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Preparation and field study of combined vaccine against *Clostridium perfringens* type A and bovine viral diarrhea virus in camels

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Purpose: The key objective of this study was to formulate a local combined inactivated gel adjuvanted vaccine containing bovine viral diarrhea virus (BVDV)-1, BVDV-2 viruses and *Clostridium perfringens* type A toxoid. The study evaluated its ability to enhance protective active immune response in camels' calves against these infectious pathogens under field conditions.
Materials and Methods: The local BVDV cytopathic strains and a local strain of toxigenic *C. perfringens* type A were used in vaccines formulation. Vaccines A and B were monovalent vaccines against *C. perfringens* and both strains of BVDVs, respectively. While the vaccine C was the combined vaccine used against the three agents. All vaccines were adjuvanted with Montanide gel. Sterility, safety, and potency tests were applied on the formulated vaccines. Virus neutralization and toxin anti-toxin neutralization tests were used to evaluate the immune responses.
Results: Both monovalent (vaccine A) and combined vaccines (vaccine C) showed a protective level (4.5 and 3 IU/mL, respectively) against *C. perfringens* from the 2nd-week post-vaccination. The titer declined to 3 and 2 IU/mL, respectively at the 5th-month post-vaccination. The titer against BVDV, the monovalent vaccine (vaccine B) reached the beak (1.95 IU/mL) at the 1st-month post-vaccination and lasted till 6th-month post-vaccination (0.92 and 0.94 IU/mL) for BVDV-1a and BVDV-2, respectively.
Conclusion: Vaccination of camels with the combined vaccine adjuvanted by Montanide gel containing *C. perfringens* type A toxoid and BVDV strains with 6-month intervals is recommended to protect camels safely and efficiently against such infections in the field.

Keywords: *Clostridium perfringens* type A enterotoxin, Bovine viral diarrhea viruses, Inactivated vaccines, Montanide gel, Camelidae

Introduction

Thousands of tons of meat, milk, hides, and weber are produced by Egyptian camels [1]. Camel meat and milk contain less cholesterol and fat than other animal meats, and its milk is safe for people who are allergic to bovine milk [2]. Camel breeding has an economic and socio-cultural influence in Saharan and sub-Saharan countries, such as Egypt, due to their goods, as well as their unique use in transportation and visitors' field trips through badlands [3]. While camels are susceptible to many viral diseases, they do not show extreme clinical symptoms due to their toughness [4], and thus play an important role in the epizootiology of some livestock diseases [5].

Camels' neonatal calf diarrhea (NCD), also known as calf scours and calf enteritis is a multifactorial disease that has a significant impact on the camel industry due to low productivity and high mortality. It is caused by a combination of infectious and non-infectious causes, mostly viral and bacterial infections. Even though a single pathogen may be the primary cause of diarrhea in calves, co-infection is often present [6,7].

One of the main viral causes of this syndrome is the bovine viral diarrhea virus (BVDV), which is a small enveloped RNA virus that is currently classified in the genus Pestivirus, it includes BVDV genotype 1 and 2, border disease virus, and classical swine fever virus, as well as the genera Flavivirus and Hepacivirus, which belong to the family Flaviviridae [8]. BVDVs are divided into two antigenically distinct genotypes, BVDV-1 and BVDV-2 [9], each of which has one of two biotypes: cytopathogenic or non-cytopathogenic. BVDV-1 may also be divided into at least 11 phylogenetic groups [10]. Primary postnatal BVDV infection causes a number of subclinical enteric, respiratory, and reproductive diseases in cattle [11,12]. Certain BVDV-2 strains, on the other hand, have been found to cause an extreme type of bovine viral diarrhea (BVD) in veal calves [13,14]. Furthermore, trans-placental BVDV infection during the first trimester of pregnancy can cause miscarriage, fetal death, congenital defects, or the birth of persistently infected animals that function as continuous virus shedders [11,15,16].

Comprehensive monitoring of animals for BVDV infection annually and prior to joining a herd, as well as vaccination, are the mainstays of BVDV control. The primary goal of BVDV vaccination is to provide fetal protection against a wide variety of antigenically distinct strains, with the goal of eliminating BVDV carriers. There are currently no vaccines available that provide effective prevention of antigenically homologous and heterologous BVDV infections [17,18]. The genetic diversity of local and imported vaccinal BVDV strains versus circulating field BVDVs in Egypt is uncertain. This could be a risk factor, since a vaccinated herd could still be vulnerable to BVDV infection.

The main bacterial cause of NCD in camels is Clostridial microorganisms. Their pathogenicity is dependent on the infected host secreting harmful exotoxins. Acute and subacute enterotoxaemia, as well as hemorrhagic enteritis, have been reported in camels caused by *Clostridium perfringens* types A, C, and D [19,20].

C. perfringens infections are spread through the soil, where spores produced in a carcass, its sections, or feces are immune to all environmental influences and can survive for an indefinite period of time. Camels kept in desert paddocks are constantly exposed to spores and vegetative forms of *C. perfringens*. Despite routine removal of feces from paddocks, *C. perfringens* could still be ingested by touch carrier animals by polluted food or water [20]. Clostridial infections are rarely predictable and sometimes unsuccessful when treated with antibiotics. As a result, prophylactic vaccine formulas containing one or more Clostridial bacterin or toxoid are commonly used to prevent such infections [21,22]. *C. perfringens* was extracted from camels' stools and meat swabs samples at rates of (26.7%) and (2.7%), respectively, during a survey conducted in Egypt in summer 2008 [23]. *C. perfringens* types A, B, C, D, and E toxin genes were detected by multiplex polymerase chain reaction (PCR) with rates of 65%, 10%, 2.5%, 2.5%, and 0%, respectively, and the highest percentage of isolates belong to *C. perfringens* type A in camels age between 3–5 years [24]. Also, Basma and AM [25] detected the causative bacteria in the meat samples collected from apparently healthy camels with an incidence of 33.7%.

As the trend of using different microorganisms in a combined vaccine is greatly simplify the prophylaxis control of diseases of livestock besides saving costs, efforts, and time during vaccination [26-28].

The key objective of this study was to formulate a local combined inactivated Montanide gel adjuvanted vaccine containing BVDV-1, BVDV-2 viruses and *C. perfringens* type A toxoid as the primary viral and bacterial pathogens responsible for serious NCD amongst camels' calves. In addition, the study was designed to evaluate its ability to enhance protective active immune response in Camels' calves against these infectious pathogens under field conditions.

