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Research Article

Use of Rabbits as an Alternative Host for Quality Control of Combined Bovine Enteric Vaccine

Alaa A El-Kholy*,[1], Effat L El-Sayed[1], Elham F El-Sergany[1], Wahid M Ghatas[1], Nermeen G Shafik[2] and Selim S Salama[2]

[1]Veterinary Serum and Vaccine Res. Instit., ARC, El-Sekka El-Beda St., P.O. Box 131, P.C. 11381 Abbassia, Cairo, Egypt; [2]Central Laboratory for Evaluation of Veterinary Biologics, Egypt
***Corresponding author:** aaelfouly@yahoo.com; svri@idsc.net.eg; vsvri@idsc.gov.eg

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ABSTRACT

This endeavor addresses the utility of rabbits versus susceptible calves, for relative potency evaluation of a newly prepared combined inactivated oil adjuvanted vaccine containing: Bovine *Rotavirus*, Bovine *Coronavirus*, *Escherichia Coli* K99 and alpha Toxoid of *Clostridium perfringens* type A. The new vaccine, namely Entero-4 was prepared and inoculated into six groups, each of seronegative three calves and five rabbits, by two different doses (3ml and 4ml /animal) using both intramuscular and subcutaneous routes. Two weeks later, all vaccinates received a similar booster dose. Seroconversion was monitored post vaccinations in sera of all vaccinates by Virus Neutralization, Micro Agglutination and Toxin-Antitoxin Neutralization Tests along with an indirect Enzyme linked immunosorbent assay. The prepared vaccine was proved safe and potent, manifested by high mean antibody titers specific to all viral and bacterial components as detected by all tests, relatively in sera of vaccinated calves and rabbits, using different routes and doses. Calves vaccinated with either doses of 3 ml or 4 ml seroconverted similarly, suggesting a better economic use of the 3 ml as an initial and a booster dose with revaccination every 9 months. Nevertheless, our data signified the validity of rabbits, as laboratory animals, to evaluate the relative potency of bovine enteric vaccines. That could initiate a potential to utilize rabbits as a versatile and cheaper alternative for potency verification of other veterinary vaccines, overcoming expensive and difficult availability of seronegative naive animal hosts.

Key words: Bovine *Coronavirus* (*BCoV*), Bovine *Rotavirus* (*BRV*), *Clostridium perfringens* type A, *E. Coli* K_{99} (ETECK99), ELISA, Entero 4 vaccine, Relative potency in rabbits

INTRODUCTION

Neonatal calf diarrhea (NCD), also known as calf scours and calf enteritis, is a worldwide, cost-effective, multifactorial clinical manifestation, associated with a variety of infectious and non-infectious causes (Snodgrass *et al*., 1986; Constable, 2004). Infectious causes of NCD include bacteria (*Escherichia coli* and *Clostridium perfringens*), viruses (bovine *rotavirus* and *corona virus*), and intestinal parasites that often occurs in combination and might be associated with other nutritional or environmental factors (Sihvonen and Miettinen, 1985; Gumusova *et al*., 2007; Uhde *et al*., 2008; Malik *et al*., 2012).

Bovine *rotaviruses* (*BRV*) are a major cause of acute gastroenteritis, malabsorptive diarrhea, dehydration and death in newborn calves up to 8 weeks of age (Estes and Kapikian, 2007; Robaiee and Al-Farwachi, 2013). Recent molecular studies have suggested interspecies transmission of group A rotaviruses and genetic reassortment of rotaviruses, particularly in developing countries, where different domestic animal species as well as humans live in close contact (Park *et al*., 2006; Dhama *et al*., 2009; Cashman *et al*., 2010; Martella *et al*., 2010). Bovine *corona* virus (*BCoV*) is an important viral pathogen associated with neonatal calf diarrhea, winter dysentery in adult cattle and respiratory tract disorders in bovines of all ages, resulting in significant economic losses worldwide due to mortalities and decreased productivity of the survivors (Saif, 2010; Hansa *et al*., 2013).

Enterotoxigenic *E. coli* (ETEC) are the most commonly isolated pathotypes from cases of NCD. Newborn calves that are left to suckle their dams are exposed to *ETEC* infection shortly after birth, particularly

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from birth to 7 days of age, as *E. Coli* are normally found in manure of healthy cows. Consequently, most of *ETEC* attach to and colonize the microvilli of small intestine mucosa through their pili or fimbrial adhesions, projections from the bacterial cell surface namely *K99*, causing enterotoxin-derived enteritis and secretory diarrhea, dehydration and death (Younis *et al*., 2009; Nataro and Barry, 2013).

Clostridium perfringens (*C. perfringens*) type A is an economically important pathogen that has been associated with several disease syndromes in cattle, including Clostridial enteritis in neonates manifested by hemorrhagic abomasitis and abomasal ulceration (Songer and Miskimins, 2005); hemorrhagic enteritis in adult cattle and calves (Songer, 1996) and necro-haemorrhagic enteritis along with sudden death in newborn and veal calves specially during feeding period (Goossens *et al*., 2017).

Many studies have been conducted towards development of vaccines for protecting newborn calves against the commonly isolated pathogens of NCD including *BRV, BCoV, ETEC* and *C. Perfringens*. Dams are to be inoculated at late pregnancy with such vaccines, providing passive immunity to their offspring via colostrum (Daoud *et al*., 2003).

Use of rabbits as a relatively cheap and easy to handle lab animal is ongoing in researches of drug testing, production of pathogens-specific antisera, studies of pathogenesis and infectivity of many animal pathogens (Valera *et al*., 2008). Furthermore, many authors succeeded to use rabbits for evaluation of different vaccines (Tsenova *et al*., 2006; Allam *et al*., 2012; Effat *et al*., 2016).

