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PREPARATION OF ELISA KIT FOR DETECTION OF ANTIBODIES TO BOVINE HERPES VIRUS 1

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SUMMARY

In the present study, an ELISA assay was developed for detection of antibodies against BHV-1. Colorado strain of BHV-1 was propagated on MDBK cell and characterized by using SNT and FAT. The virus was titrated after and before concentration by polyethylene glycol (PEG). The protein content of the concentrated virus was determined. In the optimization steps of ELISA kit, the best results were at antigen concentration of 5μg/ml and 1/25 dilution of serum. The efficiency of the prepared ELISAwas studied by comparison the results with those of SNT when applied both tests on 86 serum samples. 85 % of these samples were positive by ELISA and only 61% were positive by SNT. Comparison between the prepared ELISA and SNT as well as commercial ELISA kit was carried out. A slight difference was reported. Application of the prepared ELISA kit was employed on the screening of 354 serum samples collected from 5 different localities, El Behira (vaccinated cattle), Sharkia, Menofia, El Fayoyum and Kafr El Sheikh. The prevalence percent of anti BHV-1 antibodies were 95%, 88%, 80%, 38%, and 48%, respectively. Also analysis of the obtained ELISA results revealed that samples of cattle were of high prevalence percentage than those of buffalo. In conclusion, the present study reports the development of a locally prepared ELISA kit for detection of anti BHV-1 antibodies and its success in a preliminary field application trial.

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INTRODUCTION

Bovine herpes virus (BHV-1) is double stranded DNA virus belonging to Family Herpesviridae; Subfamily Alpha Herpes virus; Genus Varicellovirus (Roizman, 1982; Brown, 1989; and Wyler et al., 1989). The viral DNA encode for 33 structural proteins, from which 10 are classified as glycoprotiens. Three major glycoproteins gI (gB), gIII (gC) and gIV (gD) are all responsible for inducing neutralizing antibodies and could be recognized by CD4 and CD8. The gIII (gc) is responsible for haemagglutinating activity of the virus (Tikoo et al., 1995). The seven minor glycoprotiens (gE, gI, gG, gK, gL, gM and gM) are sharing common function as they are responsible for virus multiplication in cell culture and play an important role in the pathogensis and spread of viral infection to various cell types (Rijsewijk et al., 1995; Baranowski et al., 1996).

BHV-1 causes different forms of the disease with great economic losses in animal industry. Respiratory form or infectious bovine rhinotracheitis one of respiratory disease complex and shipping fever had been estimated to cause losses of $500 million per year in U.S.A cattle industry (Bowlan and Shewen, 2000). BHV-1 causes 2 different forms of disease associated with reproduction: Infectious Pustular Vulvovaginits (IPV) and Infectious Balanopositis (IBP). These forms reported to lead to great economic losses in production and fertility (Gibbs and Rweyemamu, 1977).

BHV-1 is also spread to nervous system from local infection, which probably occurred by entry of virus to peripheral neural cell at nerve ending and transport of naked nucleocapsid within axon by retrograde axonal flow to nucleus in the body in trigeminal and sacral ganglion leading to establishment of latency (Ackerman and Wyler, 1984). Latent infection is reactivated by wide variety of stimuli, which responsible for transport of virus in cattle population (Kutish et al, 1990).

Enzyme Linked Immunosorbent Assay (ELISA) has a great important in diagnosis of BHV-1 infection. It is considered to be technically superior as a routine diagnostic test for detection of BHV-1 antibody in bovine sera (Cho and Bohac, 1985). It shows higher sensitivity than traditional serum neutralization test and similar sensitivity to modified SNT (Bitsch, 1978). In addition, it is successfully used to detect antibody in serum as well as milk samples (Krause et al., 1989). IBR Free European countries use gE blocking ELISA together with marker vaccine to differentiate between vaccinated and infected animal (Van oris-chot et al., 1997).

The aim of present study was to develop an ELISA kit to be locally used in field diagnosis of IBR infection and also to evaluate the success of the currently applied IBR vaccine program.

MATERIAL AND METHOD

Virus and Cell Culture

Colorado strain of BHV-1 was kindly provided by Prof. Dr. Hussein Aly Hussein Department of Virology, Faculty of Veterinary Medicine, Cairo University.