Materials and Methods

Ethical approval

The Medical and Veterinary Research Ethics Committee at Egypt's National Research Centre approved the study's care and use of laboratory animals (approval no., 20/053).

Viruses and cells

The local Egyptian BVDV, namely "Iman" strain [29], a cytopathic BVDV-1a [30] with a titer of $10^{6.5}$ tissue culture infectious dose 50% ($TCID_{50}$)/mL and the reference cytopathic BVDV-2, namely "125C" strain, with a titer of 10^{7} $TCID_{50}$/mL,

were used in vaccine formulation. They were kindly supplied by the Veterinary Sera and Vaccines Researches Institute (VSVRI), Abbassia, Cairo, Egypt. Both virus entities were propagated and titrated on Madin-Darby bovine kidney (MDBK) cell line which has been proved free of any extraneous contamination; particularly non-cytopathic bovine virus diarrhea. The MDBK cells were grown at 37°C, in minimum essential medium with Earle's salts 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, they were tested for their identity by virus neutralization test (VNT) using the respective specific reference antisera and by the reverse transcription-PCR as previously described [14].

Bacterial strains

The local strain of toxigenic *C. perfringens* type A was supplied from Anaerobic Bacterial Vaccines Department, VSVRI, Abbassia, Cairo, Egypt [31].

Experimental animals

Mice

A total of 40 Albino Swiss mice were obtained from Laboratory Animal Breeding Farm at VSVRI and used to study the safety of the prepared vaccine, minimal lethal dose 50% (MLD_{50}) of *C. perfringens* alpha toxin, and toxin-antitoxin neutralization test.

Camels

The study was run on 30 clinically apparently healthy adult male camels which were reared in the camel station of Desert Research Center, Ras-Sedr city, South Sinai, Egypt and in the experimental station of VSVRI, Cairo, Egypt. Before vaccination, all animals were proved seronegative to both viral and bacterial strains incorporated in the prepared vaccine and confirmed to be free from internal and external parasites. All animals were kept in a hygienic atmosphere during the period of experimentation.

A group of camels in the station were kept as non-vaccinate control group. The animals were injected the same dose but with sterile saline. Samples were collected in coordination with other experimental animals.

Vaccine preparation

BVD viruses

Briefly, confluent log-phase monolayers of MDBK cells grown in two roller bottles were inoculated separately with the cytopathic strains of BVDV-1a (Iman strain) and BVDV-2 (125C strain) at multiplicity of infection of 0.5 and incubated at 37°C. When the infected cells showed 70%–80% cytopathic effect, the culture fluid were harvested after two consequent freezing and thawing cycles, then clarified. The viruses were titrated and inactivated individually by stirring with 0.01 M binary ethyleneimine (BEI) 10% volume per 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. On final vaccine formulation both BVDV-1a and BVDV-2 concentration were adjusted so that a vaccine dose should contain at least 10^6 $TCID_{50}$ of each [32].

C. perfringens

C. perfringens type A seed was grown into cooked meat medium as a primary toxin production medium; incubated at 37°C in anaerobic conditions for 4 hours, then transferred into main toxin production medium and re-incubated for 4 hours [31]. The toxin was separated and concentrated from the bacterial culture after its MLD_{50} being determined [33]. The toxin was then inactivated by adding formaldehyde 37% at a concentration of (0.5% v/v) for about 7 days until it was completely inactivated. The toxoid generated was altered to have an 80 MLD_{50}/vaccinal dosage. The inactivated viral and bacterial components were mixed altogether in equal volumes, then the sterile Montanide gel (Seppic, Courbevoie, France) was added to the formulated mix in a ratio of 20% of the total formula as instructed by the manufacturer.

Quality control of the vaccine

Sterility test

In-process sterility testing was performed on various stages of vaccine manufacturing (including viral and bacterial propagation, inactivated fluids, and the finished product) to ensure that the vaccine was free of contaminants [32].

Safety test in mice

The vaccine's safety was investigated using 40 adult albino mice. Each group of ten mice was separated into four groups. The produced vaccines (*C. perfringens*, BVDV, and combined vaccine) were inoculated intraperitoneally with 0.2 mL/mice in the first three groups, while physiological saline was administered with the same dose and route in the last group as control non-vaccinated group [32].

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Field potency of the prepared vaccines in camels

Eighteen camels (18/30) were randomly assigned into three equal groups (six camels each): Group A was vaccinated intramuscularly (3 mL/animal) with *C. perfringens* type A toxoid; group B was vaccinated intramuscularly (3 mL/animal) with inactivated BVDV genotypes 1a & 2 vaccine; and group C was vaccinated intramuscularly (3 mL/animal) with the combined BVDV-1a, BVDV-2 and *C. perfringens* toxoid inactivated vaccine.

All camels' calves were bled and sera were collected on the days of initial vaccination (zero day); on the day booster vaccinations (2 weeks post-vaccination); 2 weeks post-booster vaccination (PBV); 1-month PBV then, monthly up to 7 months post-vaccination. Sera were inactivated at 56°C for 30 minutes and stored at -20°C to be examined by serology.

Challenge trial of the prepared vaccines in camels

Twelve camels were used in challenge test after vaccination by combined vaccine (vaccine C). Camels were assigned into two groups (six animals each). The first group was challenged with alpha toxin of *C. perfringens* type A at three different periods after 2nd dose of vaccination (2, 5, and 7 months). The challenge performed in two animals in different periods, and before challenge blood samples were taken for evaluation of antibody titer. The second group (six animals) was challenged with BVDV in the same manner of the previous group.

Serological investigations

Virus neutralization test

The VNT was done on log phase MDBK cell cultures grown in microtiter plates using two-fold dilutions of each serum sample, in quadruplicates, incubated with 100 $TCID_{50}$ of each virus (BVDV-1a and BVDV-2) and a 100 µL MDBK cells, separately [34]. After 2–3 days of incubation at 37°C, the final readings were recorded. The virus neutralizing antibody titers of serum samples were expressed as $\log_{10}$ $TCID_{50}$ of the reciprocal serum dilution that protected ≥50% of cells in the microtiter plate following the calculation procedure of [35]. In this study, a serum sample with a titer of <1:4 or <0.6 $\log_{10}$ $TCID_{50}$ was regarded as negative where it was the lowest final dilution tested.

