Preparation of diagnostic lysate antigens for rapid evaluation of local entero-3 vaccine

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Studies were conducted to determine the utility of lysate antigens for rapid evaluation of the local entero-3 vaccine, antigens were prepared from cell cultures infected with bovine rota virus (BRV) and bovine corona virus (BCV) as well as from Enterotoxigenic *E. coli* strain K99. Prepared antigens were tested with field serum samples collected from both late pregnant entero-3 vaccinated cows and their offsprings using different serological assays including: microagglutination test, indirect ELISA and immunofluorescent antibody technique. Results of this endeavor were correlated to that of the standard virus neutralization test. The locally prepared antigens were proved useful for vaccine evaluation. Moreover, these antigens are recommended for both detection and assessment post vaccination or post infection of sero-conversion against BRV, BCV and *E. coli*.

The first few weeks in the life of newborn calves are considered the most critical period, during which a considerable number of animals can be lost. Neonatal calf diarrhea remains one of the most important disease problems facing livestock production, causing great economic losses every year in Egypt and worldwide (Acres *et al.,* 1986 and Mortin *et al.,* 1978). The etiology of neonatal calf diarrhea is a multifactorial complex involving many infectious agents. Bovine rota virus (BRV) and Bovine corona virus (BCV) being the most dominant causative agent in combination with the enterotoxigenic *E. coli* (Sondgrass *et al.,* 1986 and Saif and Thiel, 1990).

In Egypt, bovine rotavirus was isolated for the first time by Shalaby *et al.,* (1981), from faeces of newborn diarrheic calves. Since that time, many studies were conducted to assess the incidence of infection (Shalaby *et al.,* 1987; Iman *et al.,* 1995 and Perk *et al.,* 2000). Previous studies proved that the incidences of pathogens affecting bovine neonates in Egypt were BRV (37.3 %), BCV (18.2 %) and *E. coli* (25.4 %) (Farid *et al.,* 1992) and 19.1% for others such as Clostridia, Cryptosporidia and non-infectious causes including environmental, managements, hygienic and nutritional factors (Perk *et al.,* 2000). If calves are infected at birth, it is almost impossible to actively immunize them prior to their exposure to virulent field virus. Therefore, the best way to reduce economic losses among neonatal calves is to vaccinate dams at late

pregnancy thus increasing their colostral antibody level that is passively transferred to suckling calves (Saif *et al.,* 1983).

Currently, some inactivated vaccines, containing BRV, BCV and *E. coli* K99, are used in the Egyptian field either prepared locally e.g. Entero-3 (Daoud *et al.,* 2003) or imported e.g. Scour-Guard. Traditional evaluation of such vaccines requires special procedure for both viral and bacterial antigens, which are complicated, time consuming, laborious and expensive.

The present work aimed to save time, effort and expenses using simple approach for preparation of reliable BRV, BCV and *E. coli* lysate antigens such antigens could be utilized in microagglutination, indirect ELISA and immunofluorescent antibody techniques for rapid and accurate evaluation of the vaccine.

Material and Methods

Viruses and cells. The local isolate of bovine rotavirus (Shalaby *et al.,* 1981) with a titer of $10^{6.7}$ $TCID_{50}$ and the reference bovine corona virus (Nebraska strain, with a titer of $10^{6.0}$ $TCID_{50}$) were used. Both virus entities were propagated and titrated on Madin-Darby Bovine Kidney (MDBK) cell culture. The MDBK cells were grown at 37°C, in minimum essential medium with Earle's salts (MEME) supplement with 10% fetal calf serum. Prior to experimental work, both viruses were tested for their identity by serum neutralization test and indirect immunoflourescent antibody technique (Palmer *et al.,* 1982) using the respective specific

reference antisera (Glasgow Animal Clinic, Bacterial strain. Reference strain of the enterotoxigenic *E. coli* strain K99 was used in this study. Bacterial Strain (*E. coli* K99) was identified by the microagglutination test (Collins *et al.*, 1988) using the respective monoclonal antiserum (Glasgow Animal Clinic, Kentucky, USA).

