Preparation and evaluation of combined inactivated vaccine containing rota, corona viruses, Escherichia coli bacterin and Clostridium perfringens type Ctoxoid (Entero-4)

A. M. Daoud[1], R. A. Diab[1], S. M. Aboul Saoud[1], S. M. Zeidan[1], F. F. Zaki[2]

[1]*Veterinary Serum and Vaccine Research Institute, Abbassia, Cairo, Egypt*
[2]*Central Laboratory for Evaluation of Veterinary Biologics, Abbassia, Cairo, Egypt*

A conclusive study was conducted for preparation and evaluation of combined inactivated entero-4 vaccine containing bovine rotavirus (BRV), bovine coronavirus (BCV), *E. coli* K_{99} and toxoid of *C. perfringens* type "C".

Laboratory and field evaluations were conducted on laboratory animals, calves and late pregnant cows with monitoring the active and passive antibodies in vaccinated cows and their offspring respectively. Laboratory evaluation proved purity, safety and high efficacy of the vaccine without interference between different vaccine ingredients. Field evaluation gave satisfactory results when pregnant cows vaccinated at late stage of pregnancy with high neutralizing antibody titers against BRV, BCV and *C. perfringens* as well as high *E. coli* agglutinating titers. Maternal immunity passively protected offspring during the critical period of age and remained protected till the end of sampling time (30th day post parturition).

Diarrheal diseases are the most important causes of death especially during the early few weeks of life in which a considerable number of newly born animals could be lost (Acres *et al.,* 1977).

Neonatal calf diarrhoea has a complex etiological agents (Radostitis, 1991), as bovine rotavirus (BRV) and bovine coronavirus (BCV), being the most dominant causative agent in combination with other bacterial agents as enterotoxigenic *E. coli* (Snodgrass *et al.,* 1986 and Saif and Theil, 1990), and *Clostridium perfringens* type C that cause a highly fatal hemorrhagic enteritis among calves less than 10 days of age (Niilo, 1980 and Quigley *et al.,* 1995).

Previous studies proved that the percentage incidence of pathogens affecting neonates in Egypt were 37.3% for BRV, 18.2% for BCV and 25.4% for *E. coli* (Farid *et al.,* 1992). Clostridia as well as other non- infectious causes including; environment, management, hygiene and nutritional factors were represented 19.1% of neonatal affections (Perk *et al.,* 2000).

Since, infection repeatedly occurs at birth, it is almost impossible to actively immunize them prior to exposure to virulent field viruses or bacteria.Therefore, the best way to reduce economic losses in calves is the active immunization of pregnant dams during late stage of pregnancy to increase the level of immunoglobulins which are passively transferred to calves (Saif *et al.,* 1983). It is postulated that colostral antibodies in the intestine can neutralize field viruses and bacteria and guard against diseases. Depending on the balance between the infective dose and the antibody titers, animals can be infected or developed immunity without showing clinical diseases.

The previously mentioned data about diarrhoea in combination with the dramatic economic losses made the development of effecious inactivated vaccine containing the most destructive viral and bacterial agents is necessary. Thus, the present work was planned to widen the locally prepared enter-3 vaccine (Daoud *et al.,* 2003) that contains bovine rota, coronaviruses and *E. coli* K_{99} vaccine by incorporation of *C. perfringens* type C toxoid.

Material and Methods

Viruses. Local isolates BRV and reference strain of BCV was used for preparation and evaluation of the vaccine. The viruses were kindly supplied by the Central Laboratory for Evaluation of Veterinary Biologics.

Bacteria. Reference strain of enterotoxigenic *E. coli* K_{99} strain was kindly supplied from Animal Reproduction Research Institute, Giza, Egypt.

Reference strain of toxigenic *C. perfringens* type C was supplied from med.l school, Edinburgh Univ., UK.

Hyperimmune sera. Specific hyperimmune sera against BRV, BCV, *E. coli* and *C. perfringens* type Cwere kindly supplied from the Central Laboratory for Evaluation of Veterinary Biologics, Abbassia, Cairo, Egypt.

Vaccine preparation. Binary ethyleneimine (BEI) inactivated rota and coronaviruses and formalin inactivated *E. coli* and *C. perfringens* type C were adjuvanted with aluminium hydroxide according to the method described by (Kasem *et al.,* 1999; Daoud *et al.,* 2003 and Fayez and Zeidan, 2004).

Evaluation of the prepared vaccine.

Sterility test. Different steps of vaccine preparation which includes viruses or bacterial propagation for seed production, fluid harvestion, fluid inactivation and final gel adjuvanted vaccinel product were subjected to srerility test according to the method described by British Veterinary Pharmacopoeia (1993).

