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A direct Neutralizing Peroxidase linked antibody assay (NPLA) for evaluation of immune response to a commercial bovine ephemeral fever vaccine

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Bovine ephemeral fever has major economic significant losses due to drop in milk production in dairy herds and reduction in conditions of prime animals or disruption of stock movements and markets. This study was conducted for evaluation of immune response to a commercial bovine ephemeral fever vaccine in vaccinated calves using the Neutralizing Peroxidase Linked Antibody assay (NPLA) in comparison with micro neutralization test (MNT). The antibody titers were estimated weekly for the first 3 weeks after primary vaccination then monthly after booster vaccination by NPLA and MNT. The geometric mean of antibodies titers for the first 3 weeks by NPLA were 5.2, 12.8, and 24.8 respectively. While MNT for the first 3 weeks were 5.6, 10.8 and 21.6 respectively. After booster vaccination the geometric mean of antibodies titers were monitored monthly for 11 months. The monthly geometric mean of antibody titers by NPLA were 364.8, 396.8, 384, 332.8, 332.8, 345.6, 153.6, 153.6, 89.6, 76.8 and 76.8 respectively. Using MNT were 300.8, 345.6, 332.8, 307.2, 281.6, 256, 160.0, 179.2, 89.6, 57.6 and 76.8 respectively. In conclusion, although there is no significant difference between the NPLA and MNT; The NPLA is an accurate, sensitive and specific method for detection and titration of the neutralizing antibodies to BEF virus induced by the commercially available BEF vaccine. Moreover the NPLA is not affected by the non- specific cytopathogenic effect in cell culture. The level of immunity in the vaccinated calves was found to be protective for 6 months and further studies are needed.

Keywords: NPLA, bovine ephemeral fever, antibodies, live attenuated vaccine.

INTRODUCTION

Bovine ephemeral fever (BEF) or the alternative name (3-day sickness) is a vector born viral disease affecting cattle and occasionally buffaloes of highly economic impact including severe reduction in milk production in dairy herds and loss of condition in beef cattle (Walker, 2005). The seasonal incidence of the disease was associated with vector multiplication in tropical and subtropical areas with globe distribution in Africa, the Middle East, Asia and Australia (Walker and Klement, 2015). The causative agent (BEFV) is a cone-shaped single stranded, RNA virus belongs to genus Ephemerovirus in the family Rhabdoviridae. The main clinical signs of BEF including sudden fever (bi-phasic fever) accompanied with anorexia, salivation, muscle stiffness, ocular and nasal discharge, ruminal

stasis and recumbence (Walker, 2005). Sternal and lateral recumbenc, and ataxia may be found in complicated cases that ended by death due to pneumonia (Zheng and Qui, 2012). The morbidity is often high with low mortality as well as rapid recovery usually occurs (Nandi and Neg 1999). In outbreaks the morbidity and mortality rates increased in high manner as recorded in Saudi Arabia during 1990 and 1996 (Farag et al., 1998); Egypt 2000 in which subcutaneous emphysema was pronounced with recumbence (Zaghawa et al., 2000). The prevention and control measures basically depend on vector control, vaccination and treatment of affected cattle (Aziz-Boaron et al., 2013; Akakpo, 2015 and Wallace and Viljoen 2005).

Problems with conventional vaccines are due to the rapid loss of antigenicity and pathogenicity of BEFV when passaged in cell culture (Tzipori and Spradbrow, 1973) as well as presence of defective interfering particles (Della-Porta and Snowdon, 1979). These interfering particles constitute problems for the purification and characterization of BEFV (Uren, 1989). Micro neutralization test is the gold standard test for detection and titration of neutralizing antibodies to BEF virus that is used for evaluation of the vaccination program and different studies for sero-survay (Ting et al., 2014). The test has disadvantage of time consuming and confusion with the non-specific cytopathogenic effect (CPE) so, several authors apply the NPLA for detection of the residual virus effect in the neutralization test as Heyera et al., (1987) for BVD virus, Zaghawa et al., (1990) for canine distemper, seal distemper and rinderpest viruses and Terpstra et al., (1984) for hog cholera. This study was conducted to investigate the use of Neutralizing Peroxidase Linked Antibody assay (NPLA) for evaluation of a commercial bovine ephemeral fever vaccine in vaccinated calves.