This endeavor was planned to prepare and evaluate a local inactivated oil adjuvanted combined vaccine (Entero-4) for protecting calves against *BRV* and *BCoV* along with *ETEC*K99 and *C. perfringens* type A. Besides, utility of rabbit as a cheaper and easier to handle lab animal was explored as a surrogate for calves in verifying the vaccine relative potency.

MATERIALS AND METHODS

Ethical approval

All procedures performed according to Egyptian ethical standards of the national research committee. Approved from Institutional Animal Care and Use Committee (IACUC) Cairo University under number 1221 /2013 review and approve all activities involving the use of vertebrate animals prior to their initiation.

Viruses and cells

The local strain of bovine *rotavirus* (*BRV*) with a titer of $10^{7.8}$ $TCID_{50}$/ml and the reference strain Mebus of bovine *coronavirus* (*BCoV*) with a titer of $10^{8.5}$ $TCID_{50}$/ ml, were kindly supplied by the Rinder Pest Like Diseases Department, (RPLD), Veterinary Sera and Vaccines Researches Institute (VSVRI), Abbassia, Cairo, Egypt. Both virus entities were propagated and titrated on Madin-Darby bovine kidney (MBDK) cell culture which has been proved free of any extraneous contamination. The MBDK cells were grown at 37ºC, in minimum essential medium with Earle's salts (MEME) 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, both viruses were tested for their identity by serum neutralization test using the respective specific reference antisera (Glasgow Animal Clinic, Kentucky, USA).

Bacterial strains

Pure cultures of the *ETEC*K99 and *C. perfringens* type A vaccine strains, used in formulation of the Entero-4 vaccine, were obtained from Aerobic and Anaerobic Bacterial Vaccines Departments, respectively, VSVRI, Abbassia, Cairo, Egypt.

Experimental animals

Mice

One hundred and twenty Albino Swiss mice were obtained from Laboratory Animal Breeding Farm at VSVRI and were used to study the safety of the prepared vaccine, minimal lethal dose (MLD) of *C. perfringens* alpha toxin, and toxin neutralization test (TNT).

Calves

Eighteen clinically apparently healthy mixed bred calves aging 6-8 months, supplied by VSVRI, were used to study the safety and potency of the newly prepared vaccine.

Rabbits

Thirty adult Bosket rabbits of about 2.5 kg body weight each, were used to evaluate the relative potency, safety and dose / route responses of the vaccine under testing (five rabbits/group).

Before vaccination, all calves and rabbits were proved seronegative to both viral and bacterial strains incorporated in the prepared vaccine and were examined for internal and external parasites. All animals were kept in a hygienic animal facility at the VSVRI during the period of experimentation.

Vaccine preparation

The current Entero-4 vaccine was formulated to comprise two inactivated virus antigens *BRV* and *BCoV*, together with two inactivated bacterial antigens, *E.Coli K99* culture and *C. perfringens type A* toxoid in addition to an oil adjuvant (Montanoide ISA, 206).

Briefly, Confluent monolayers of MDBK cells grown in Roux bottles were inoculated separately with *BRV* and *BCoV* at multiplicity of infection of 0.5 and incubated at 37°C, in the presence of 1 mg/ml of trypsin. After 70-80 % of the infected cells showed Cytopathic effect (CPE), the culture fluid was harvested after two freeze-thaw cycles, clarified and titrated. Then, viruses were inactivated individually by stirring with 0.01 M binary ethyleneimine (BEI; 10% v/v) at 37°C for overnight. Sodium thiosulphate 20% was then added with a final concentration of 2% to stop the action of BEI (Daoud *et al*., 2003). On final vaccine formulation both *BRV* and *BCoV* were measured so that a vaccine dose should contain least titer of 10^6 $TCID_{50}$ of each (OIE, 2016).

The *E. coli K99* strain was grown on its selective medium (Minca-vitox supplemented medium) and incubated for 24 h at 37°C, producing a final culture suspension that was inactivated by adding Formaldehyde

37% as 0.5% (v/v). The final *E. coli K99*antigen content was considered to be not less than 1 x $10^{9.5}$CFU per vaccine dose.

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 (El-Helw *et al*., 2017). The toxin was separated and concentrated from the bacterial culture after its minimum lethal dose (MLD) being determined (Fu *et al*., 2004). Then, it was inactivated by adding Formaldehyde 37% (0.5%; v/v) for about 7 days until complete toxin inactivation. The produced toxoid was adjusted to contain 80 MLD/ vaccinal dose. The inactivated viral and bacterial components were mixed altogether in equal volumes, then the sterile Montanoide oil 206 (Seppic, France) was added to the formulated mix in a ratio of 1:1 (Daoud *et al*., 2003; Nataro and Barry, 2013).

Quality control of the vaccine

Sterility testing

Different steps of vaccine preparation (includes virus and bacterial propagations, inactivated fluids and the final product) were subjected to in process testing for sterility according to (OIE, 2016) to prove that prepared vaccine was free from any contaminants.

Safety according to (OIE, 2016)

Safety in mice

Twenty adult albino mice were used to study the safety of the vaccine. The mice were divided into two groups each of 10 mice. The first group was inoculated intraperitoneally (ip) with 0.2 ml/mice with the prepared vaccine whereas the other one was inoculated with physiological saline using the same dose and route and kept as placebo control group.

Safety in calves and Rabbits

Six calves and Ten rabbits were used for vaccine safety testing where each animal species was assigned into two groups. Half of animals of the first group was inoculated intramuscularly (im) twice with the vaccine double high dose (8 ml) and the other half was similarly inoculated with the same dose subcutaneously(sc). All animals were observed up to 21 days afterwards for development of any clinical abnormalities.