Safety test. 50 adult Swiss albino mice were divided into 5 groups to study the safety of the prepared inactivated vaccines, which include monovalent *E. coli* K_{99}, *C. perfringens* type C, bivalent rota/corona vaccine and polyvalent rota/coronaviruses, *E. coli* K_{99} and *C. perfringens* type C vaccine. Each mouse in each group inoculated intraperitoneally with a dose of 0.2ml of each vaccine, 10 mice were kept as non-vaccinated control.

Entero-4 vaccine was also tested for its safety in applicant host by inoculation of two susceptible calves intramuscularly with 50 ml (10 vaccinal doses). Inoculated and control animals were kept under observation for 10 days.

Efficacy tests.

A. In calves: Efficacy of the prepared monovalent *C. perfringens* type C, entero-3 (BRV, BCV and *E. coli*) and entero-4 (BRV, BCV, *E. coli* and *C. perfringens* type C) vaccines were conducted in three calf groups A, B and C respectively, five calves each. The vaccination was conducted through i/m inoculation (2 ml of monovalent, 4 ml of enter-3 and 5 ml of entero-4) with two doses at three weeks interval. Calves were bled at 0, 1, 2, 4, 6 and 8 weeks post vaccination, sera were tested for both neutralizing antibodies of BRV, BCV and *C. perfringens* and agglutinating *E. coli* antibodies according to the methods described by (Collins *et al.,* 1988; European Pharmacopoeia, 2001 and Daoud *et al.,* 2003).

B. In pregnant cows. Fifteen pregnant Holstein cows in private dairy herds in Alexandria Governorate were assigned into two groups. Group I (12 cows) was inoculated by deep intramuscular injection, at one week before drying-off or 8 - 12 weeks before calving with 5 ml of the combined vaccine. Two weeks later, cows were boostered with identical inoculum. Group II (3 cows) was left as non-vaccinated control.

Serum samples were collected from different tested cows group at both vaccination, and parturition time. First milking colostrum was obtained from tested cows and treated as described by (Saif *et al.,* 1983).

Sera of newly born calves were also collected at 1st, 2nd, 3rd and 4th weeks post parturition. Both serum and colostral samples were subjected for serum neutralization test (Dauvergene *et al.,* 1983), antitoxin neutralization test (Gadalla *et al.,* 1971), and microagglutination test (Collins *et al.,* 1988) for evaluation of the immunogenicity for rota/ coronaviruses, *C. perfringens* type C and *E. coli* K_{99} respectively.

Results and Discussion

Neonatal diarrhoea is one of the most important problems of cattle industry allover the world (Radostitis, 1991), including Egypt (Shalaby *et al.,* 1981 and Perk *et al.,* 2000). The present situation in Egypt dictates development of control and preventive measures through preparation and application of a highly effective conclusive vaccine containing most destructive viral and bacterial etiological agents e.g. BRV, BCV, *E. coli* and *C. perfringens* type C. As the hazard of calf loss is usually expected during the first two weeks of life (Perk *et al.,* 2000), thus the correct protection should be directed to active immunization of late pregnant cow dams to amplifying the magnitude of immunoglobulins in their colostrum nd milk (Myer, 1980). The preliminary studies for preparation of combined gel adjuvanted inactivated vaccine containing BEI inactivated rota/coronaviruses, and formalin inactivated *E. coli* K_{99} and *C. perfringens* type C

(entero-4), gave satisfactory results regarding sterility and complete neutralization with respective reference antisera. Also neither aerobic nor anaerobic bacterial growth was seen in the inoculated media and remained

Table (1): Serum neutralizing antibody titers against bovine rota and coronaviruses in calves post vaccination with entero-3 (BRV, BCV and E. coli) and entero-4 (BRV, BCV, *E.coli* and *C. perfringens* type C) vaccines.

Applied Vaccine	Group	Calf number	Log_{10} Rota, Corona antibody titers at weeks PV											
			0		1		2		4		6		8	
			R	C	R	C	R	C	R	C	R	C	R	C
Entero-3	B	5	0.3	0.0	0.6	0.3	0.9	0.6	1.8	1.5	2.4	2.1	2.1	2.1
		6	0.0	0.0	0.3	0.3	0.6	0.9	1.5	1.5	2.1	2.4	2.1	1.8
		7	0.3	0.0	0.6	0.3	0.6	0.9	1.8	1.8	2.7	2.7	2.4	2.4
		8	0.0	0.3	0.3	0.6	0.6	0.6	1.5	1.5	2.4	2.1	2.1	2.1
	Mean		0.15	0.075	0.45	0.375	0.675	0.75	1.65	1.575	2.4	2.325	2.175	2.1
Entero-4	C	9	0.3	0.0	0.6	0.3	0.9	0.9	1.8	1.8	2.4	2.4	2.1	2.1
		10	0.3	0.0	0.6	0.6	0.9	0.9	1.8	1.8	2.7	2.7	2.4	2.4
		11	0.3	0.3	0.3	0.6	0.6	0.9	1.5	1.5	2.4	2.4	2.4	2.1
		12	0.0	0.3	0.3	0.3	0.6	0.6	1.5	1.5	2.4	2.1	2.1	2.1
	Mean		0.225	0.15	0.45	0.45	0.75	0.825	1.65	1.65	2.475	2.4	2.25	2.175

Table (2): *E. coli* K99 microagglutination antibodies in calf sera post vaccination with entero-3 (BRV, BCV and *E. coli*) and entero-4 (BRV, BCV, *E. coli* and *C. perfringens* C) vaccines.

