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Comparative studies on Application of Enzyme–Linked Immunosorbent Assays and Serum Neutralization Test for Measuring of Bovine Ephemeral Fever Virus Antibodies in Vaccinated Animals

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SUMMARY

Two models of ELISA were developed for serological detection of Bovine Ephemeral fever (BEF) antibodies in the sera of vaccinated Buffaloes in comparison with serum neutralization test. Blocking ELISA (B. ELISA) was developed and standardized for detection of BEF antibodies. In this test, the binding capacity of rabbit BEF hyper immune sera to BEFV antigen was blocked in the presence of positive BEF vaccinated Buffaloes sera.

The sensitivity of both ELISA models was compared with serum neutralization test (SNT) using a total of 160 serum samples obtained from vaccinated Buffaloes where it was found that the sensitivity and specificity of B- ELISA and I-ELISA were (98.4% & not estimated) and (100% & 100%), respectively. These finding suggested that the both ELISA models were considered an inexpensive, rapid and sensitive serological methods that would be suitable for measuring of BEFV antibodies in vaccinated Buffaloes.

INTRODUCTION

Bovine ephemeral fever (BEF) is an acute viral infection of cattle and water buffalo that occurs in many countries and results in considerable economic losses. Affected animals show high fever, reduced milk production, abortion and lameness or paralysis (walker, 2005) In Egypt, the disease was first described by (Rabagliati, 1924) and in summer 1991, a typical form of the disease had been recorded in different governorates in lower Egypt (Hassan *et al.*, 1991). A second outbreak of BEF occurred in summer 2000, where it included several governorates in lower and Upper Egypt and characterized by 50% morbidity and 2.5% mortality, (Zaghawa, 2000). A severe outbreak was

occurred in Bahr El-Baker (Hassan, 2000) and another one was recorded in cattle and buffaloes in Dakahlia and Damietta Governorates during the summer of 2004 (Daoud *et al.*, 2005 and Younis *et al.*, 2005) while (Nayel, 2006) recorded an outbreak affecting Friesian and local breeds of cattle in Menufia Governorate. Local live and inactivated BEF vaccines were prepared successfully and showed good protection rates for vaccinated cattle and buffaloes (Daoud *et al.*, 2001a&b; Sayed-Ahmed, 2005 and Younis *et al.*, 2005 and El-Behwar *et al.*, 2010).

Currently, the micro-neutralization test is the standard method used for the serological detection of BEFV antibodies. Cell culture and asepsis are a strict requirements, it can prove difficult to carry out under some laboratory conditions (St-George, 1988).

The present work aims to compare between the simplicity and sensitivity of ELISA with SNT in order to improve the performance of ELISA in terms of application.

MATERIALS AND METHODS

1-Animals and vaccination:

Ten healthy native buffalo calves between 6-8 months of age and sero-negative for BEF were vaccinated with the local produced inactivated cell culture BEF vaccine (Daoud *et al.*, 2001). Each animal received 2 doses of 2ml of the vaccine injected S/C in the neck side at 2 weeks interval. Another 2 calves were kept without vaccination as test control. Serum samples were obtained from vaccinated and unvaccinated calves 2-3 weeks after the second dose for optimal post vaccination BEF-sero monitoring.

2- BEF Virus strain:

The BEF local strain propagated on BHK-21 cell culture with a titer of $10^{7.5}$ $TCID_{50}$ /ml (Azab *et al.*, 2002) was used in vaccine preparation and antigen preparation for ELISA and application of SNT.

3-Preparation of BEF antigen for use in ELISA:

The virus suspension was purified following the method described by (Walker *et al.*, 1991).

4- Preparation of BEF hyper immune serum:

It was prepared in 2 Boscat rabbits using purified virus BEF-AVS 2000 (local) strain which was mixed with an equal volume of complete Freund's adjuvant.Rabbits were firest immunized subcutaeneously with the virus antigen containing approximately 250 µg/ dose of antigen. A series of three boosters administered,mixing the virus with an equal volume of incomplete Freund's adjuvant at two weeks intervals according to Ching Hsieh *et al.*, (2006).