Potency in calves and Relative Potency in Rabbits

The test was conducted using susceptible calves and rabbits which were randomly assigned into 4 groups each of three calves and five rabbits. The tested vaccine was inoculated in 4 groups by two different routes (im and sc) and two doses (3 ml and 4 ml / animal). Two weeks later, all vaccinated animals in the four groups received a booster vaccination with the same dose and route.

All calves and rabbits were bled and sera were collected on the day of initial vaccination (Zero day); on the day booster vaccinations (2 weeks post vaccination); two weeks post booster vaccination (PBV); 1 month PBV then, monthly up to 12 months PBV**.** Sera were inactivated at 56°C for 30 min and stored at -20°C to be examined by serological assays.

Serological investigations

Virus neutralization test (VNT)

The VNT was done on log phase MDBK cell cultures grown in microtitration plates using two-fold dilutions of each serum sample, in quadruplicates, incubated with 100 tissue culture infective dose fifty ($TCID_{50}$) of each virus (*BRV* and *BCoV*) and a 100 µl MDBK cells, separately (Robson *et al*., 1980). After 2-3 days of incubation at 37ºC, the final readings were recorded. The virus neutralizing antibody (VN-Ab) titers of serum samples were expressed as $\log_{10}$ $TCID_{50}$ of the reciprocal serum dilution that protected ≥50% of cells in the microtitration plate wells following the calculation procedure of Reed and Muench, (1938). In this study, a serum sample with a titer of <1:4 or <0.6 $\log_{10}$ $TCID_{50}$ was regarded as 0 (negative) as it was the lowest final dilution tested.

Micro agglutination test (MAT)

The MAT was conducted to determine the geometric mean agglutinating antibody (GMA) titers against *E. Coli* K99 in sera of vaccinated groups, using V-shape bottomed microtitration plates as one row of 12 wells was utilized to each sample. High, low, and negative control sera of known titers as well as an antigen control were included in each test. Each serum sample was initially diluted 1:10 and a two-fold serial dilution was made in phosphate buffered saline (PBS), pH 7.2. In each well of one row per sample, a 100 µl of diluted serum sample was added to a 50 µl of PBS and a 50 µl of safranin O-stained K99 sonicated antigen so that the starting dilution of each sample was 1:20The plates were sealed and contents were mixed for 20s, then incubated for 24 h at 37°C. On reading, agglutination was indicated by a mat of stained cells covering the well bottom or by a diminished button of cells in the well center surrounded by a slightly opaque diluent. The GMA titer of ≥ four folds of pre-vaccination titer (0 day) was regarded as protective for both rabbits and calves (Wassel *et al*., 1999).

Toxin neutralization test (TNT)

It was done following the procedure described by Hammer *et al*. (2008) to verify the antitoxin titers against *C. perfringens* type A in sera of vaccinated calves and rabbits. Briefly, L+/2 dose of *C. perfringens* type A alpha toxin was determined (one L+/2dose 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 hr to allow neutralization. Two mice were injected iv 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 divided by 2 was regarding as the antitoxin titer which was expressed as international unit (iu)/ ml. An antitoxin titer of <1 iu /ml was regarded as negative and non-protective.

Indirect ELISA

Seroconversion of vaccinated calves and rabbits against both viral and bacterial components of the prepared vaccine were estimated by a simple indirect ELISA as was described by (Voller *et al*., 1978) after

some modifications. The antigens (*BRV* and *BCoV*, sonicated *E. coli K99* antigen and alpha toxin *C. perfringens* type A) were prepared at concentration of 5-10 µg/ml Each antigen was diluted in PBS (pH 7.2) containing 0.03 % Triton X-100. 96-well Immunolon-I microtitration plates (Nunc, IL, USA) were coated with 100 µl/well of each diluted antigen (about 5-10 µg/ml total protein as determined by the Bradford method) separately, and incubated at 4ºC for overnight. The plates were washed in PBS containing 0.1% Tween 20 (PBST), blocked by adding 100 µl/well blocking buffer (PBS, pH 7.2 containing 5% non-fat milk and 5% bovine serum albumin, BSA) and incubated for 2 h at 37ºC. The plates were decanted and washed three times as before. Each bovine or rabbit serum sample was diluted to 1:10 in PBS (pH 7.2) and inoculated as 100 µl/well in quadruplicates. Each plate included a positive and a negative sera as well as a blank control. The plates were incubated 2 h at 37ºC then, decanted and washed. The diluted conjugate (1:2000), horse radish peroxidase-labeled anti-bovine, Sigma-Aldrich Union, USA.® (for bovine serum samples) or (1:5000) anti-rabbit IgG, Sigma-Aldrich Union, USA.® (for Rabbit serum samples), (KPL, MD, USA), was added as 100 µl/well. After 1 h incubation at 37 ºC, the plates were decanted, washed then, 100 µl/well of the TMB-ELISA substrate (KPL, MD, USA) was added and the plates were agitated until the color developed (15–20 min). The reaction was halted by adding 50 µl/well of stopping solution (KPL, MD, USA). The plates were read using a computer-assisted microplate reader (Vmax kinetic microplate readers, Molecular Devices) at wavelength 450 nm. The absorbance values (OD) of 0.7 and 0.75 were determined as the cut off points for both *BRV* and *BCoV* in calves and Rabbit sera, respectively. Whereas, the estimated cut-off values of *E.coli K99* were 0.8 for calves sera, 0.4 for rabbit sera, and that of *C. perfringens type A* were 0.75 and 0.55 for sera from calves and rabbits, respectively. The resulted OD value of a serum sample ≥ those estimated cut-off values were regarded as positive.

