated respiratory virus vaccine (Pneumo-3) adjuvanted by Montanide oil (ISA 206). Glob Vet 2012;8:254-8.
29. El-Bagoury GF, El-Nahas EM, Abd-Elfadiel MR, Ghaley HM. Evaluation of maternal antibody in calves borne from cow dam vaccinated with inactivated Pnumo-5 vaccine. Benha Vet Med J 2012;23:9-15.
30. De Gregorio E, Tritto E, Rappuoli R. Alum adjuvanticity: unraveling a century old mystery. Eur J Immunol 2008;38:2068-71.
31. El-Fadil A, Maha R, El Hawary RI, Warda FF. Keeping quality of inactivated respiratory virus vaccines (Pneumo-5). Benha Vet Med J 2017;33:193-9.
32. Cibulski SP, Silveira F, Mourglia-Ettlin G, et al. Quillaja brasiliensis saponins induce robust humoral and cellular responses in a bovine viral diarrhea virus vaccine in mice. Comp Immunol Microbiol Infect Dis 2016;45:1-8.
33. Silveira F, Cibulski SP, Varela AP, et al. Quillaja brasiliensis saponins are less toxic than Quil A and have similar properties when used as an adjuvant for a viral antigen preparation. Vaccine 2011;29:9177-82.
34. Fleck JD, Kauffmann C, Spilki F, Lencina CL, Roehe PM, Gosmann G. Adjuvant activity of Quillaja brasiliensis saponins on the immune responses to bovine herpesvirus type 1 in mice. Vaccine 2006;24:7129-34.