MATERIALS AND METHODS

Animals and vaccination schedule:

Fifteen Friesian calves aged 6-8 months were selected to determine the immune response of calves to live attenuated BEFV vaccine. The 1st group consisted of 10 calves that vaccinated with the vaccine by 2 doses of 2 ml s/c with 3 weeks a part. The second group consisted of 5 non-vaccinated calves that were kept as a controls.

2.2. Serum samples:

Serum samples were collected from all animals for antibodies detection before vaccination (zero time), and weekly for 4 successive weeks after primary vaccination then monthly till 11 months after booster dose administration.

2.3. Bovine ephemeral fever vaccine:

A freeze-dried suspension of live attenuated virus produced from Websters; was used in this study. Each 2 ml dose contains a standard strain (BB2271-919) at ≥ $10^{4.3}\mathrm{TCID}_{50}$ (Ultravac BEF Vaccine, Zoetis®).

2.4. Vero cell subculture:

Vero cell line (African green monkeys renal epithelial cells) was supplied kindly by the Animal Health Institute, (Rhiyadh, Ministry of Agriculture KSA) and was used for serum neutralization test. The cells were cultivated at 37°C, 5% CO2 and 70-80% humidity. Modified Eagle Medium (ATCC. 30-2002™ VWR) was used supplemented with 5% fetal calf serum (Sigma®) and 50 µg/ml Gentamicin (Thermo Scientific®).

2.5. BEF virus:

The BEF virus used in this study for MNT and NPLA was isolated from a cow at Al-Hasa region of KSA (Zaghawa et. al., 2017).

2.6. Peroxidase conjugated BEF antibodies:

Hyperimmune serum was prepared in rabbits according to (Hsieh et al., 2009) in which rabbits were inoculated by 3 doses (0.5 ml) of the virus after mixing with complete freund adjuvant with 2 weeks interval. Rabbit immunoglobulin fraction of anti BEF virus serum was isolated by precipitation with sodium sulphate at 37°C according to Zaghawa et al., (1990). The protein content of the resulting solution was determined by according to Lowery et al., (1951). Prior to conjugation with horse radish peroxidase, the pH of the protein was adjusted to 9.3 - 9.5 by o.o2M sodium carbonate buffer. Conjugation of horse radish peroxidase with anti-BEF immunoglobulin (Ig) was accomplished by the periodate (p-) according to Boorsma and Streefkerk (1979). The conjugate was tested in Vero cells in microtiter plates seeded with 6000 cell per well with both positive and negative control in check board method to determine the optimal dilution for use.

2.7. Microneutralization test (MNT): Cybinski et al. (1992).

Two fold serum serial dilution from 1/ 2 to 1/ 1024 were prepared in maintenance medium (MEM). Four wells per dilution were performed. In each well 50µl of the diluted serum were mixed with an equal volume of diluted virus($200\ \mathrm{TCID}_{50}$). The mixture was incubated for 30 minutes at 37 °C in CO_2 incubator. Known BEFV positive serum, BEFV negative serum and cell culture medium were set up in each test for control purposes. Vero cells were diluted to contain 10^6 cells / ml then , 100 µl of this suspension was transferred to each well. Incubation was followed for 5-7 days and evaluation was done with inverted microscope for observing the CPE in each well. Antibody titers were expressed as the reciprocal value of the highest serum dilution giving a positive reaction. The ND50 was calculated using the method of Reed and Meunch (1938).

2.8. Neutralizing peroxidase linked antibody assay:

Neutralizing peroxidase linked antibody assay (NPLA) for detection of anti – BEFV antibodies was carried out according to (Zaghawa, 1998) with slight modification. Two fold serum serial dilution from 1/ 2 to 1/ 1024 were prepared in maintenance media (MEM). Four wells per dilution were performed. In each well 50µl of the diluted serum were mixed with an equal volume of diluted virus($200\ \mathrm{TCID}_{50}$). The mixture was incubated for 30 minutes at 37 °C in CO_2 incubator. Known BEFV positive serum, BEFV negative serum and cell culture medium were set up in each test for control purposes. Vero cells were diluted to contain 10^6 cells / ml then 100 µl of this suspension was transferred to each well. After 2 days of incubation at 37°C in CO_2 incubator, washing one time with PBS-tween and fixation at 80°C for 4 hour was followed. Anti-BEF virus peroxidase conjugate was diluted 1:200 and 50 µl was added to each well. Incubation and washing were followed as above. Subsequently 50µl of freshly prepared substrate solution (2-amino 9-ethylcarbazole was dispersed in each well. After 15-30 second the substrate was discarded and replaced by 100 PBS-tween. The plates were evaluated through an inverted microscope in which the positive wells showed reddish to dark brown stained cells while the negative well remained unstained. Antibody titers were expressed as the reciprocal value of the highest serum dilution giving a positive reaction. The ND50 was calculated using the method of **(**Reed and Meunch 1938).