Statistical analysis

All resulted parameters were analyzed for statistical difference by analysis of variance (ANOVA). When a significant difference was found, it was compared pair wise using a Tukey Post. Hoc test for multiple comparisons of observed means. Differences were considered significant at $P<0.05$. All statistical analyses were performed using SPSS-15.0 program for Windows, (*Barton and Peat*, 2014). Moreover, correlations of relative potency among data recorded by ELISA to VNT for *BRV and BCoV*, MAT for *E-Coli K99* and TNT for *C. perfringens* type A were analyzed using linear regression test.

RESULTS

The studies for preparation of polyvalent combined inactivated vaccine gave satisfactory results where it was proved sterile, free from any bacterial, fungal and Mycoplasma contaminants upon cultivation on suitable synthetic media. The sterility results were in harmony with that obtained from animals' inoculation tests (mice, rabbits and calves) which provided clear evidence of vaccine safety. The inoculated mice, rabbits and calves remained clinically normal without deaths or development of local reactions. Also, body temperatures were within normal values and no abnormalities or illness could be observed in all animals during the experiment.

Concerning serological investigations, the results illustrated in Tables (1, 3 and 4) showed that the mean virus neutralizing antibody (VN-Ab) titers and ELISA OD values against *BRV* and *BCoV*, in all vaccinated groups, began to rise by the 2nd week post vaccination (WPV) followed by a significant increase reaching their peaks on the 2th month post booster vaccination (MPBV), then gradually decreased till the end of the experiment. No significant difference could be obtained among all vaccinated groups whether calves or rabbits were vaccinated sc or im with a dose of 3 ml or 4 ml of Entero-4 vaccine ($P>0.05$). Linear regression equation revealed considerable relationship between the mean VN-Ab titers against *BRV* and *BCoV* measured by VNT and OD values measured by ELISA as R^2 values were 0.95, 0.96, 0.96 and 0.89 for calves vaccinated sc with 3 ml dose, im with 3 ml dose, sc with 4 ml dose, and im with 4 ml dose, respectively.

Table 1: Relative mean seroconversion in sera of rabbits and calves against viral antigenic components (*BRV and BCoV*) post vaccination with Entero-4

Dose	Route	Time	Mean Seroconversion							
			BRV				BCoV			
			VNT*		ELISA**		VNT		ELISA	
			rabbit	calves	rabbit	calves	rabbit	calves	rabbit	Calves
3 ml	sc	14 D	0.55±0.01	0.63±0.02	0.75±0.02	0.95±0.01	0.6±0.09	0.50±0.01	0.72±0.08	0.88±0.06
		2 W	1.70±0.02	1.62±0.03	1.66±0.1	1.89±0.01	1.65±0.02	1.73±0.01	1.91±0.12	1.99±0.06
		4 W	1.90±0.12	1.86±02	1.96±0.04	2.15±0.02	1.80±0.02	1.95±0.02	1.98±0.03	2.04±0.09
	im	14 D	0.78±0.12	0.72±0.21	1.30±0.03	1.02±0.03	0.70±0.01	0.67±0.01	0.93±0.04	0.98±0.04
		2 W	1.72±0.11	1.81±0.04	1.97±0.07	2.03±0.06	1.5±0.02	1.89±0.04	1.81±0.11	1.93±0.03
		4 W	1.85±0.03	1.89±0.06	2.15±0.02	2.21±0.03	1.8±0.03	1.95±0.03	1.99±0.14	2.10±0.12
4 ml	sc	14 D	0.9±0.04	0.84±0.02	1.01±0.02	0.9±0.01	0.9±0.01	0.9±0.01	0.96±0.04	1.20±0.9
		2 W	1.6±0.03	1.87±0.05	1.89±0.01	1.78±0.02	1.7±0.03	1.95±0.02	1.88±0.1	2.11±0.3
		4 W	1.85±0.03	1.96±0.03	2.23±0.06	2.09±0.04	1.9±0.1	2.1±0.02	2.24±0.06	2.35±0.06
	im	14 D	0.8±0.04	0.78±0.02	0.97±0.01	1.08±0.01	0.9±0.04	0.85±0.01	1.15±0.07	1.20±0.3
		2 W	1.87±0.05	1.81±0.02	2.15±0.02	2.26±0.03	1.7±0.04	1.8±0.1	1.98±0.04	2.25±0.3
		4 W	1.93±0.02	1.9±0.02	2.34±0.07	2.29±0.04	1.9±0.03	2.1±0.05	2.5±0.02	2.43±0.1

D = Days post 1st dose. W= Weeks post booster dose. sc = Subcutaneous route.im = Intramuscular route. VNT= Virus Neutralization Test* VN-Ab titers: expressed as Log $_{10}TCID_{50}$ / ml. ELISA= Enzyme linked immunosorbent assay. **ELISA antibody titers expressed as OD value.

Table 2: Relative means seroconversion in sera of rabbits and calves against bacterial antigenic components (*E-Coli and C. perfringens type A*) post vaccination with Entero-4