Madin-Darby Bovine Kidney (MDBK) cells was grown in Minimum essential Media (MEM) with 5-10% fetal calf serum.

Antigen Preparation

Virus concentration by Poly Ethylene Glycol 6000 (PEG 6000) was done according to Virology Methods Manual (1996). The harvested infected cells complete CPE were disrupted by 3 cycles of freezing and thawing then clarified by centrifugation at 3000 rpm for 20 minutes at 4°C. The supernatant was stirred with 2-3% NaCl and 7% PEG 6000 overnight at 4°C then centrifuged at 10.000 rpm for 20 minutes. The precipitate was resuspended in TES buffer followed by centrifugation at 13.000 rpm for 4 minutes. The protein content of the concentrated virus was determined using spectrophotometer at 260 and 280nm wavelengths. The $TCID_{50}$ of the prepared virus was calculated before and after concentration using the method of Reed and Muench, (1938).

Serum samples

354 serum samples were randomly collected from different localities. The complete data of these serum samples are presented in the following table.

Localities	Buffalo	Cattle	Total
Menofia	-	42	42
El-Fayoum	33	57	90
Sharkia	-	90	90
Kafr El-Sheikh	28	62	90
Vaccinated Dame at Abou El-Matamer Behira	-	42	42
Total	61	293	354

Optimization of ELISA (check board titration)

ELISA plate was coated with three different antigen concentration (3ug, 5ug and 10ug/ml) then blocked with 10% non-fat dry milk in PBS. The different concentration of antigen was reacted for 1 hour at 37°C with standard positive and negative sera from the commercially available bovine rhinotracheitis antibody test kit from IDEXX, USA. After washing, the plate was incubated for 1 hour at 37°C with peroxidase conjugated goat antibovine IgG (KPL, USA) diluted 1/1000. Three cycles of washing were applied. The reaction was developed with H_2O_2/ ABTS. Further color development was stopped by SDS (0.5%) then the plate was read at 405nm wavelength. Determination of the optimum reactivity of 42 serum samples was performed with dilution 1/25 and 1/50 against 5ug/ml antigen.

Serological tests

1- Prepared indirect ELISA

The test was applied according to the standard

technique descried by (Edward and Giats, 1978) with some modification. Briefly, ELISA plates were coated with 5µg/ml BHV-1 concentrated antigen then blocked with 10% non-fat dry milk in PBS. The serum samples were diluted 1/25 and incubated in the plates for 1 hour at 37°C. After washing, the plate was incubated for 1 hour at 37°C with peroxidase conjugated goat antibovine IgG (KPL) diluted 1/1000 followed by three cycles of washing. The reaction was developed with H_2O_2/ ABTS. Further color development was stopped by SDS (0.5%) then the plate was read at 405nm wavelength.

2- Serum neutralization test (Beta Procedure)

The test was conducted according to the standard technique described by (Bitsch, 1978). In a 96 well diluting plate, the serum samples were diluted two fold serial dilution in maintenance medium then incubated for 1 hour with equal volume of 100 $TCID_{50}$ of BHV-1. 100µl from diluting plate were transferred to corresponding wells of 96 well tissue culture plate containing monolayer of MDBK cells except the last row, which was left as virus and cell controls. The tissue culture plates were incubated at 37°C in 5% CO_2 tension with daily observation until the development of CPE in virus control wells. The wells showing complete absence of CPE were recorded and the neutralizing end point and titer were determined for each serum samples.

3- Commercial ELISA kit (Herd check ELISA kit, IDEXX, USA)

The test was applied as described by Manufacture Company. Briefly, The serum samples were diluted 1/25 and transferred to the coated plate and incubated. After washing, the plate was incubated for 30 minutes at 37°C with peroxidase conjugated goat antibovine IgG followed by four cycles of washing. The reaction was developed by adding 100µl/well TMB. Further color development was stopped then the plate was read at 650nm wavelength.

RESULTS

Titration and protein content of prepared antigen

The BHV-1 was subjected to titration using MDBK cells. The results revealed that the preconcentrated virus titer was $10^{5.5}$ $TCID_{50}$ / ml and by concentration the virus titer was increased to $10^{6.7}$ $TCID_{50}$ / ml. The determination of protein content showed that the concentrated antigen contains 1.92 mg/ml.