2.9. Statistical analysis:

Statistical analysis was performed using Chi-square test (X^2) and multiple analysis of variance (ANOVA) according to (Hill, 1979).

RESULTS AND DISCUSSION

Bovine ephemeral fever (BEF) is an acute febrile vector-borne disease causes mild to severe illness in cattle and buffaloes. The disease has widely distributed in different parts in the world from the southern of Africa to the Nile River Delta, across the Middle East, South and South-East Asia, Australia, China, Taiwan, the Korean Peninsula and southern Japan (Walker and Klement, 2015). The disease is associated with high economic impact due to cessation in milk production, loss of condition in beef cattle and the immobilization of water buffaloes used for draught (Walker, 2005; Aziz-Boaron et al., 2014). Evaluation for Positive and negative residual BEF virus CPE detected by MNT and NPLA was done with inverted microscope for observing the CPE in each well as shown in figure (1).

In this study the humeral immune response of 10 calves vaccinated with live attenuated bovine ephemeral fever vaccine was estimated in addition to 5 control non-vaccinated calves. All animals were injected with 2 doses of live attenuated BEFV with 3 weeks interval at which the neutralizing antibody titer was estimated using Microneutralization test (MNT) and Neutralizing peroxidase linked antibody test (NPLA). The neutralizing antibody titers elevated after 1st, 2nd and 3rd week with geometric mean of (5.2, 12.8, and 24.8) by the NPLA and (5.6, 10,8 and 21.6) by the MNT respectively table (1). The statistical analysis showed that with chi-square= 34.76 (p=1.0) significant at 0.01 or neutralization peroxidase linked antibody with chi-square=14.22 (p=3.53) significant at 0.05. Although, there was no significant differences between the two tests NPLA and MNT in antibodies with P value (0.0596). Our findings are in agreement with previous study of Daoud et al., (2001) who demonstrated successful protective immunity level in animals vaccinated with live BEF vaccine. Also the obtained results are in accordance with those described by Hsieh et al., (2005 and 2006) who reported that the level of serum neutralizing antibody titer was significantly increased by 8 to 128 fold within one month post vaccination of inactivated BEFV vaccine. Parallel

to this in Egypt, inactivated oil BEF vaccine adjuvant with Montanide ISA206 oil was prepared and induced protective immune response after 2 weeks with protective level till 40 weeks with single vaccine dose, with no adverse reactions (Manal, 2005; Bagoury et al., 2014).

The results of the immune response of calves after booster dose with live attenuated BEF vaccine showed significant higher antibody titers for three successive months (1st, 2nd and 3rd) with geometric mean of (364.8, 396.8 and 384) by NPLA, and (300.8, 345.6 and 332.8) by the MNT respectively table (2). These results are supported by Tzipori and Spradbrow (1973), Della-Porte and Snowdon (1977) and Tzipori and Spradbrow, (1978) who recorded that long-lasting immune response for more than one year after the use of two doses of BEFV live vaccines, although post vaccination reactions and potential impairment of vaccine potency by heat or light act as obstacles for using live attenuated vaccines widely .

In this study statistical non-significant decrease in the titer of neutralizing antibodies was noticed at the 4th , 5th and 6th months postboostering of (332.8, 332.8 and 345.6) and (307.2, 281.6 and 256.0) by the MNT respectively. A marked and gradual decrease in antibody titers was recorded (153.6, 153.6 and 89.6) by the NPLA and (160.0, 179.2 and 89.6) by the MNT respectively table (2).