Dose	Route	Time	Mean Seroconversion							
			E.coli				*C. perfringens type A*			
			GMA*		ELISA**		TNT***		ELISA	
			rabbit	calves	rabbit	calves	rabbit	calves	rabbit	Calves
3 ml	sc	14 D	3.03	20.16	0.43±0.01	0.91±0.01	0.2±0.1	0.4±0.04	0.88±0.03	1.39±0.06
		2 W	24.25	40.32	0.78±0.06	1.15±0.02	4.8±0.2	4.56±0.15	1.67±0.06	1.76±0.01
		4 W	48.5	322.54	0.97±0.04	2.39±0.01	6±0.3	5.03±0.21	1.82±0.04	2.13±0.04
	im	14 D	4.59	32	0.47±0.02	1.17±0.03	0.1±0.2	0.2±0.02	0.81±0.02	1.28±0.02
		2 W	48.5	322.5	0.81±0.03	2.14±0.02	4.8±0.3	4±0.21	1.74±0.01	1.69±0.02
		4 W	337.79	256	1.33±0.09	2.23±0.01	6.1±0.4	5.5±0.31	1.88±0.03	2.07±0.01
4 ml	sc	14 D	4	4	0.42±0.02	1.14±0.02	0.1±0.03	0.3±0.01	0.89±0.02	1.43±0.03
		2 W	36.75	25.39	0.83±0.1	1.19±0.01	4.9±0.4	5.1±0.44	1.83±0.05	1.78±0.02
		4 W	55.71	50.79	1.03±0.02	2.4±0.02	5.9±0.3	5.5±0.25	1.9±0.06	2.19±0.04
	im	14 D	3.03	3.17	0.55±0.2	1.21±0.02	0.2±0.02	0.2±0.03	0.85±0.03	1.47±0.01
		2 W	42.22	40.31	0.96±0.03	2.23±0.01	5±0.3	4.5±0.36	1.84±0.03	1.79±0.05
		4 W	445.72	406.37	1.41±0.02	2.21±0.03	6.2±0.3	6.0±0.61	1.93±0.07	2.14±0.05

D = Days post 1st dose. W= Weeks post booster dose. sc = Subcutaneous route. im = Intramuscular route. *GMA: geometric mean agglutinating antibody titer /ml. ELISA= Enzyme linked immunosorbent assay; **ELISA antibody titers expressed as OD value. TNT= Toxin Neutralization Test; ***Serum neutralizing anti-alpha toxin titers expressed as iu / ml.

Table 3: Monitoring seroconversion against *BRV* in sera of calves post vaccination with Entero-4 as tested by virus neutralization test (VNT) and indirect ELISA

	Seroconversion							
Dose	3 ml				4 ml			
Route	sc		im		sc		im	
Test / Time	VNT*	ELISA**	VNT	ELISA	VNT	ELISA	VNT	ELISA
0 Day	0.3±0.03	0.62±0.01	0.3±0.03	0.45±0.01	0.3±0.01	0.6±0.03	0.45±0.05	0.69±0.02
2WPBV	1.12±0.02	0.89±0.04	1.51±0.03	1.23±0.04	1.17±0.03	1.08±0.04	1.69±0.05	1.41±0.1
1 MPBV	1.80±0.02	1.45±0.1	1.90±0.02	1.83±0.05	1.75±0.01	2.09±0.09	1.94±0.02	1.91±0.07
2 MPBV	2.2±0.1	2.17±0.11	2.20±0.06	2.25±0.1	2.27±0.06	2.23±0.11	2.40±0.05	2.35±0.03
3 MPBV	2.1±0.1	2.40±0.12	1.96±0.11	2.33±0.02	2.10±0.1	2.40±0.1	2.15±0.05	2.39±0.1
4 MPBV	1.95±0.05	2.25±0.13	1.9±0.10	2.17±0.14	2.16±0.06	2.19±0.09	2.1±0.17	2.42±0.05
5 MPBV	1.80±0.04	2.10±0.09	1.86±0.05	2.16±0.11	1.98±0.10	2.15±0.13	1.91±0.01	2.10±0.06
6 MPBV	1.7±0.02	2.0±0.09	1.68±0.06	1.98±0.14	1.91±0.03	2.01±0.01	1.88±0.03	2.10±0.04
7 MPBV	1.65±0.02	1.88±0.2	1.57±0.64	1.86±0.12	1.81±0.01	1.98±0.02	1.80±0.03	2.06±0.1
8 MPBV	1.5±0.03	1.76±0.07	1.55±0.02	1.69±0.12	1.65±0.03	1.68±0.01	1.78±0.03	1.81±0.04
9 MPBV	1.46±0.04	1.65±0.04	1.36±0.04	1.61±0.1	1.55±0.02	1.55±0.05	1.59±0.04	1.49±0.02
10 MPBV	1.35±0.05	1.52±0.03	1.35±0.05	1.5±0.08	1.5±0.05	1.38±0.05	1.51±0.01	1.25±0.1
11 MPBV	1.25±0.05	1.35±0.02	1.25±0.05	1.26±0.09	1.34±0.02	1.44±0.05	1.51±0.05	1.18±0.05
12 MPBV	1.2±0.1	1.19±0.02	1.18±0.07	1.03±0.1	1.25±0.05	1.31±0.06	1.22±0.02	1.10±0.04

WPBV= Weeks post booster vaccination. MPBV= Months post booster vaccination. sc= Subcutaneous route. im= Intramuscular rout. VNT= Virus Neutralization Test; *Mean VN-Ab titers expressed as $log_{10}TCID_{50}$/ml. ELISA= Enzyme linked immunosorbent assay; **Mean ELISA antibody titers expressed as OD value.

Whereas, the estimated mean GMA Ab titers and OD values against *E. coli* K99 in sera of all vaccinated groups revealed a significant increase by the 1stMPBVwith maximum titers and OD values on 2nd MPBV (Tables 2 and 5). Similarly, the mean *C. perfringens* type A antitoxin titers, as recorded in sera of all vaccinated groups, showed the highest level by the 2nd MPBV. These antitoxin titers continued within the permissible limits for vaccine potency requirements (4 iu /ml in at least 80 % of vaccinated animals that were seronegative prior to vaccination) (USDA, 2002).