Animals and serum samples. Ten late pregnant Holstein cows, in private dairy farm at El-Nubarria, Alexandria in addition to their offspring calves (n=10) were used in this study. Before vaccination, cows were proved seronegative to BRV and BCV by virus neutralization test (Dauvergene *et al.*, 1983) and to *E. coli* by microagglutination test (Collins *et al.*, 1988). They were vaccinated twice with the local inactivated entero-3 vaccine at 8 and 5 weeks before parturition. All cows and their offsprings were clinically normal throughout the experimental period. Serum samples were collected from vaccinated cows at the times of 1st vaccination zero time, 2nd vaccination and calving. Offspring calves were bled on the 1st, 2nd and 3rd weeks post natal (after parturition).

Preparation of bovine rota and corona lysate antigens. Confluent monolayers of MDBK cells grown in Roux bottles were inoculated separately with BRV and BCV at multiplicity of infection of 1 (m.o.i=1), and incubated at 37°C, in the presence of 1 mg/ml of trypsin. When about 65-75 % of infected cells showed CPE, the culture fluid was removed and virus-infected cells were solubilized directly with lysis buffer. The translucent cell lysate was scraped off, transferred into clean strile tubes, sonicated on ice at 60% intensity and clarified by low speed cooling centrifugation. The whole virus cell lysate (WVCL) were kept at - 20°C until used.

Preparation of *E. coli* lysate antigen. As an attempt to solubilize all possible immunoreactive antigenic components, the *E. coli* strain K99 was grown in its selective media (Minca-Vitox medium and trypticase soya broth). Growth continued for 24 h at 37°C and the bacterial cells were collected by centrifugation at 6000 rpm, 4°C for 30 min. The bacterial cell pellets were washed twice in sterile normal saline then, solubilized with lysis buffer thus producing translucent lysates. The whole bacterial cell lysates (WBCL) were kept at -20°C until used.

Virus neutralization test (VNT). The VNT was conducted for standard evaluation of seroconversion of late pregnant cows and their offsprings following local Entero-3 vaccination.

Kentucky, USA).
All sera were assayed for presence of specific antibodies to BRV and BCV in 96-well flat-bottom tissue culture plates (Nunc.) following the procedure described by Dauvergene *et al.*, (1983). The virus neutralizing antibody (VN-Ab) titers of serum samples were expressed as $\log_{10}$ of the reciprocal serum dilution that protected $\geq$ 50 % of microtitration plate wells following the calculation procedure of (Reed and Muench, 1938).

Microagglutination test (MAT). This test was done as was described by (Collins *et al.*, 1988) for determination of anti-*E. coli* strain K99 antibody titers.

Indirect Immunofluorescent assay (IFA). The IFA technique was carried out in a duplicate per serum sample according to (Palmer *et al.*, 1982) with some modifications. Briefly, the slide wells were coated separately with the BRV, BCV and *E. coli* k99 lysate antigens, air-dried, reacted with serum samples for 1h at 37°C, and probed with fluorescein isothiocyanate (FITC, Sigma) labeled anti-bovine IgG (diluted to 1:50 in Phosphate buffer saline (PBS, pH 7.6). The slides were mounted with glycerol (4:1 in PBS) and examined microscopically for greenish yellow fluorescence using a fluorescence microscope. A sample was considered positive when at least two virus-specific fluorescent cells were observed in any slide well.