Due to the questionable protective effect of BEF vaccines many trials were done as the use of inactivated BEF vaccine at the time of vaccination by saponins to achieve the advantages of live attenuated and inactivated vaccines (Vanselow et al., 1995 and Daoud et al., 2001). In Japan, another protocol was done through administration of live-attenuated virus followed by an inactivated vaccine that stimulated a strong and effective neutralizing antibody response in comparison with live-attenuated vaccine alone or two doses of the inactivated vaccine with no adverse effects on animals health and production (Inaba et al., 1974). Recently, effective vaccination was achieved using inactivated recombinant G glycoprotein vaccines of BEFV (Bai et al., 1993; Walker and Klement, 2015). Although different formulations of experimental and commercial vaccines were developed for control of BEFV, the evaluation of their efficacy of theses vaccines needs further investigations by a more reliable evaluation methods (Walker and Klement, 2015).

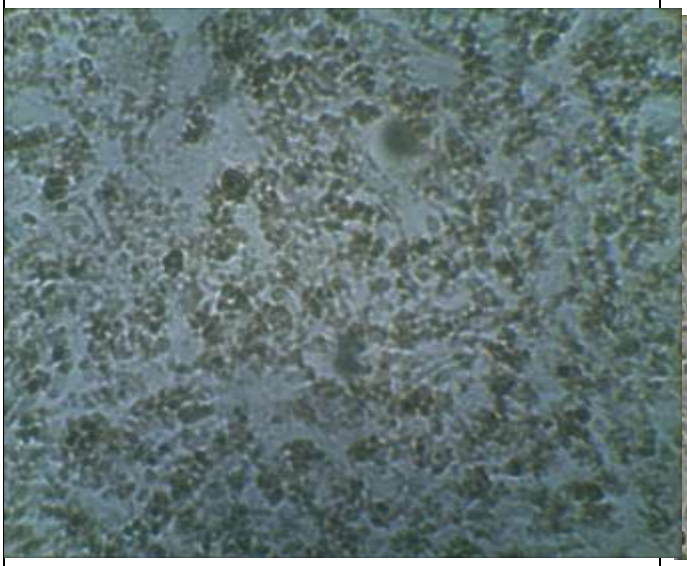
Table 1: Humeral immune response of calves after primary vaccination with life attenuated bovine ephemeral fever vaccine.

	Reciprocal Neutralizing antibody titers						
	Weeks after vaccination						
No	0	1st week		2nd week		3rd week	
		NPLA*	MNT**	NPLA	MNT	NPLA	MNT
1	≤ 2	4	4	8	6	16	**16**
2	≤ 2	**4**	4	8	**8**	8	**8**
3	≤ 2	**6**	8	16	**16**	32	**16**
4	≤ 2	**4**	4	8	**6**	16	**16**
5	≤ 2	**6**	8	16	**8**	32	**32**
6	≤ 2	**8**	8	16	**8**	32	**32**
7	≤ 2	**8**	8	16	**16**	32	**16**
8	≤ 2	**4**	4	16	**16**	32	**32**
9	≤ 2	**4**	4	16	**16**	32	**32**
10	≤ 2	**4**	4	8	**8**	16	**16**
Mean		5.2	5.6	12.8	10.8	24.8	21.6
11—15 Control a neutrlizing antibody titer all period =≤ 2							

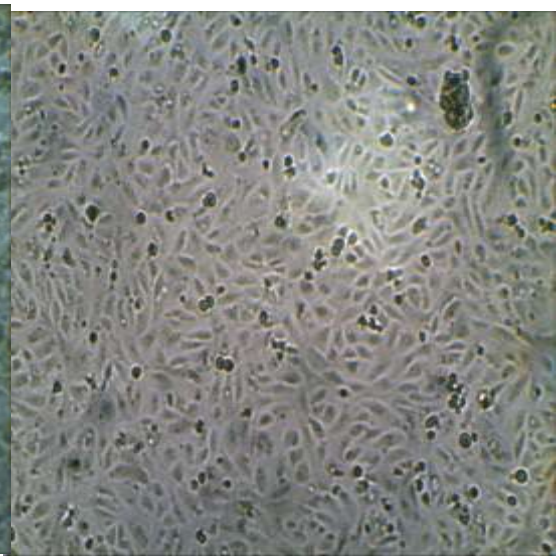
MNT: Microtitre neutralization test with chi-square= 34.76 (p=1.0) significant at 0.01
NPLA: neutralization peroxidase linked antibody with chi-square=14.22 (p=3.53) significant at 0.05.
No significant difference between the MNT and NPLA with P value (0.0596).