The resulted GMA antibody (Ab) titers against *E. Coli K99* in all groups of claves and rabbits vaccinated sc or im with 3 ml or 4 ml dose showed a significant increase on 4th WPBV ($P<0.05$). Whereas, GMA Ab highest titers were recorded at 1-2 MPBV and 2-3 MPBV in calves vaccinated im and sc with the 3 ml dose, respectively (Table 5). Comparing the GMA Ab titers against *E. Coli K99* measured by MAT in all groups of calves and rabbits either vaccinated sc or im with a dose of 3 ml or 4 ml, there were no significant differences ($P>0.05$) (Table 2).The linear regression correlation between mean GMA Ab titers measured by MAT and OD values measured by ELISA against *E. Coli K99* were determined as $R^2= 0.95$, 0.97, 0.92 and 0.92 for groups of calves vaccinated sc with 3 ml dose, im with 3 ml dose, sc with 4 ml dose, and im with 4 ml dose, respectively.

On the other hand, the mean antitoxin titers against *C. perfringens* type A alpha toxin measured by TNT in all groups of claves and rabbits vaccinated sc or im with 3ml or 4 ml dose showed a significant increase on the 4th WPBV ($P<0.05$). However, no significant difference ($P>0.05$) could be found on comparison of the mean antitoxins titers recorded among all vaccinated groups up to 4 WPBV (Table 2). The antitoxin Peak titers were recorded at 1 MPBV (5.5 iu / ml) and 2 MPBV (6.03 iu/ml)

Table 4: Monitoring seroconversion against *BCoV* in sera of calves post vaccination with Entero-4 as tested by virus neutralization test (VNT) and indirect ELISA

Seroconversion								
Dose	3 ml				4 ml			
Route	sc		im		sc		im	
Test / Time	VNT*	ELISA**	VNT	ELISA	VNT	ELISA	VNT	ELISA
0 Day	0.45±0.03	0.69±0.01	0.3±0.03	0.45±0.09	0.3±0.01	0.55±0.01	0.6±0.1	0.68±0.01
2 WPBV	1.73±0.02	1.99±0.08	1.75±0.07	1.93±0.11	1.95±0.09	2.11±0.11	1.8±0.11	2.25±0.09
1 MPBV	1.95±0.1	2.04±0.1	2.10±0.1	2.10±0.08	2.10±0.15	2.35±0.1	2.1±0.12	2.43±0.1
2 MPBV	2.15±0.11	2.2±0.11	2.25±0.1	2.44±0.06	2.40±0.1	2.69±0.02	2.20±.09	2.72±0.03
3 MPBV	2.10±0.2	2.41±0.12	1.89±0.09	2.38±0.03	2.25±0.01	2.40±0.03	2.10±0.1	2.61±0.03
4 MPBV	1.89±0.03	2.19±0.1	1.87±0.16	2.17±0.1	1.95±0.14	2.23±0.07	2.01±0.08	2.44±0.1
5 MPBV	1.65±0.09	1.98±0.1	1.65±0.08	1.95±0.1	1.80±0.08	1.98±0.09	1.80±0.2	2.21±0.11
6 MPBV	1.55±0.07	1.80±0.07	1.58±0.05	1.74±0.04	1.70±0.1	1.86±0.03	1.80±0.1	2.13±0.09
7 MPBV	1.50±0.04	1.79±0.05	1.53±0.04	1.84±0.04	1.65±0.07	1.89±0.17	1.76±0.08	1.98±0.07
8 MPBV	1.50±0.03	1.67±0.04	1.50±0.04	1.80±0.03	1.65±0.06	1.84±0.08	1.66±0.06	1.87±0.2
9 MPBV	1.45±0.04	1.59±0.04	1.48±0.03	1.67±0.06	1.55±0.04	1.81±0.1	1.58±0.15	1.87±0.02
10 MPBV	1.35±0.03	1.64±0.03	1.35±0.02	1.65±0.03	1.50±0.02	1.78±0.1	1.50±0.11	1.81±0.11
11 MPBV	1.26±0.04	1.54±0.07	1.29±0.1	1.59±0.04	1.35±0.03	1.66±0.08	1.35±0.09	1.70±0.07
12 MPBV	1.19±0.03	1.49±0.08	1.20±0.05	1.52±0.02	1.23±0.02	1.59±0.02	1.25±0.12	1.65±0.08

WPBV= Weeks post booster vaccination. MPBV= Months post booster vaccination. sc= Subcutaneous route. im= Intramuscular rout. VNT= Virus Neutralization Test * Mean VN-Ab titers expressed as $\log_{10}TCID_{50}$/ml. ELISA= Enzyme linked immunosorbent assay ** Mean ELISA antibody titers expressed as OD value.

Table 5: Mean seroconversion against *E. Coli K99* in sera of calves post vaccination with Entero-4 as tested by micro-agglutination test (MAT) and indirect ELISA

Seroconversion								
Dose	3 ml				4 ml			
Route	sc		im		sc		im	
Test / Time	*GMA	**ELISA	GMA	ELISA	GMA	ELISA	GMA	ELISA
0 Day	3.17	0.76±0.08	5.03	0.74±0.07	4	0.70±0.31	3.17	0.78±0.08
2 WPBV	20.16	0.91±0.01	32	1.17±0.03	25.39	1.14±0.02	40.31	1.21±0.02
1 MPBV	40.32	1.15±0.02	322.5	2.14±0.022	50.79	1.19±0.01	406.37	2.23±0.01
2 MPBV	322.54	2.39±0.011	256	2.23±0.01	406.37	2.4±0.02	256	2.21±0.03
3 MPBV	256	2.27±0.042	161	2.3±0.032	256	2.36±0.03	161.26	1.98±0.02
4 MPBV	203.19	2.02±0.056	128	2.17±0.01	203.18	2.13±0.02	80.63	1.90±0.07
5 MPBV	128	1.7±0.018	50.79	1.69±0.02	101.59	1.81±0.02	50.79	1.77±0.1
6MPBV	50.79	1.61±0.02	50.79	1.66±0.01	50.79	1.7±0.10	50.79	1.73±0.01
7 MPBV	40.32	1.56±0.01	25.39	1.59±0.04	25.39	1.54±0.06	25.39	1.60±0.03
8 MPBV	25.39	1.22±0.012	25.39	1.51±0.02	25.39	1.27±0.05	25.39	1.58±0.02
9 MPBV	25.39	0.96±0.03	12.69	1.38±0.01	20.15	0.91±0.02	12.69	1.45±0.01
10 MPBV	20.16	0.81±0.04	12.69	1.31±0.03	20.15	0.87±0.01	12.69	1.28±0.04
11 MPBV	12.17	0.79±0.01	10.07	1.26±0.02	12.69	0.8±0.03	12.69	1.23±0.02
12 MPBV	10.08	0.78±0.09	5.03	0.89±0.01	6.34	0.79±0.03	6.3	0.93±0.07