Indirect enzyme-linked immunosorbent assay (ELISA). ELISA was applied according to methods described by (Rose *et al.*, 1986). 96-well flat-bottomed ELISA plates (Immunoblon 1) were coated separately with 100 µl of the BRV, BCV and *E. coli* k99 lysate antigens, adjusted to concentrations of 0.6 mg/ml in carbonate-bicarbonate buffer (pH 9.6) for each and kept overnight at 4°C. Serum samples (n=60), diluted 1:10 in PBS (pH 7.4), were added as 100 µl / well in duplicates for each. Each plate contained positive and negative sera as well as a blank control. After an hour incubation at 37°C, PBS (pH 7.4) washing of the plates, a 100 µl / well of the horse radish peroxidase labeled anti-bovine IgG conjugate was added to each well and allowed to react for 1 h at 37°C. A freshly prepared, ortho-phenylene diamine, (sigma) substrate working solution was added (50 µl /well). Plates were incubated at 37°C for 15 min., with gentle agitation in dark place. The reaction was halted by adding 50 µl/well of 1.25 M sulphuric acid and the optical density (OD) was estimated at wave length

of 490 nanometer using the microplate ELISA auto-reader (Dynatech). The cut off mean absorbance values for BRV, BCV and *E. coli* k99 lysate antigens were 0.06, 0.03 and 0.07 respectively

Results and Discussion

Neonatal calf diarrhoea represents an important cumbersome, exerting an economic impact on bovine industry worldwide (Radostitis, 1991), including Egypt (Shalaby *et al.*, 1981; Kassem *et al.*, 1995 and Perk *et al.*, 2000). Currently, a trivalent inactivated vaccine (either the local entero-3 or the imported Scour gaurd), containing BRV, BCV and *E. coli* K99, is being used in Egypt for immunization of late pregnant cows to passively protect their offsprings via the elicited colostral antibodies. Traditional evaluation of such vaccines depends mainly on virus neutralization test (VNT) which is considered the standard test. However, it is gradually being replaced by ELISA and fluorescent antibody technique that offered advantages of rapid results and reduced costs (Rose *et al.*, 1986). In the present endeavor, reliable BRC, BCV and *E. coli* lysate antigens were prepared using simple procedure antigen lysates are utilized in microagglutination, indirect ELISA and immunofluorescent antibody techniques for rapid evaluation of such vaccines, versus the standard VNT.

Post-vaccination seroconversion of late pregnant cows and their offsprings, vaccine was monitored by VNT (Table 1). Results revealed that virus neutralizing antibody (VN-Ab) titers elicited against BRV and BCV were increased by the 2nd week post-vaccination ($\log_{10}$ mean VN-Ab titer was 1.68 for both viruses), reaching the highest titer (2.28 and 2.37 for BRV and BCV, respectively) by the calving time. The present results are in agreement with that obtained by (Daoud *et al.*, 2003 and Mahmoud and Zeidan, 2004). They concluded that there was a remarkably increase in the mean neutralizing antibody titers by the time of the 2nd dose that sharply increased by the time of parturition. Offsprings of vaccinated cows were passively seroconverted to BRV and BCV exhibiting high VN-Ab titers during the experimental period (Table 1). These results were in harmony with that obtained by (Daoud *et al.*, 2003) who concluded that VN-Ab titers of the newly born calves were sharply increased after ingestion of colostrum from entero-3 vaccinated cows. Saif *et al.* (1983) postulated that lactogenic immunity plays a significant role
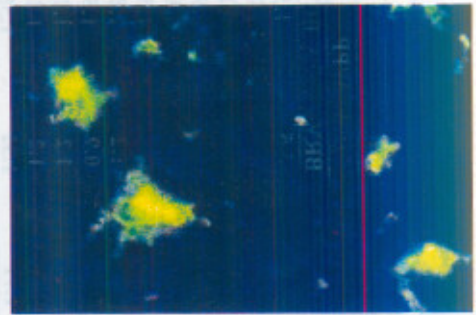

Fig. (1): Indirect immunofluorescent antibody technique. Positive results showing specific fluorescent cytoplasmic granules.

in protection of calves against infection.