Check board titration

The check board assay revealed that the optimum reactivity of BHV-1 antigen with standard BHV-1 positive serum was occurred at concentration 5µg/ml as shown in figure (1). Whereas, the optimum reactivity of the 42 tested serum samples was observed at dilution 1/25 as shown in figure (2).

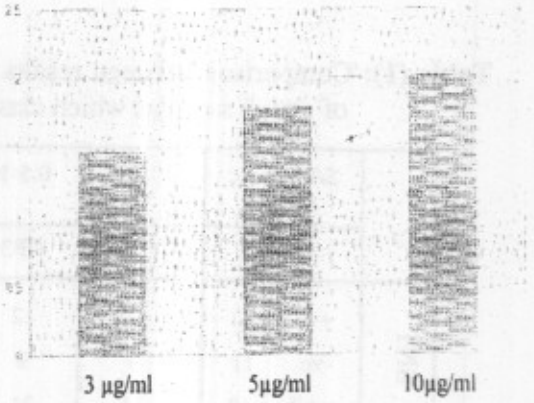

Fig. (1): Comparison of different antigen concentration reacted with the standard BHV-1

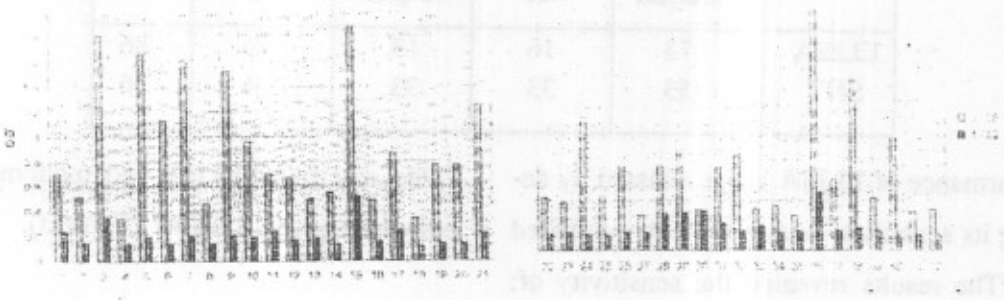

Fig.(2): Comparison between 2 different dilutions of serum samples against prepared antigen (5µg/ml) concentration BHV-1 in an ELISA.

Serological tests results

86 serum sample were tested by the prepared ELISA and classified according to its S/P ratio into seven groups and then tested by SNT for comparison between them in screening of BHV-1 antibodies in field serum samples (Table.1). The results revealed that in group 2-3 which were of low positive in ELISA result, number of serum samples were negative by SNT appear to be higher than other group with higher S/P ratio. The overall comparison between ELISA and SNT indicates high positive percent of ELISA 85% than positive percent in SNT (61%) (Table 2 and Figure3).

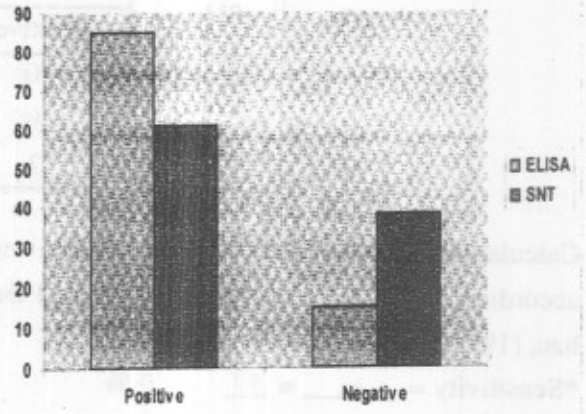

Fig.(3): Percent of positive and negative serum sample detected by ELISA and SNT.

Table (1): Comparison between results of ELISA and SNT for screening of seven groups of serum samples which classified according to S/P ratio in ELISA test.

	S/P ratio < 0.25	0.25-0.5	0.5-1	1-1.5	1.5-2	2-2.5	2.5<	
	GP1	GP2	GP3	GP4	GP5	GP6	GP7	Total
SNT	+ve 2	7	12	9	5	5	13	53
	-ve 11	4	9	3	2	2	2	33
	total 13	11	21	12	7	7	15	86

Table (2): summarizes the obtained results of overall comparison between the positive samples by ELISA when screened by SNT.