5- Indirect ELISA:

A checkerboard titration was performed to optimize the working dilution of the coating antigen where BEF negative and positive bovine sera and horseradish peroxidase labeled rabbit-anti-bovine IgG (HRP-IgG) (BioDov-Tech) were included. Antigen dilutions were 1:5, 1:10, 1:20, 1:40, 1:80, 1:160 and 1:320 and serum dilutions were 1:10, 1:20, 1:40 and 1:80 etc. The dilutions that gave the maximum difference in absorbance at 490 nm between positive and negative serum (P/N) were selected for test proper. The reaction temperature, time and other conditions were optimized as described by Bakheit *et al.*, (2004).

After optimization, the indirect ELISA was performed according to Fu-Ying Zheng *et al.*, (2010)

6-Blocking ELISA:

It was carried out according to Voller *et al.*, (1979) and the results were expressed as percentage of inhibition and calculated as follow:

$$\text{Percentage of inhibition} = 100 - \frac{(OD_{\text{test serum reaction}} \times 100)}{(OD_{\text{no serum reaction}} \times 100)}$$

To standardize test condition, the antigen was calibrated against the hyper immune serum using two dilutions of the antigen, hyper immune serum and standard positive and negative sera. The sera were titrated to determine the dilution required in the preliminary screening of test samples.

7-Serum Neutralization test (SNT):

SNT was conducted as described by Cybinski *et al.*, (1978) while serum and virus titers were calculated according to Reed and Muench, (1938).

Controls included positive serum (diluted from 1/5 to 1/640), negative serum (1/4 dilution), virus control (inoculating directly on cells with 100 $TCID_{50}$ BEFV) and cell control (normal cells) were used in the test proper. The control positive serum had four replicates with every dilution. The negative serum, virus control and cell control steps were performed in quadruplicate. Serum samples were considered positive to BEF antibodies or negative when the two well of cells mixed with serum at a 1/4 dilution, either exhibited or did not exhibit CPE. When only one well demonstrated CPE, the result was considered equivocal. Serum samples of control negative (unvaccinated) and vaccinated Buffaloes were tested by B-ELISA using a cut off 0 - 30% of inhibition from BEFV-serum neutralization test negative sample and > 30-100%from positive samples.

Also indirect ELISA (I-ELISA) was applied where ELISA plates were coated with BEFV antigen against multiple serum dilution (double fold) starting with 1/10. It was optimal that sera with Positive / Negative value ≥1.8 were considered positive, P/N

value ≤ 1.6 negative, and that between 1.8 and 1.6 was ambiguous.

RESULTS AND DISCUSSION

Table (1): BEF virus antibodies in vaccinated calves' serum as detected by SNT and ELISAs

Serological test	Positive/ total	Percentage of +ve samples	Percentage of –ve samples
SNT	128/160	80%	20%
I-ELISA	128/160	80%	20%
B-ELISA	126/160	78.8%	21.2%

*BEF: Bovine Ephemeral Fever
*SNT: Serum Neutralization Test
*I- ELISA: Indirect Elisa
*B-ELISA: Blocking ELISA

Table (2): Evaluation of the performance of ELISAs relative to SNT for detection of BEF virus antibodies in sera of vaccinated buffalo calves.