Results of VNT (Table 1) were in accordance with that obtained by ELISA on testing the same serum samples (Table 3), using the locally prepared BRV and BCV lysate antigens. Accordingly, ELISA could be used for evaluation of the entero-3 vaccine utilizing these antigens. Mean anti-*E. coli* agglutinating antibody titers of entero-3 vaccinated cows and their offsprings, using the *E. coli* lysate antigen in microagglutination test (MAT), were illustrated in Table (2). It was evident that the vaccinated cows and their offsprings remained with high anti-*E. coli* agglutinating antibody titers throughout the experiment. The MAT results were parallel to the ELISA results (Table 3). These results coincided with that reported by (Collins *et al.*, 1988 and Daoud *et al.*, 2003 and 2005) who reported that mean anti-*E. coli* agglutinating antibody titers of vaccinated cows increased by the 4th week post-vaccination and reached their maximal titers by the 6th week post-vaccination. Detection of specific antibodies against the prepared BRV, BCV and *E. coli* K99 lysate antigens using indirect immunoflurescent antibody technique (IFA) was satisfactory (Table 4, Fig. 1). The IFA gave discrete positive results with sera of the vaccinated cows and their offsprings. These results were supported with that obtained by VNT, indirect ELISA and microagglutination tests.

In conclusion, locally prepared lysate antigens, from BRV and BCV infected MDBK cells as well as from *E. coli* K99 bacterial cells, are economic and reliable for rapid evaluation of entero-3 vaccine. Nevertheless, they are recommended for both detection and assessment of post-vaccination or post-infection antibodies against BRV, BCV and *E. coli*.

Table (1): Virus neutralizing antibody (VN-Ab) titres expressed as Log_{10} against bovine rota virus (BRV) and bovine corona virus (BCV) in sera of entero-3 vaccinated late pregnant cows and their offsprings using viral lysate antigens.

Cows No.	Log_{10} VN-Ab											
	Pregnant cows						Offspring calves					
	1st vaccination		2nd vaccination		Calving		1st WPP		2nd WPP		3rd WPP	
	BRV	BCV	BRV	BCV	BRV	BCV	BRV	BCV	BRV	BCV	BRV	BCV
1	0.3	0.3	1.8	1.5	2.4	2.1	2.1	2.1	1.8	1.8	1.5	1.5
2	0.3	0.0	1.5	1.5	2.1	2.4	1.8	2.1	1.5	1.8	1.2	1.2
3	0.6	0.3	1.8	1.8	2.4	2.4	1.8	1.8	1.8	1.5	1.5	1.2
4	0.3	0.3	1.5	1.5	2.1	2.1	1.8	1.8	1.8	1.5	1.5	0.9
5	0.9	0.3	1.8	1.8	2.4	2.4	2.1	2.1	1.8	1.8	1.8	1.8
6	0.3	0.3	2.1	1.8	2.7	2.4	1.8	2.1	1.5	1.8	1.2	1.5
7	0.6	0.6	1.5	2.1	2.1	2.7	1.8	2.4	1.5	2.1	1.2	1.8
8	0.0	0.3	1.5	1.5	2.1	2.4	1.5	2.1	1.2	1.8	0.9	1.5
9	0.3	0.3	1.8	1.5	2.4	2.4	2.1	1.8	1.8	1.5	1.2	1.2
10	0.3	0.3	1.5	1.8	2.1	2.4	1.8	1.8	1.5	1.5	1.2	1.2
Mean	0.39	0.3	1.68	1.68	2.28	2.37	1.86	2.01	1.62	1.71	1.32	1.38

WPP: Weeks Post Parturition

Table (2): Anti-*E. coli* K99 microagglutinating antibody titres in sera of entero-3 vaccinated pregnant cows and their offsprings as determined by microagglutination test (MAT) using bacterial cell lysate antigen.