Toxin anti-toxin neutralization test

It was done following the procedure described to verify the antitoxin titers against *C. perfringens* type A in sera of vaccinated calves [36]. Briefly, L+/2 dose of *C. perfringens* type A alpha toxin was determined (1 L+/2 dose of toxin is defined as the smallest amount of toxin that can be combined with 0.5 unit of the standard antitoxin and cause death when injected into mice). Serum samples were two-fold serially diluted and an equal volume of alpha toxin dose (L+/2) was added to each serum dilution, then the mixture was incubated at 37°C for 1 hour to allow neutralization. Two mice were injected intravenously with 0.2 mL from each serum/toxin dilution mixture and observed for 24 hours. The reciprocal of the highest dilution of serum that caused death of all mice multiplied by 2 was regarded as the antitoxin titer which was expressed as IU/mL. An antitoxin titer of <1 IU/mL was regarded as negative and non-protective.

Statistical analysis

The normality of antibody titers against alpha toxin of *C. perfringens* and BVDV in different groups of vaccinated camels was checked by Shapiro-Wilk test. The titers in different groups are skewed and not normal distribution, so performed Mann-Whitney rank test as non-parametric test to differentiate between the two groups of vaccinated camels (monovalent and combined) and consider the significance between groups when p-value <0.05

Sensitivity and specificity of antibody titer against alpha toxin of *C. perfringens* type A and BVDV were evaluated by creating receiver operator characteristic (ROC) curve and determined the area under the ROC curve (AUC). An AUC of 0.7–0.9 was considered moderately accurate, AUC of >0.9 highly accurate, and AUC of 1 was perfect [37].

The Youden Index (J)=maximum (sensitivity+specificity-1) was used to identify the optimal cutoff values for detection of protective titer which able to protect camels against alpha toxin of *C. perfringens* type A and BVDV. The maximum value of the Youden Index is 1 (perfect test) and the minimum is 0 when the test has no value. Graphically, Youden Index (J) is the maximum vertical distance between the ROC curve and the diagonal line.

The ROC curves and values were created and estimated to assess the accuracy after perform challenge at different intervals post the 2nd dose of vaccination (2 months, 5 months, and 7 months). The values could help in differentiating between antibody titers which able and unable to protect animals at different periods post-vaccination. The calculation was carried out using R program for Windows ver. 4.0.3 (The R Foundation for Statistical Computing, Vienna, Austria).

Results

The Shapiro-Wilk test for normality of the data for vaccine A and vaccine B showed p=0.02161 and p=0.009579, respectively, indicating that the data is not normally distributed. Over the estimation duration, the mean antibody titer against alpha toxin in a group of camels vaccinated with vaccine A was (3.16±0.428 IU/mL). There was no difference in the mean titer of camels vaccinated with vaccine C, which was (3.33±0.46 IU/mL) over the estimate period (Z[94]=-0.58641, p=0.5606).

Challenge trial of the prepared vaccines in camels

Animals in both groups observed with any symptoms or illness was recorded and evaluated. The ROC and Youden Index (J) were calculated to determine the cutoff values of antibody titer which able to protect camels against infection.

Sensitivity and specificity for vaccine A

Estimated cutoff value for vaccine A revealed 3 IU/mL with estimated 95% confidence interval (CI) of sensitivity (0.714), specificity (0.833), and AUC (0.895 [0.766–1.023]) as showed in Table 1 and Fig. 1.

Sensitivity and specificity for vaccine B

The approximate cutoff value for antibody titer against alpha toxin of *C. perfringens* type A for vaccine B was 2 IU/mL, with a 95% CI of sensitivity (0.888), specificity (1), and AUC (0.981 [0.942–1.021]) (Table 1, Fig. 2).

Table 1. Sensitivity and specificity of different prepared vaccines describing significance of challenge trial

Vaccine type	Antibody titer	Threshold (IU/mL)	Sensitivity (95% CI)	Specificity (95% CI)	AUC
Vaccine C	Alpha toxin of *C. perfringens*	>2	0.88 (0.75–0.96)	1.0 (0.292–1.02)	0.98 (0.94–1.02)
Vaccine A & C	Alpha toxin of *C. perfringens*	>2	0.91 (0.82–0.95)	0.77 (0.39–0.97)	0.92 (0.84–1.01)
Vaccine C	BVD-1	>1.1	0.82 (0.74–0.88)	1.0 (0.94–1.01)	0.94 (0.88–1.01)
Vaccine A & C	BVD-1	>1.1	0.88 (0.79–0.91)	1.0 (0.97–1.01)	0.97 (0.94–0.99)
Vaccine C	BVD-II	>0.9	0.94 (0.89–0.99)	1.0 (0.97–1.01)	0.96 (0.91–1.01)
Vaccine A & C	BVD-II	>0.9	0.92 (0.85–0.97)	1.0 (0.95–1.01)	0.97 (0.94–1.01)

CI, confidence interval; AUC, area under the receiver operator characteristic curve; *C. perfringens, Clostridium perfringens*, BVD, bovine viral diarrhea.

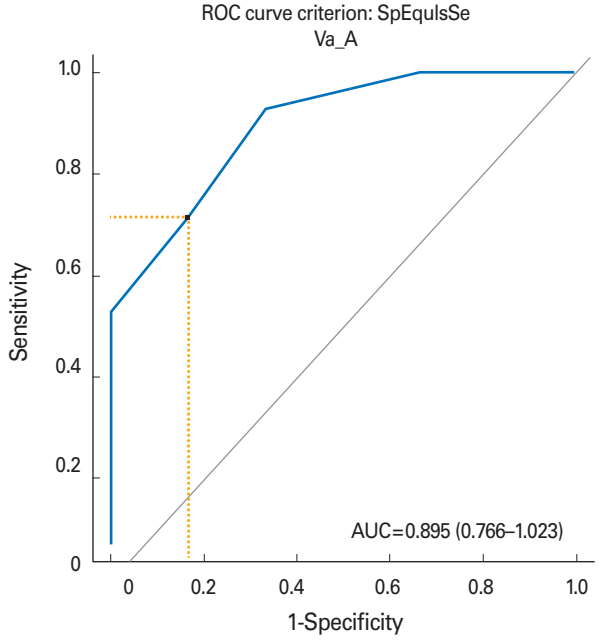

Fig. 1. Sensitivity and specificity test for group of camels vaccinated with vaccine A showing area under the receiver operator characteristic (ROC) curve (AUC).