Table (2) : Humeral immune response of calves after booster dose with life attenuated bovine ephemeral fever vaccine

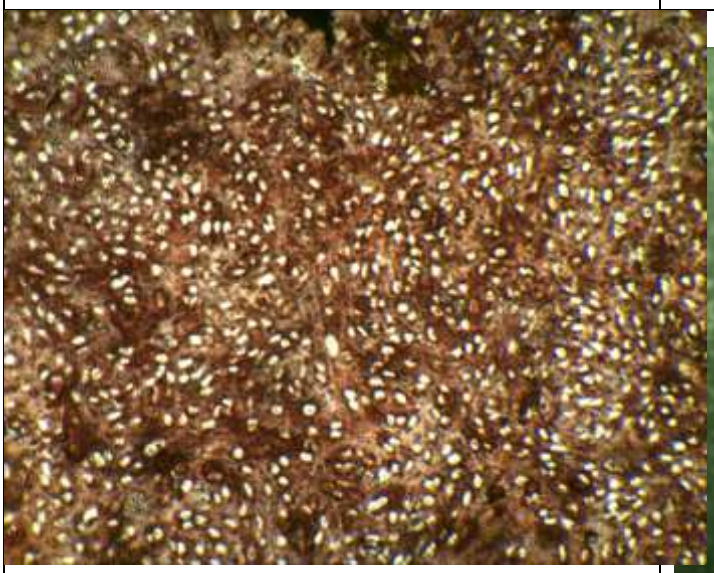
Months after revaccination (booster dose)	Reciprocal Neutralizing antibody titers means		Months after revaccination (booster dose)	Reciprocal Neutralizing antibody titers means	
	MNT	NPLA		MNT	NPLA
0	33.6	49.6	6	**256**	345.6
1	**300.8**	364.8	7	**160**	153.6
2	**345.6**	396.8	8	**179.2**	153.6
3	**332.8**	384.0	9	**89.6**	89.6
4	**307.2**	332.8	10	**57.6**	76.8
5	**281.6**	332.8	11	**76.8**	76.8



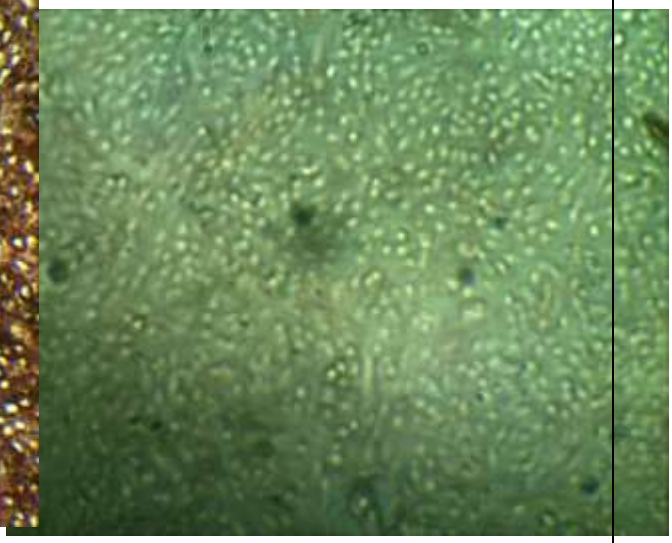
Positive residual BEF virus CPE detected by MNT in Vero cells.Mag. 10^x



Negative residual BEF virus CPE detected by MNT in Vero cells.–Mag. 10^x



Positive residual BEF virus detected by NPLA in Vero cells.



Negative residual BEF virus detected by NPLA in Vero cells.

Figure 1 : Humeral immune response of calves after revaccination (Booster dose) with life attenuated bovine ephemeral fever vaccine.

CONCLUSION

In conclusion, The NPLA is an accurate, sensitive and specific method for detection and titration of the neutralizing antibodies to BEF virus. Moreover the NPLA do not affected by the non- specific cytopathogenic effect in cell culture. The level of immunity in the vaccinated calves is protective for 6 months and further studies are needed

CONFLICT OF INTEREST

The authors declared that present study was performed in absence of any conflict of interest.

AUTHOR CONTRIBUTIONS

All listed authors have made substantial contributions to the research design, the acquisition, analysis, or interpretation of data; and to drafting the manuscript or revising it critically; and that all authors have approved the submitted version.

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