Animal number	Microagglutinating antibody titers					
	Pregnant cows			Offspring calves		
	1st vacc.	2nd vacc.	Calving	1st WPP	2nd WPP	3rd WPP
1	16	512	1024	1024	1024	512
2	16	256	512	2048	1024	512
3	32	512	1024	1024	1024	512
4	16	256	512	1024	1024	512
5	16	256	512	1024	512	512
6	64	512	1024	2048	1024	1024
7	16	256	512	1024	512	512
8	8	256	512	1024	512	512
9	16	512	1024	1024	1024	512
10	16	512	1024	1024	1024	512
Mean	21.6	384	768	1228.8	870.4	563.2

Vacc. = Vaccination   WPP = week post parturition

Table (3): ELISA absorbance values for anti-bovine rota virus (R), anti-bovine corona virus (C) and anti-*E. coli* K99 (E) antibodies in sera of Entero-3 vaccinated pregnant cows and their offsprings using prepared lysate antigens

Tested	Pregnant cows									Offspring calves								
Animal No.	1st vaccination			2nd vaccination			Calving			1 WPP			2 WPP			3 WPP		
	R	C	E	R	C	E	R	C	E	R	C	E	R	C	E	R	C	E
1	0.06	0.02	0.06	0.265	0.231	0.283	0.333	0.299	0.301	0.311	0.282	0.306	0.292	0.231	0.261	0.281	0.201	0.250
2	0.03	0.03	0.05	0.311	0.301	0.311	0.459	0.391	0.351	0.397	0.299	0.311	0.295	0.261	0.276	0.275	0.250	0.246
3	0.02	0.02	0.07	0.261	0.231	0.251	0.321	0.231	0.351	0.310	0.276	0.315	0.231	0.211	0.241	0.221	0.201	0.231
4	0.04	0.03	0.03	0.273	0.221	0.241	0.352	0.261	0.357	0.231	0.210	0.221	0.231	0.221	0.241	0.211	0.211	0.232
5	0.04	0.02	0.06	0.241	0.201	0.231	0.310	0.311	0.352	0.311	0.310	0.322	0.251	0.202	0.215	0.231	0.200	0.205
6	0.03	0.03	0.05	0.262	0.210	0.251	0.395	0.351	0.321	0.373	0.309	0.312	0.253	0.221	0.231	0.236	0.203	0.222
7	0.03	0.03	0.06	0.275	0.241	0.255	0.386	0.364	0.351	0.365	0.342	0.312	0.239	0.201	0.225	0.219	0.190	0.210
8	0.03	0.03	0.03	0.282	0.231	0.244	0.361	0.321	0.331	0.341	0.337	0.339	0.232	0.221	0.231	0.207	0.203	0.207
9	0.06	0.03	0.03	0.371	0.302	0.357	0.446	0.396	0.399	0.411	0.397	0.339	0.295	0.282	0.276	0.281	0.270	0.250
10	0.02	0.02	0.05	0.397	0.321	0.361	0.496	0.354	0.396	0.455	0.337	0.371	0.282	0.262	0.253	0.240	0.240	0.233
Mean	0.036	0.026	0.049	0.294	0.249	0.279	0.386	0.328	0.351	0.351	0.310	0.315	0.260	0.231	0.245	0.241	0.217	0.229

WPP = week post parturition

Table (4): Detection of anti-bovine rota virus (BRV), anti-bovine corona virus (BCV) and anti-*E. coli* K99 (E) antibodies in sera of entero-3 vaccinated pregnant cows* and their offsprings** by indirect immunofluorescent antibody technique (IFA)using prepared lysate antigens.

Examined sera	Number of samples	Positive IFA		
		BRV	BCV	*E. coli* K99
Cows' sera*	15	15	15	15
Offsprings' sera**	5	5	5	5
Cumulative positive percentage			20/20 = 100%	

* = Randomly selected serum samples collected at the times of 2nd vaccination and calving.

** = Randomly selected serum samples collected at the time of 2nd week post-natal.

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