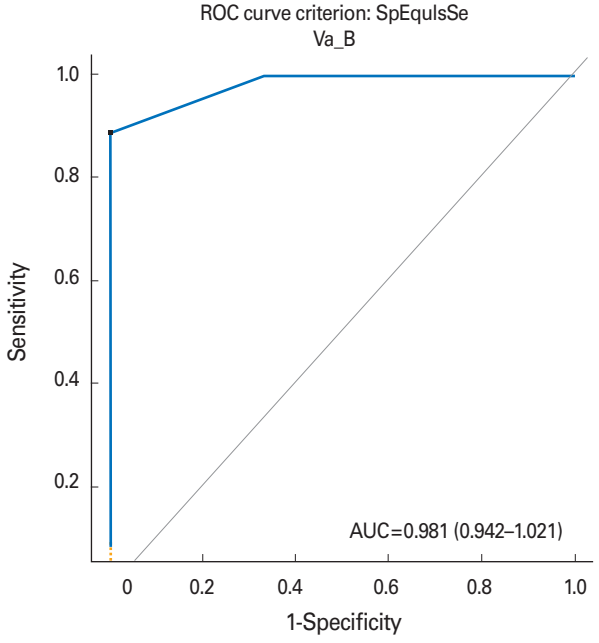

Fig. 2. Sensitivity and specificity of antibody titer against alpha toxin of *Clostridium perfringens* toxinotype A vaccinated by vaccine B. ROC, receiver operator characteristic; AUC, area under the ROC curve.

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Sensitivity and specificity for vaccines A and C

Estimated cutoff value of two vaccines for antibody titer against alpha toxin of *C. perfringens* toxinotype A was 2 IU/mL with estimated 95% CI of sensitivity (0.908), specificity (0.777), and AUC (0.922 [0.836–1.008]) (Table 1, Fig. 3).

The normality of antibody titers against BVD-1 in camels vaccinated with vaccines B and C was tested using the Shapiro-Wilk test, which revealed that p=0.001826 and p=0.001799 for both groups, indicating that the data did not follow a normal distribution. The Wilcoxon Mann-Whitney test was used to compare the two groups.

We discovered that antibody titer against BVD-1 in camels vaccinated with vaccine B, where the mean titer over the estimation period was (1.25±0.16 IU/mL), did not vary from camels vaccinated with vaccine C, where the mean titer over the estimation period was (1.24±0.16 IU/mL; Z [94]=0.1026, p=0.9201).

We discovered that antibody titer against BVD-2 in camels vaccinated with vaccine B, where the mean titer over the estimation period was (1.29±0.16 IU/mL), had no difference from camels vaccinated with vaccine C, where the mean titer over the estimation period was (1.28±0.16 IU/mL; Z[94]=0.08, p=0.9346).

The estimated cutoff value for antibody titer against BVD-1 in the vaccine B group was 1.1 IU/m/L, with a sensitivity of 93.75%, a specificity of 100%, and an AUC (0.991 [0.975–1.007]) (Fig. 4).

The estimated cutoff value for antibody titer against BVD-1 in group vaccinated with vaccine C was 1.1 IU/mL, with a sen-

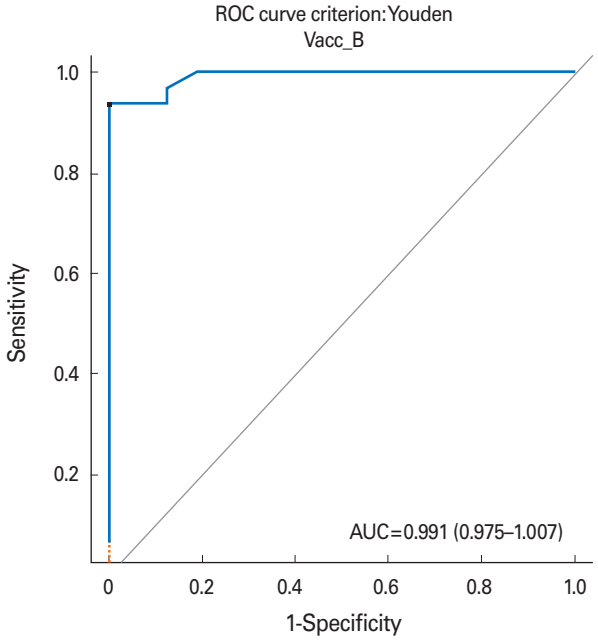

Fig. 4. The cutoff value for antibody titer against bovine viral diarrhea-1 in the vaccine B group. ROC, receiver operator characteristic; AUC, area under the ROC curve.

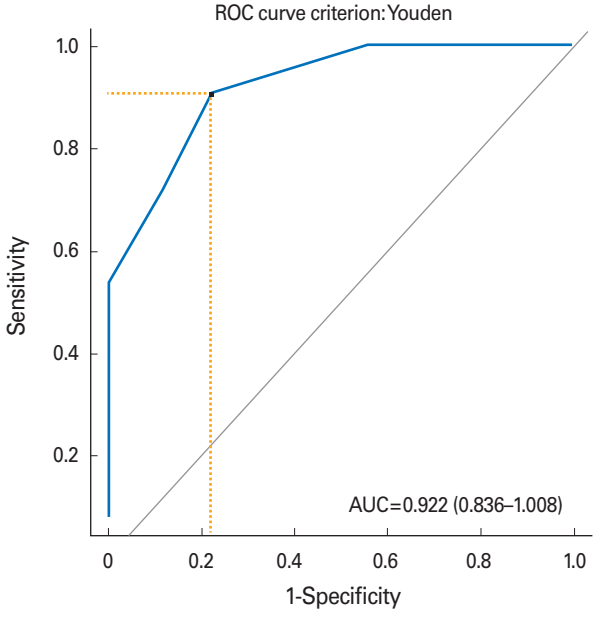

Fig. 3. The antibody titer sensitivity and specificity test for both groups of animals vaccinated with vaccines A and C. ROC, receiver operator characteristic; AUC, area under the ROC curve.