Applied vaccine	Group	Calf No.	*E. coli* K99 microagglutination antibody tites at weeks post vaccination					
			0	1	2	4	6	8
Entero-3	B	5	4	16	32	128	512	1024
		6	4	16	32	128	512	1024
		7	2	32	64	128	512	1024
		8	2	32	128	256	1024	2048
	Mean		3	24	64	160	640	1280
Entero-4	C	9	4	32	64	128	512	1024
		10	4	32	128	256	1024	1024
		11	4	16	64	128	512	1024
		12	2	16	32	128	512	2048
	Mean		3.5	24	72	160	640	1280

Table (3): Neutralizing *C. perfringens* beta antitoxin titres in calves post vaccination with monovalent and polyvalent vaccine (entero-4).

Applied vaccine	Group	Calf No.	*C. perfrigens* beta antitoxin IU/ml titres post vaccination (weeks)					
			0	1	2	4	6	8
Monovalent *C. perfringens* vaccine	A	1	0	1	5	25	30	35
		2	0	2	7	30	30	35
		3	0	3	4	20	25	25
		4	0	1	4	20	25	30
	Mean		0	1.75	5	23.75	27.5	31.25
Entero-4	C	9	0	2	4	25	30	35
		10	0	2	6	30	30	35
		11	0	2	5	20	30	30
		12	0	1	5	20	30	35
	Mean		0	1.75	5	23.75	30	33.75

Table (4): Neutralizing rota and corona antibody titres in pregnant cows' sera, colostrums and their pffsprings sera post vaccination with entero-4 (BRV, BCV, *E. coli* K99 and *C. perfringens* type "C") vaccine.

Animal No.	Log_{10} Neutralizing antibody titres in													
	Pregnant cow's sera at				Colostrum		Offspring's sera at weeks Post parturition							
	Vaccination time		Parturition time				1		2		3		4	
	R	C	R	C	R	C	R	C	R	C	R	C	R	C
1	0.6	0.0	2.4	2.1	2.7	2.4	2.1	2.1	1.8	1.8	1.5	1.5	1.2	1.2
2	0.3	0.0	2.1	2.4	2.4	2.4	1.8	2.1	1.5	1.8	1.2	1.2	0.9	0.9
3	0.6	0.3	2.4	2.4	2.4	2.7	1.8	1.8	1.5	1.5	1.2	1.5	1.2	0.9
4	0.9	0.6	2.1	2.1	ND	ND	1.8	1.8	1.8	1.5	1.5	0.9	1.2	0.9
5	0.3	0.3	2.4	2.4	2.7	2.7	2.1	2.1	1.8	1.8	1.8	1.8	1.5	1.2
6	0.3	0.3	2.7	2.4	2.4	2.4	1.8	2.1	1.5	1.8	1.2	1.5	1.2	1.2
7	0.3	0.3	2.1	2.7	ND	ND	1.8	2.4	1.5	2.1	1.2	1.8	0.9	1.5
8	0.0	0.6	2.1	2.4	2.1	2.4	1.5	2.1	1.2	1.8	0.9	1.5	0.9	1.2
9	0.6	0.3	2.4	2.4	2.4	2.4	2.1	1.8	1.8	1.5	1.2	1.2	1.2	0.9
10	0.0	0.0	2.1	2.4	ND	ND	1.8	1.8	1.5	1.5	1.2	1.2	1.2	1.2
Mean	0.39	0.27	2.28	2.37	2.44	2.48	1.86	2.01	1.59	1.71	1.29	1.41	1.14	1.11

R: Rota C: Corona ND: Not Done

Table (5): *E. coli* microagglutination antibody titers in pregnant cow's sera, colostrums and offspring's sera post vaccination of pregnant cows with polyvalent BRV, BCV, *E. coli* K99 and *C. perfringens* type C vaccine.