WPBV= Weeks post booster vaccination. MPBV= Months post booster vaccination. sc= Subcutaneous route. im= Intramuscular rout. * Mean GMA=geometric mean agglutinating antibody titers /ml. ELISA= Enzyme linked immunosorbent assay ** Mean ELISA antibody titers expressed as OD value.

in sera of calves vaccinated with 3 ml dose im and sc, respectively (Table 5). The mean antitoxin titer was found below the minimal protective level (1 iu / ml) in sera of vaccinated calves after 10 MPBV by im route and at 12 MPBV by sc route. The linear regression correlation between mean antitoxin Ab titers measured by TANT and OD values measured by ELISA against *C. perfringens type A α-toxin* were determined as R^2 0.94, 0.98, 0.92 and 0.93 for groups of calves vaccinated sc with 3 ml dose, im with 3 ml dose; sc with 4 ml dose, and im with 4 ml dose, respectively. Regarding ELISA as a confirmatory test, the results revealed that there was increase in the levels of OD values in sera of all vaccinated animals and persisted for a longer period of time as shown in Tables (1, 2, 3, 4, 5 and 6). Regarding seroresponses of rabbits versus calves, there were high correlations between results obtained by ELISA and VNT for *BRV* and *BCoV* (R = 0.96 – 0.99) as well as by ELISA and TNT (R = 0.88 – 0.99). However, the correlations of results obtained by ELISA and GMA were variable as the lowest correlation was in groups vaccinated with 3 ml by im route (R = 0.41), moderate in groups vaccinated sc with 4 ml (R = 0.77) and high in other groups (R = 0.84 – 0.99).

DISCUSSION

Neonate Calf Diarrhea usually occurs in calves less than one month of age causing major economic losses mainly due to decreased performance, high morbidity, mortality, the expenses of medication and labor to treat the sick animals (House, 1978; Myers *et al*., 1984). Although livestock industry has attained considerable progress in various aspects of herd husbandry, NCD is still challenging due to the multi-factorial nature and

Table 6: Mean seroconversion against *C. perfringens type A*in sera of calves post vaccination with Entero-4 as tested by toxin neutralization test (TNT) and indirect ELISA

Seroconversion								
Dose	3ml				4ml			
Route	S/C		IM		S/C		IM	
Test / Time	*TNT	**ELISA	TNT	ELISA	TNT	ELISA	TNT	ELISA
0 Day	0.13±0.06	0.71±0.05	0.1±0.03	0.59±0.03	0.13±0.06	0.72±0.05	0.2±0.12	0.67±0.02
2 WPBV	4.56±0.15	1.39±0.06	4±0.21	1.28±0.02	5.1±0.44	1.43±0.03	4.5±0.36	1.47±0.01
1 MPBV	5.03±0.21	1.76±0.01	5.5±0.31	1.69±0.02	5.5±0.25	1.78±0.02	6±0.61	1.79±0.05
2 MPBV	6.03±0.29	2.13±0.04	5±0.2	2.07±0.01	6.63±0.25	2.19±0.04	5.6±0.56	2.14±0.05
3 MPBV	5.56±0.25	1.98±0.06	4±0.26	1.97±0.04	6±0.4	1.99±0.01	4.5±0.36	1.95±0.01
4 MPBV	3.96±0.15	1.91±0.05	3.5±0.17	1.89±0.03	5±0.31	1.96±0.01	3.5±0.17	1.91±0.01
5 MPBV	4.56±0.15	1.65±0.03	3±0.21	1.49±0.02	5±0.42	1.71±0.02	3.5±0.23	1.75±0.02
6 MPBV	4±0.17	1.53±0.05	2.6±0.26	1.43±0.01	4.63±0.21	1.69±0.01	3±0.21	1.59±0.01
7 MPBV	3.5±0.2	1.45±0.04	2.1±0.26	1.37±0.02	4±0.31	1.56±0.01	2±0.31	1.47±0.01
8 MPBV	3.5±0.12	1.33±0.03	2±0.31	1.27±0.03	4±0.2	1.43±0.04	1.5±0.31	1.41±0.03
9 MPBV	2.5±0.12	1.16±0.05	1±0.4	1.19±0.04	3±0.31	1.27±0.01	1.5±0.36	1.35±0.01
10 MPBV	2±0.15	1.07±0.04	0.5±0.15	1.09±0.05	2±0.26	1.14±0.01	1±0.36	1.21±0.01
11 MPBV	1±0.06	0.94±0.06	0.5±0.12	0.86±0.03	1±0.15	1.01±0.01	0.8±0.35	1.03±0.05
12 MPBV	0.36±0.15	0.84±0.07	0.2±0.15	0.82±0.02	0.5±0.1	0.86±0.01	0.4±0.15	0.91±0.07