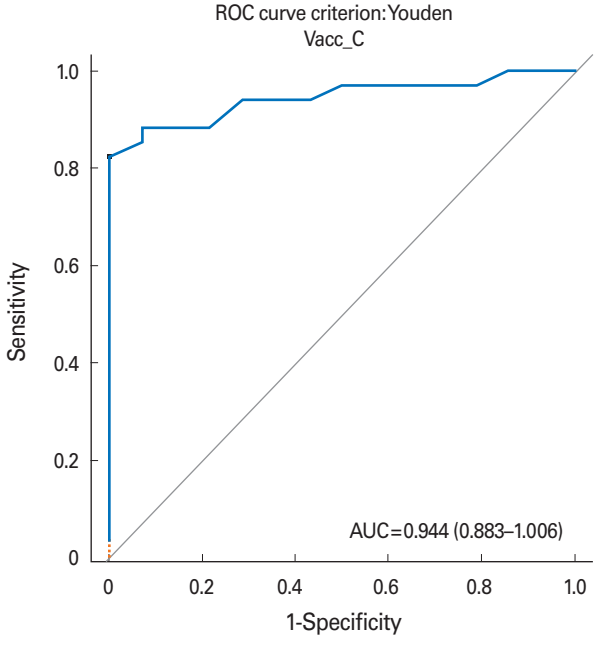

Fig. 5. The cutoff value for antibody titer against bovine viral diarrhea-1 in the vaccine C group. ROC, receiver operator characteristic; AUC, area under the ROC curve.

sitivity of 82.35%, and a specificity of 100%, and an AUC (0.944 [0.883–1.006]) (Fig. 5).

In both groups of camels vaccinated with vaccines B and C, the estimated cutoff value of antibody titer against BVD-1 was 1.1 IU/mL, with a sensitivity of 87.8%, a specificity of 100%, and an AUC (0.969 [0.939–0.999]) (Fig. 6).

The estimated cutoff value for antibody titer against BVD-2 in the vaccine B was 1.19 IU/mL, with a sensitivity of 93.75%, a specificity of 100%, and an AUC (0.98 [0.95–1.011]) (Fig. 7).

The estimated cutoff value for antibody titer against BVD-2

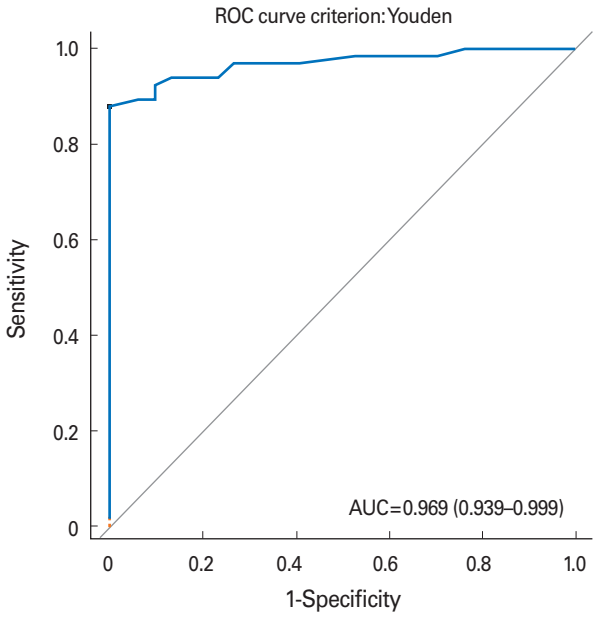

Fig. 6. The cutoff value of antibody titer against bovine viral diarrhea-1 in both groups of camels vaccinated with vaccine B and vaccine C. ROC, receiver operator characteristic; AUC, area under the ROC curve.

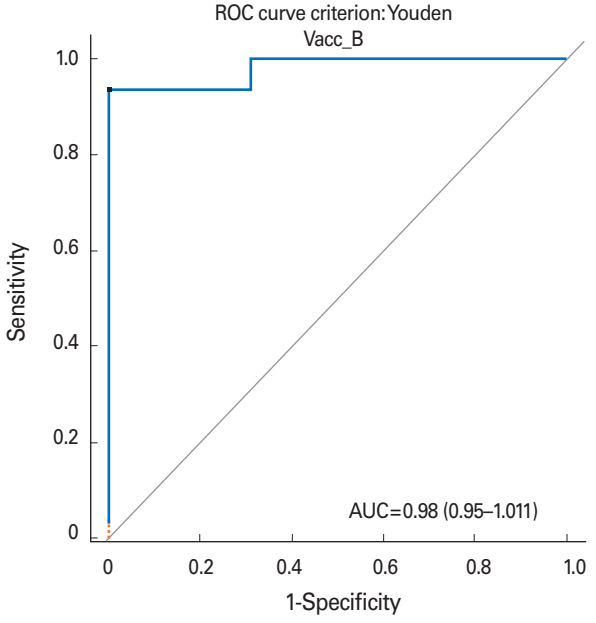

Fig. 7. The cutoff value for antibody titer against bovine viral diarrhea-2 in the vaccine B group. ROC, receiver operator characteristic; AUC, area under the ROC curve.

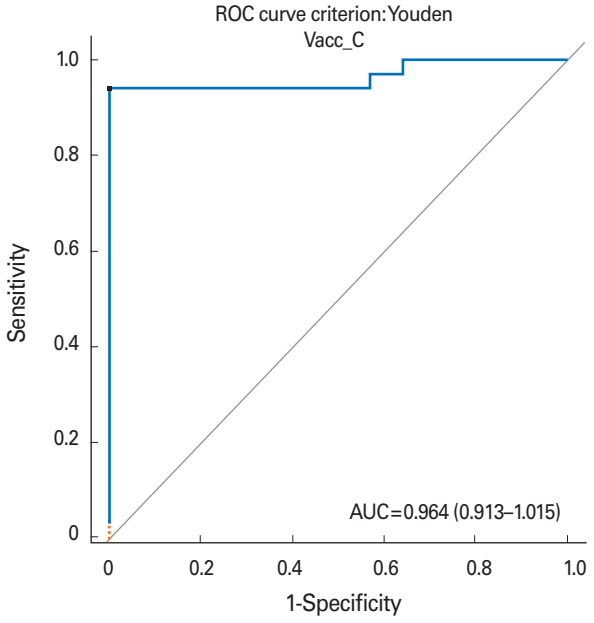

Fig. 8. The cutoff value for antibody titer against bovine viral diarrhea-2 in the vaccine C group. ROC, receiver operator characteristic; AUC, area under the ROC curve.