	No.of +ve samples	Percent of +ve	No.of -ve samples	Percent of -ve	Total
ELISA	73	16	13	15%	86
SNT	53	33	33	38%	86

The performance of ELISA was evaluated by determining its sensitivity and specificity compared to SNT. The results revealed the sensitivity of; 78%, specificity 84.6% and modern correlation between both tests 68.6% (Table.3).

Table (3): Determination of sensitivity, specificity and correlation of the prepared ELISA.

SNA	ELISA		Total
	Positive	Negative	
Positive	$75^{(a)}$	$2^{(b)}$	59
Negative	$16^{(c)}$	$11^{(d)}$	27
Total	73	13	86

Calculation of sensitivity and specificity were according to Vecchio, (1966) and Cho and Bohac, (1985)

$$*\text{Sensitivity} = \frac{a}{(a+c)} = \frac{57}{73} = 78\%$$

$$*\text{Specificity} = \frac{d}{(b+d)} = \frac{11}{13} = 84.6\%$$

a = positive ELISA

b = No.of serum sample negative ELISA and +ve SNT

c = No. of serum sample positive ELISA and -ve SNT

d = No. of serum sample negative in both test

*Correlation $(a + b) / (a + b + c + d) = 68.6\%$

Table (4) and Figure (4) show the results of comparing the prepared ELISA with the commercially available ELISA kit and SNT. The results revealed minor difference between commercially used kit and the prepared on the same SNT titer. Field application of prepared ELISA on 354 serum samples for BHV-1 screening in different localities in Egypt indicates the prevalence percent of antibodies to BHV-1 in Behira (vaccinated) (95%); Sharkia (88%); Menofia (80%); moderate percent in Kafr Elsheikh (48%) and Fayoum (38%) (Table5). BHV-1 antibodies were screened in 2 different hosts indicate virus is circulating between cattle and buffalo as shown in Table (6).

Table (4): Results of comparison between the results of the prepared ELISA and commercial ELISA as well as SNT.

Code No. of serum samples	ELISA expressed as S/P Ratio	Commercial Kits	SNT
1	0.31 -ve	0.02 –ve	-ve
53	0.06 -ve	0.22 –ve	-ve
12	0.39 +ve	0.41 +ve	1/16
5	0.44 +ve	0.51 +ve	1/32
77	0.5 +ve	0.54 +ve	1/16
90	0.74 +ve	0.81 +ve	1/32
28	0.72 +ve	0.79 +ve	1/32
11	1.37 +ve	1.42 +ve	1/64
35	1.41 +ve	1.49 +ve	1/64
90	1.67 +ve	1.52 +ve	1/64
30	1.8 +ve	1.92 +ve	1/64
11	2.98 +ve	2.32 +ve	1/128
72	3.52 +ve	3.6 +ve	1/16*
19	3.65 +ve	3.75 +ve	1/128
129	3.46 +ve	3.54 +ve	1/16*

* These two samples are not representative due to great difference between results of the prepared ELISA, commercial ELISA and SNT.

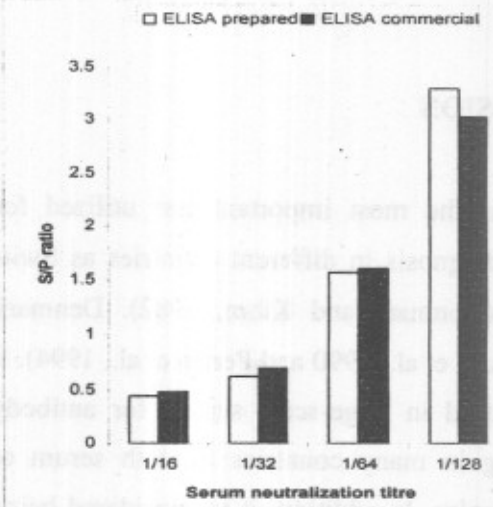

Fig.(4): Comparison between the results of the prepared ELISA and commercial ELISA.

Table(5): Results of ELISA screening of serum samples collected from different localities in Egypt for detection of BHV-1 antibodies.