Animal No.	*E. coli* K99 microagglutination antibody titers in						
	Pregnant cow's sera at		Colostrum	Offspring's sera			
	Vaccination time	Parturition time		1	2	3	4
1	16	1024	1024	1024	1024	512	512
2	16	512	1024	2048	1024	512	512
3	32	1024	1024	1024	1024	512	256
4	16	512	ND	1024	1024	512	512
5	16	512	512	1024	512	512	256
6	64	1024	1024	2048	1024	1024	512
7	16	512	ND	1024	512	512	265
8	8	512	1024	1024	512	512	265
9	16	1024	1024	1024	1024	512	265
10	16	1024	ND	1024	1024	512	265
Mean	21.6	768	950	1228.8	870.4	563.2	358.4

sterile for 15 days post inoculation. These results are in harmony with that obtained from animal inoculation tests which confirmed vaccine safety as the inoculated mice and safety tested calves remained clinically normal without deaths or elevation of temperature.

Concerning with serological interference studies between ingredients of entero-3 vaccine (rota, coronaviruses and *E. coli* K_{99}) and *C. perfringens* type C vaccine in calves are represented in (Tables 1-3). Mean neutralizing antibody titre (NAT) against BRV and BCV in entero-3 (group B) and entero-4 (group C) potency tested calves were increased by the 4^{th} week following vaccination to 1.65, 1.575 and 1.65, 1.65 log_{10}respectively. Maximal response occurred at the 6^{th} week following the 1^{st} vaccination with a titre of 2.4, 2.32 and 2.475,

Table (6): *C. perfringens* beta antitoxins in pregnant cows' sera, colostrums and offspring's sera post vaccination of pregnant cows with entero-4.

Animal No.	Neutralizing *C. perfringens* beta antitoxin antibody titers in						
	Pregnant cow's sera		Colostrum	Offspring's sera at weeks Post parturition			
	Vaccination time	Parturition time		1	2	3	4
1	5	35	40	35	25	15	10
2	0	35	35	30	20	10	5
3	0	35	40	30	20	15	10
4	0	40	ND	30	20	10	5
5	5	35	35	25	15	10	5
6	5	40	40	30	20	15	10
7	0	30	ND	25	15	10	5
8	0	30	35	25	15	10	5
9	5	40	40	30	20	10	5
10	0	30	ND	25	15	10	5
Mean	2	35	37.5	28.5	18.5	11.5	6.5

ND: Not Done

2.4 $\log_{10}$ respectively (Table 1). These results are gone in harmony with that obtained by (Daoud *et al.,* 2003). *E. coli* K_{99} microagglutinating titers obtained by entero-3 vaccinated calves (group B) and entero-4 vaccinated calves (group C) are represented in (table 2). It revealed that mean agglutinating titers in groups B and C increased by the 4th week post vaccination to 160 agglutinating unit where as maximal titer obtained by the 8th week post vaccination (1280) agglutinating unit. These results indicate that there was no immunological interference between *E. coli* bacterin and either BRV, BCV or *C. perfringens* type Cfractions in the Inactivated vaccine. These results agree with the findings of (Collins *et al.,* 1988 and Daoud *et al.,* 2003) who reported no serological interference between either rota or coronavirus fractions and the *E. coli* bacterin.

Concerning *C. perfringens* neutralizing antitoxin (Table 3), the Obtained results proclaimed that the antitoxin titres increased in monovalent vaccinated calves (group A), and entero-4 vaccinated calves (group C), by the 4th week post vaccinated to 23.75 IU and the maximal titers were obtained by the 8th week where it reached 31.25 and 33.75 IU respectively. Field vaccination of late pregnant cows with entero-4 vaccine causing elicited neutralizing antibodies against BRV, BCV and antitoxin to *C. perfringens* type C(Tables 4, 6). The mean $\log_{10}$ SNAT against BRV and BCV at time of parturition were 2.28 and 2.37 respectively while antitoxin against beta toxoid was 35 IU. These results agree with that obtained by (Fleenor and Scott 1983; Daoud *et al.,* 2003 and Fayez and Zeidan 2004) who concluded that field evaluation gave satisfactory results when pregnant cows were vaccinated at late stage of pregnancy with high level of serum neutralizing antibodies against BRV, BCV and *C. perfringens* type C.

The colostrum of vaccinated cows were proven to be very rich with immunoglobulins against different fractions of the entero-4 vaccine as it reached 2.44, 2.48 $\log_{10}$ NAT for BRV, BCV; 37.5 IU beta antitoxin titers and 950 *E. coli* agglutinating titers.

Maternal immunity was monitored in offsprings of entero-4 vaccinated cows for four weeks post parturition for detection of antibodies to BRV, BCV, *E. coli* and antitoxin against *C. perfringens* toxoid (Tables 4- 6). The obtained results proved that the maternal antibody titres in both colostrum and offsprings are highly correlated with the active titers of cows at parturition. These results are agreed with (Daoud *et al.,* 2003 and Fayez and Zeidan 2004).

It conclusion, the prepared vaccine is safe, potent and efficient and can be used for maternal vaccination as it is the only suitable method to amplify maternal immunity since high quality colostrums feeding is the most effective tool for preventing neonatal diarrhoeal diseases.

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