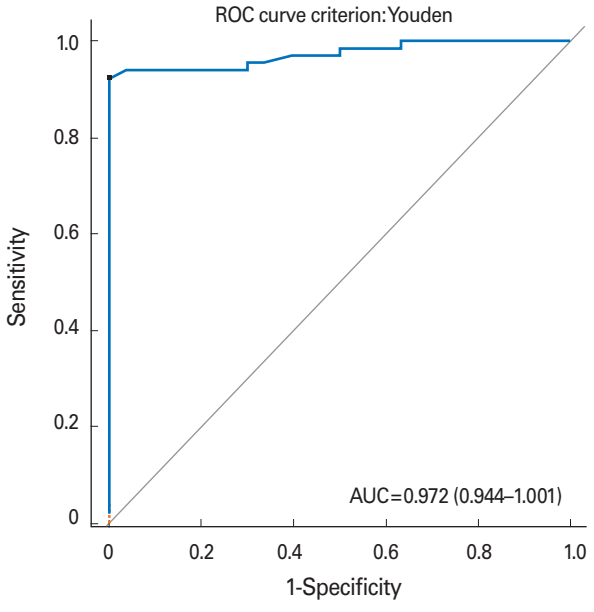

Fig. 9. The cutoff value for antibody titers against bovine viral diarrhea-1 in both groups of camels vaccinated with vaccine B and vaccine C. ROC, receiver operator characteristic; AUC, area under the ROC curve.

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in the vaccine C group was 0.9 IU/mL, with a sensitivity of 94.11%, a specificity of 100%, and an AUC (0.964 [0.913–1.015]) (Fig. 8).

The estimated cutoff value for antibody titers against BVD-1 in both groups of camels vaccinated with vaccine B and C was 0.91 IU/mL, with a sensitivity of 92.42%, a specificity of 100%, and an AUC (0.972 [0.944–1.001]) (Fig. 9).

Discussion

Since the NCD is a multifactorial and detrimental condition affecting the camel breeds leaving it with losses, the need to control and contain this syndrome by vaccination was arise. Both bacterial and viral agents are recorded in Egypt for long time in all species of farm animals [4,23].

It was clear that camels have a high percentage of *C. perfringens* type A in their gastrointestinal tracts, which can cause enterotoxaemia, diarrhea, and sudden death, particularly in young camels, and is also linked to 5%–20% cases of antibiotic-associated diarrhea [38].

The antibody response to vaccines A and C indicated that there was a protective serum neutralizing antibody titer level expressed in IU/mL against alpha toxoid of *C. perfringens* type A started at 2nd week post-vaccination (4.67±0.82 and 4.5±1.04) and reached the highest level at 3rd month in vaccine A (4.17±0.98) and 1st month in vaccine C (5.0±0.89) post-vaccination, respectively (Tables 2, 3 and Fig. 10). Based on statistical analysis of the obtained data, there was no significant difference between antibody titer against alpha toxoid of *C. perfringens* type A in vaccine A and vaccine C. These results attributed to the use of the same dose of alpha toxoid *C. perfringens* type A (60 MLD/mL) and there was no antagonist between alpha toxoid of *C. perfringens* type A and BVDV in combined vaccine. The results supported by El-Sergany et al. [39] who used a fixed dose (60 MLD/mL) for vaccination where the antibody titer reached 5 IU/mL. The prepared vaccines

Table 2. Antibody titer against alpha toxin in sera of vaccinated camel with monovalent *Clostridium perfringens* toxinotype A toxoid vaccine (vaccine A) assessed by toxin neutralization test

Camel ID	Period post 2nd dose of vaccination (IU/mL)							
	2 wk	1 mo	2 mo	3 mo	4 mo	5 mo	6 mo	7 mo
Ca-1	4	4	3	6	5	2	2	1
Ca-2	6	6	5	4	3	3	2	2
Ca-3	4	3	5	4	4	2	2	2
Ca-4	5	4	3	3	4	2	1	1
Ca-5	5	4	3	4	3	2	1	0.5
Ca-6	4	4	4	4	2	3	1	0.5
Mean±SD	4.67±0.82	4.17±0.98	3.83±0.98	4.17±0.98	3.5±1.05	2.33±0.52	1.5±0.55	1.17±0.68
Median	4.5	4	3.5	4	3.5	2	1.5	1

SD, standard deviation.

Table 3. Antibody titer against alpha toxin measured by the toxin neutralization test in serum of camels vaccinated with the combined bovine viral diarrhea and *Clostridium perfringens* type A toxoid vaccine (vaccine C)

Camel ID	Period post 2nd dose of vaccination (IU/mL)							
	2 wk	1 mo	2 mo	3 mo	4 mo	5 mo	6 mo	7 mo
Ca-13	4	4	4	4	3	2	2	1
Ca-14	5	5	5	6	4	3	2	1
Ca-15	5	5	4	4	3	2	2	1
Ca-16	4	6	5	4	3	2	2	1
Ca-17	3	4	4	4	3	2	1	0.5
Ca-18	6	6	5	5	4	3	1	0.5
Mean±SD	4.5±1.04	5±0.89	4.5±0.54	4.5±0.83	3.3±0.51	2.33±0.51	1.6±0.51	0.83±0.25
Median	4.5	5	4.5	4	3	2	2	1

SD, standard deviation.

covered the permissible limit (4 IU/mL) approved by United States Department of Agriculture in 2002 [40]. Also, results were supported by different intervals of virulent bacteria challenges on camels previously vaccinated with either vaccine.

The antibody response to vaccines B and C indicated that there was a protective serum neutralizing antibody titer level expressed in IU/mL of BVDV-1 and BVDV-2 started at 1st month post-vaccination (1.98±0.12 and 1.98±0.09) and (1.83±00.03 and 2.08±0.15), respectively. The titers reached the highest level at 2nd month in both viruses and vaccines B (1.84±0.06 and 1.83±0.04) and at 4th month in BVDV-1 in vaccine C (2.06 ±0.14) and 3rd month in BVDV-2 in vaccine C (1.63±0.03) (Tables 4, 5 and Figs. 11, 12). The specificity and sensitivity test for animals vaccinated with monovalent alpha toxoid of *C. perfringens* type A after challenge in comparison to titer showed that the cutoff value was 3 IU/mL with sensitivity and specificity (71.4%, respectively).

Animals were successfully vaccinated with multiple vaccines at the same time in experiments that included both bacterial and viral vaccines [26,27].