	No.of sample	+ve	%of +ve	-ve	%of -ve
Behira	42	40	95%	2	5%
Menofia	42	34	80%	8	20%
Fyoum	90	35	38%	55	62%
Kafr ElSheikh	90	44	48%	46	52%
	90	80	88%	10	12%

Table (6): results of screening for BHV-1 antibodies in cattle and buffalo serum collected from different localities.

	Animal	No. of sample	Positive		Negative	
			No.	%.	No.	%.
El-Fayoum	Cattle	57	31	54%	23	84%
	Buffalo	33	12	36%	20	62%
Kafr Elsheikh	Cattle	62	39	62%	23	37%
	Buffalo	28	9	32%	19	68%

DISCUSSION

ELISA is the most important test utilized for BHV-1 diagnosis in different countries as Switzerland (Bommeli and Kihm, 1982). Denmark (Ackerman, et al., 1990 and Perrin et al., 1994). It is also used in large-scale survey for antibody screening in many countries in both serum or milk samples. In addition, it is considered being more reactor test than SNT. It is technically superior test for diagnosis of BHV-1 (Cho and Bohac, 1985). In Egypt, the first isolation of BHV-1 was reported by Fathyia, (1978). The virus reported in many provinces and could not subsided till now.

The aim of the present study is to develop an ELISA for detection of antibodies to BHV-1 to be applied on large-scale survey in Future studies.

A concentration method by polyethylene glycol 6000 which considered to be cheap, rapid and effective method was applied for antigen preparation as previously described in many studies in different serological test (Ognyanov and Khara, 1976; Sologub and shulyab, 1981 and Bolton, et al., 1981).

The correct standardization and optimization of ELISA is very important to be established and used when develop a new kit. Result in (Figure 1) indicates 5 μg/ml is the optimal antigen concentration to be used and the selected concentration was in accordance with others (Liauw and Evgster, 1986 and Herring et al., 1980). Serum dilution of 1/25 gave the best results as shown in Figure (2) similar to the dilution recommended by the manufacture of currently used commercial ELISA Kit (IDEXX check herd commercial Kits).

Serum neutralization test has been evaluated and confirmed to be the standard test to which other tests are compared (Van Orischot, 2000). 86 serum samples, which were tested in the prepared ELISA and classified according to S/P ratio into seven groups, and tested by SNT. As shown in table (1), it is concluded that in groups 2 and 3 with low S/P in ELISA, higher number of negative serum samples compared to other groups. Therefore, it is confirmed that the developed ELISA was able to detect lower antibody level than SNT.

Table (2) and Figure (3) summarized the overall comparison between positive serum samples in ELISA, SNT were 85% and 61%, respectively.

Such high percentage may be due to the fact that ELISA could detect both neutralizing and non-neutralizing antibodies. This variation was also reported in previous studies (Bolton, et al., 1981 and Collins, et al., 1985) .

Calculation of sensitivity, specificity and correlation of the prepared kit to SNT revealed 78%, 84% and 69%, respectively (Table3).

The result of prepared ELISA compared to commercial ELISA as well as SNT revealed minor difference between the prepared ELISA and commercial ELISA within the same SNT titer (Table 4 and Figure 4) indicating the success of the prepared kit.

The prepared ELISA was applied on 354-serum sample belonging to different localities for screening antibody to BHV-1. The results showed that high percent (95%) obtained in Behira Governorate confirms the effect of vaccine and vaccination program currently applied. In Menofia, Fayoum, Kafr El Sheikh and Sharkia governorates, the virus appear to be circulating in absent of vaccination policy (Table 5). The percent of positive results appeared to be parallel with previous report on the same Governorates (Hassanin, 1998). The obtained data in the present study indicates presence of BHV-1 antibody in both cattle and buffalo and it is highly recommended to include the buffalo in vaccination program (Table 6).

In conclusion, this study presents a locally developed ELISA kit for BHV-1 antibody detection. The test is simple, cheap and could be used for large-scale application. The successful evaluation of currently used vaccine and vaccination program using the prepared kit needs to be addressed. On other hand, screening of antibody to BHV-1 in different localities of Egypt will determine the actual prevalence percentage of circulating BHV-1.

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