DPB= Day post boostering. MPB= Months post boostering. S/C = Subcutaneous. I/M = Intramuscular TNT= Toxin Neutralization Test.* Mean serum neutralizing anti-alpha toxin titers expressed as iu / ml. ELISA= Enzyme linked immunosorbent assay.** Mean ELISA antibody titer expressed as OD value.

complexity of the disease. A single pathogen might induce NCD however, co-infection with multiple pathogens is frequently observed in diarrheic calves. Use of antimicrobial therapeutics has been associated with emerging antibiotic resistance among several bacterial species, particularly ETEC. Moreover, they are ineffective against other pathogens causing NCD (e.g. viruses and parasites) and negatively reduce the gut beneficial bacteria. Thus, availability of reliable combined vaccines has been considered the appropriate alternative for combating against NCD (Constable, 2004; Nataro and Barry, 2013; Cho and Yoon, 2014).

In the present study, the locally produced inactivated combined Entero-3 gel vaccine comprising *BRV*, *BCoV* and *E. Coli* K_{99} (Daoud *et al*., 2003) was upgraded by inclusion of *C. Perfringens* type A toxoid in the vaccine formulation (Entero-4). The newly developed vaccine was prepared and evaluated in calves. Furthermore, likelihood of using rabbits as a lab animal model for evaluation of the vaccine relative potency, in substitution of the original host, was explored.

Results of sterility testing of the prepared vaccine indicated its freedom from any bacterial, fungal or Mycoplasma contamination on inoculated media for 15 days post-inoculation. It was also found safe in mice as well as in claves and rabbits with even the double field high dose (8 ml) and these findings meet the appreciated safety requirements (OIE, 2016). The seroconversion obtained from sera of calves and rabbits using the neutralization assays VNT (for *BRV* and *BCoV*) and TNT (for *C. perfringens type A*) as well as microagglutination assay (MAT for *E. coli* K99) were further assessed by an indirect ELISA that detects not only neutralizing and agglutinating but also other subpopulations of immunoglobulin G (Leitner *et al*., 1990).

The serological results revealed that calves vaccinated with 3 ml or 4 ml of Entero-4 exhibited relatively parallel seroresponses against both viral and bacterial components up to 12 MPBV. Nevertheless, the estimated data by all assays (VNT, TNT, MAT and ELISA) showed variable seroresponses against all viral and bacterial components in sera of vaccinated calves that were higher, yet, comparable to those in sera of vaccinated rabbits up to 4 WPBV. Mostly, the seroresponses in sera of both vaccinated rabbits and calves reached their peak titers within 1-3 months after booster vaccination regardless the vaccination dose or route. These results were consistent with that obtained by (Daoud *et al*., 2003)) who proved that the local vaccine contained *BRV*, *BCoV*, *E. coli K99* was safe and potent. Moreover, the anti- *BRV* and *BCoV* VN-Ab titers remained within the protective values of ≥0.6 Log $_{10}$ $TCID_{50}$/ml (British Veterinary Pharmacopoeia, 2010) up to the 12th MPBV.

The variable correlations of mean ELISA OD values and GMT titers between vaccinated rabbits and calves that was comparatively low in groups vaccinated im with 3 ml (R = 0.41) might be explained that MAT detects only agglutinating antibodies but ELISA detects all types of antibodies (Leitner *et al*., 1990). Regarding the efficacy of vaccination against *C. perfringens type A,* the mean alpha antitoxin titers achieved the standard vaccine potency requirements (≥4 iu /ml; USDA, 2002) at 2 WPBV in sera of both calves and rabbits. They reached their peaks on 1-2 MPBV and remained within the protective limit (>1 iu / ml; Hammer *et al*., 2008) in sera of vaccinated calves up to 10 MPBV by im route and 12 MPBV by sc route.

The obtained harmony in relative potency between rabbits and calves manifested by considerably correlated seroresponses to veterinary vaccines has been reported (Tsenova *et al*., 2006; Allam *et al*., 2012; Effat *et al*., 2016). In support to our objectives and results, rabbits have been explored as a model of homologous and heterologous *Rotavirus* infection, transmission, and protection studies. Immune response against a homologous *Rotavirus* was demonstrated after parenteral administration of inactivated virus (Conner *et al*., 1993; Desselberger and Huppertz, 2011). Moreover, neutralizing antibody to bovine *rotavirus* was found in serum samples from other species, including cattle, sheep, goats, rabbits, mice and human beings (Sato *et al*., 1981).

In view of this study, it is important for developing countries to continue upgrading the quality control and safety standards of veterinary vaccines. Besides, improving the formulation of an existing vaccine into a new better one should be advocated as a way out to conquer challenges facing control programs. Likewise, official animal health organizations have promoted exploiting alternative consistent methods to substitute the vaccine potency experiments including laboratory animal methods (Molin-Capeti *et al*., 2013). That would strongly support the objectives proposed as well as the results of this endeavor. In general, the high immune response and safety observed in both vaccinated calves and rabbits were due to reasonable antigen contents being included within the vaccine dose. It is recommended that this study will lead to the production of a reliable commercial vaccine for the protection of neonatal calves against NCD with low-costive relative potency evaluation in rabbits.

In conclusion, the prepared vaccine, namely "Entero-4", was proved safe and potent for effectual protection of calves against *BRV*, *BCoV*, *ETEC K99*, and *C. perfringens type A*, using a more economic dose of 3 ml by sc or im routes. Nevertheless, our data emphasized the reliability of rabbits, as an inexpensive and easy-handled laboratory animal, to evaluate the relative potency of bovine enteric vaccines. That initiates a potential to utilize rabbits as a versatile alternative for potency verification of other veterinary vaccines, overcoming expensive and difficult availability of seronegative naive animal hosts.

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