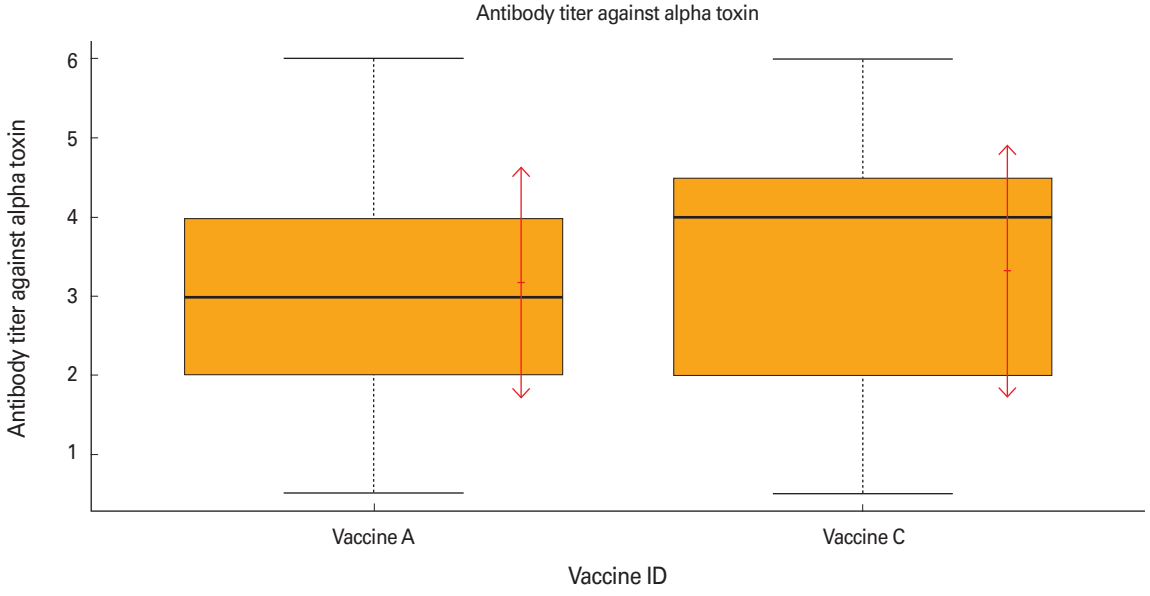

Fig. 10. Antibody titer against alpha toxin in sera of camel vaccinated with vaccine A and vaccine C.

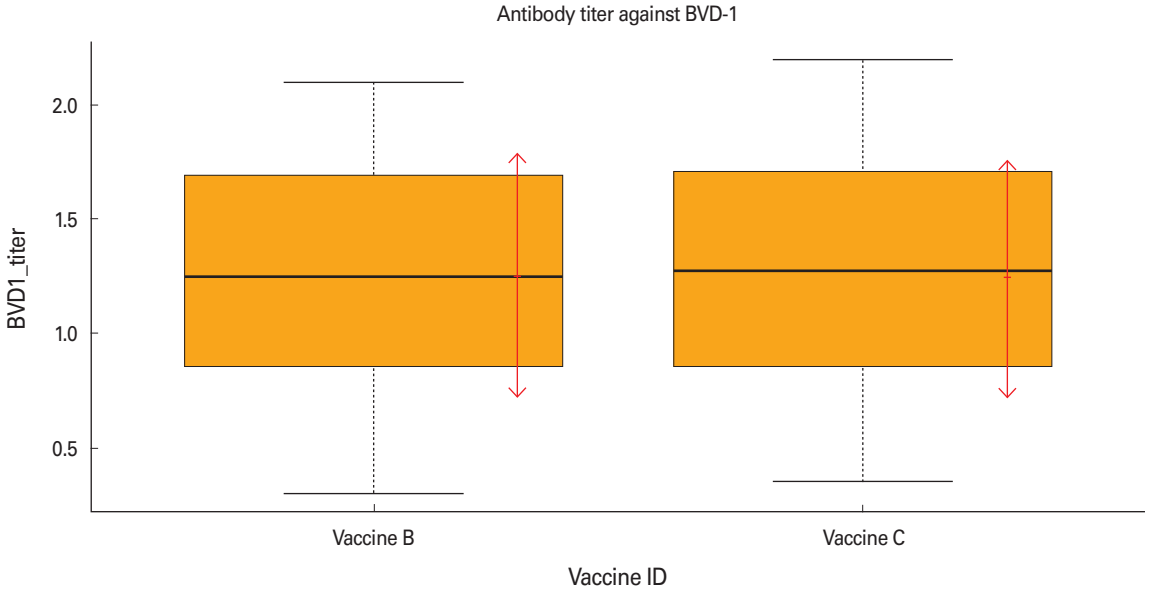

Fig. 11. Antibody titer against bovine viral diarrhea (BVD)-1 in both groups of camels vaccinated with vaccine B and vaccine C.

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Table 4. Antibody titer against BVD types 1 and 2 in sera of vaccinated camels vaccinated with monovalent BVD types 1 and 2 (vaccine B) determined by virus neutralization test

Camel ID	Period post 2nd dose of vaccination (IU/mL)															
	2 wk		1 mo		2 mo		3 mo		4 mo		5 mo		6 mo		7 mo	
	BVD1	BVD2	BVD1	BVD2	BVD1	BVD2	BVD1	BVD2	BVD1	BVD2	BVD1	BVD2	BVD1	BVD2	BVD1	BVD2
Ca-7	0.36	0.42	2	2.1	1.8	1.88	1.55	1.66	1.35	1.42	1.2	1.28	0.95	0.9	0.72	0.85
Ca-8	0.4	0.44	1.95	2.1	1.9	1.82	1.65	1.58	1.4	1.33	1	1.2	0.9	0.91	0.82	0.88
Ca-9	0.4	0.48	1.95	2	1.92	1.8	1.61	1.62	1.39	1.48	0.9	1.27	1	0.82	0.73	0.72
Ca-10	0.42	0.39	2.2	1.98	1.77	1.86	1.59	1.63	1.36	1.42	1.2	1.2	0.9	0.83	0.75	0.75
Ca-11	0.35	0.38	1.88	2.3	1.82	1.82	1.53	1.55	1.37	1.43	1.1	1.23	0.93	0.87	0.8	0.82
Ca-12	0.42	0.4	1.9	1.88	1.83	1.79	1.54	1.62	1.39	1.39	1.1	1.28	0.92	0.88	0.75	0.8
Mean± SD	0.39±0.03	0.34±0.04	1.98±0.12	1.98±0.09	1.84±0.06	1.83±0.04	1.58±0.05	1.58±0.04	1.38±0.02	1.37±0.19	1.08±0.12	1.22±0.08	0.93±0.04	0.96±0.03	0.76±0.04	0.76±0.04
Median	0.4	0.34	1.95	1.95	1.83	1.83	1.57	1.6	1.38	1.4	1.1	1.2	0.92	0.94	0.75	0.75

BVD, bovine viral diarrhea; SD, standard deviation.