TEST	SNT				
	+ve	-ve	AGREEMENT	SENSITIVITY	SPECIFICITY
I-ELISA	128	32	100%	100%	100%
B-ELISA	126	34	97.5%	98.4%	NE
Difference in agreement	2	2	Total diff.=4		

*NE: Not Estimated
*N.B.: Positive SNT titer ranges: 1.8-2.4 log 10
Negative SNT titer ranges: 0.3- 1.2 log 10
Positive ELISA titer ranges: 1.8- 2.7 log 10
Negative ELISA titer ranges: 0.6- 1.5 log 10

Optimizing the B-ELISA test conditions it was found that the optimal selected dilutions of viral antigen and hyper immune serum were 1/200 and 1/160, respectively at an absorbance between 0.4 and 0.6 where at these concentration the difference between positive and negative Buffaloes sera was significant. The optimal dilution of sera used in the preliminary screening test was also determined from the titration of positive and negative sera and it was selected at 1/5. The cut off value for positive result was determined from the titration of the positive serum and was selected at 30% inhibition at dilution of 1/256. Bovine serum samples (160) were tested using the B-ELISA with a cut off 0-30 % inhibition for negative samples and 30-100% inhibition to denote positive samples. The results were compared with SNT and B-ELISA. 128 out of 160 serum samples were positive for BEFV antibodies as determined by SNT test. 126 out of 128 SNT positive serum samples were positive by B-ELISA as shown in table (1).

These results pointed to the sensitivity of B-ELISA as antibody estimation tool in comparison with SNT as 98.4% and specificity could not be estimated respectively coming in agreement with those of Zakrzewski *et al*.,(1992).

On the other side, the checkerboard titration indicated that the optical density (OD) value that gave the maximum difference between positive and negative serum (P/N value of 3.2) was observed when the dilutions of antigen and serum were 1:100 and 1:20, respectively. Therefore, the final concentration of coating antigen was 0.5 µg/ well and the optimal dilutions of serum and HRP-IgG were 1:20 and 1:1000, respectively. Using the indirect ELISA, the absorbance of the positive control was > 0.6, and the absorbance of the negative control was < 0.2. If a value lay outside these limits, the test was repeated. Sera from vaccinated Buffaloes were tested with both indirect ELISA and SNT when the cut-off value was 1.8 of the positive sera in the SNT according to Vanselow *et al.,* (1985) who showed that animals which had BEF neutralizing antibody titers in excess 64 after vaccination and before experimental challenge were protected from developing clinical disease. It was optimal that sera with P/N value ≥1.8 were considered positive, P/N value ≤ 1.6 was negative, and that between 1.8 and 1.6 was ambiguous. The 128 SNT positive serum samples for BEF antibodies were positively I-ELISA and 32 out of 32 SNT negative sera for BEF antibodies were negatively I- ELISA (Table - 2). I-ELISA gave a calculated sensitivity of positive / total 100% (128/128) and specificity of 100% (32/32).

In conclusion, based on the obtained results the applied models of ELISA have great sensitivity and specificity compared with SNT for measuring of BEFV antibodies in sera of vaccinated Buffaloes.

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دراسة مقارنة على استخـدام اختبـارات الاليـــزا واختـبار تعـادل المصـل فى قيـــاس الاجسـام المناعـية لفـيروس مرض حمـــى الثلاثة ايـام فى الحيوانـــات المحصنـــة

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تم إستخدام صورتين مختلفين من إختبارات الإيزا للكشف عن الأجسام المناعية لحمـى الثلاثـة أيـام مقارنـة باختبـار المصل المتعـادل . وقد أوضحت النتـائج أ ن حساسية إختبـار الإليزا التنافسية ٩٨.٤% عـنـد إستخـدام مصـل عالـى العيارية محضر فى الارانـب بينما كانت حساسية إختبار الإ ليزا الغير مباشره ١٠٠% بالنسبة لحساسية إختبار المصل المتعادل كما كانت المطابقة النوعية ١٠٠% للإليزا الغير مباشرة وبالنسبه للإليزا التنافسية لم يمكن حساب المطابقه النوعيه مقارنة باختبار المصل المتعادل.

مما سبق يتضح أن إختبـار الإليزا ذو دقـة وكفاءة نوعية وحساسية عالية كما انه يتميز بسهولة إستخدامه ، وسرعتـــه يمكن من قياس المستويات المناعية لحمى الثلاثة أيام فى أمصال الحيوانات المحصنة.