Table 5. Antibody titers against BVD types 1 and 2 in sera of camels vaccinated with the combined BVD and *Clostridium perfringens* toxinotype A toxoid vaccine (vaccine C) as determined by the virus neutralization test

Camel ID	Period post 2nd dose of vaccination (IU/mL)															
	2 wk		1 mo		2 mo		3 mo		4 mo		5 mo		6 mo		7 mo	
	BVD1	BVD2	BVD1	BVD2	BVD1	BVD2	BVD1	BVD2	BVD1	BVD2	BVD1	BVD2	BVD1	BVD2	BVD1	BVD2
Ca-13	0.3	0.44	1.8	2	1.2	1.88	0.94	1.6	2.1	1.4	1.6	1.22	1.3	0.82	0.7	0.71
Ca-14	0.4	0.42	1.85	2.3	1.1	1.86	0.92	1.633	1.95	1.47	1.5	1.28	1.3	0.89	0.8	0.74
Ca-15	0.33	0.41	1.78	2.2	1.4	1.89	0.94	1.67	1.9	1.5	1.6	1.22	1.1	0.86	0.75	0.88
Ca-16	0.35	0.4	1.86	1.9	1.5	1.84	0.99	1.59	1.95	1.38	1.6	1.19	1.2	0.9	0.75	0.8
Ca-17	0.3	0.45	1.82	2.1	1.6	1.83	0.95	1.62	1.9	1.4	1.6	1.23	1.2	0.84	0.79	0.77
Ca-18	0.34	0.41	1.88	2	1.4	1.88	1	1.67	2.1	1.42	1.55	1.27	1.2	0.86	0.75	0.74
Mean± SD	0.42±0.04	0.42±0.02	1.83±0.03	2.08±0.15	1.41±0.05	1.86±0.02	0.87±0.04	1.63±0.03	2.06±0.14	1.43±0.05	1.61±0.04	1.23±0.03	1.24±0.04	0.86±0.03	0.80±0.06	0.77±0.06
Median	0.41	0.42	1.82	2.05	1.42	1.87	0.88	1.63	2.05	1.41	1.62	1.23	1.25	0.86	0.81	0.76

BVD, bovine viral diarrhea; SD, standard deviation.

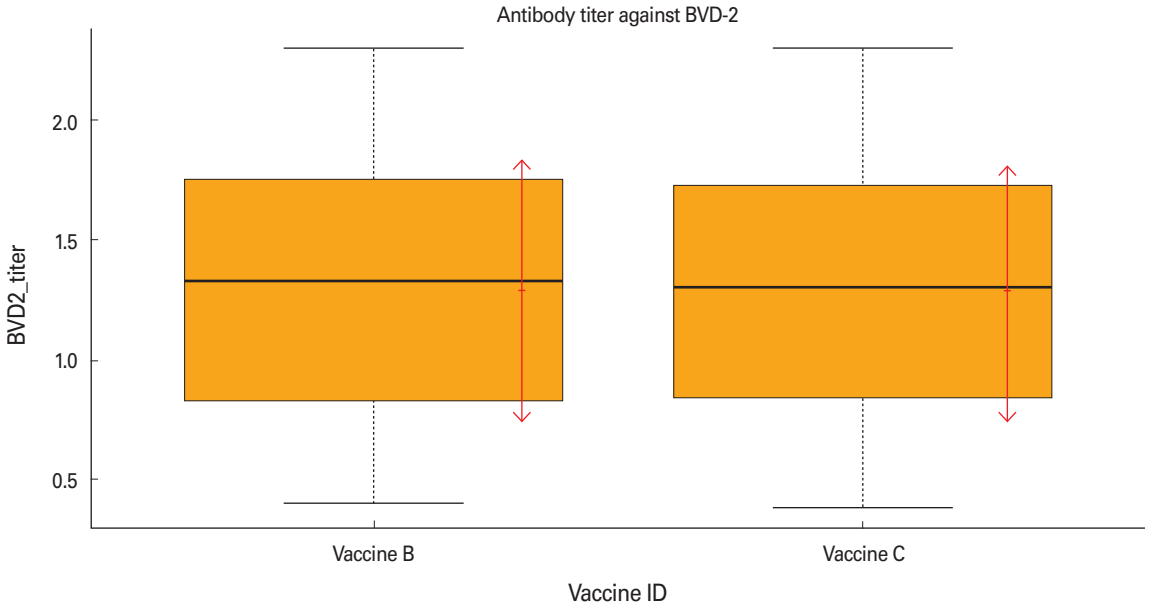

Fig. 12. Antibody titer against bovine viral diarrhea (BVD)-2 in both groups of camels vaccinated with vaccine B and vaccine C.

In the last decade, the use of killed vaccines has been considered. It was found to be safe in lab animals. These findings support the recommendation [32]. The vaccine provided a protective shield of antibodies against viral and bacterial agents that lasted for up to 7 months after vaccination [32].

In this study, the combined vaccines had higher antibody titers against the BVDV than in the monovalent vaccines. The combined vaccine had elevated levels of both bacterial and viral components. The protective titer against *C. perfringens* toxin type A and BVDV was started on the 2nd week and 2nd month after vaccination, respectively. The results were in agreement with the recommendation advised by Elbayoumy et al. [28] who strongly recommended the combined vaccines especially the polyvalent vaccines. These vaccines provide a high level of immunity as well as cover several pathogens in one place without negative impact on the immune system of the animal. On the other hand, monovalent vaccination may provide animals with partial defense, which could be related to Clostridium pathogenesis [41].

In conclusion, clostridial toxoid vaccinations are important because they protect animals from severe diseases caused by the toxoid by providing successful immunization. It is suggested that a vaccination schedule using combined vaccine of *C. perfringens* type A toxoid and the local Egyptian BVDV strains (BVDV-1a and 2) vaccine could be introduced safely and effectively as one-shot vaccine to reduce enterotoxaemia and BVD infections in camel calves, especially where there are uncontrolled movements. Vaccinating the animals at 6-month intervals is also recommended, particularly along the border regions.

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