PREPARATION OF PEROXIDASE CONJUGATED ANTISERUM AGAINST BOVINE EPHEMORAL FEVER VIRUS TO BE USED IN ELISA TEST

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ABSTRACT

The present study was designed to prepare bovine ephemeral fever antibodies conjugated with peroxidase for rapid detection of BEF virus. Five rabbits were immunized with BEF vaccine and other two rabbits were kept as control. Serum neutralization test revealed that the prepared serum had antibody titer of 1024. The immunoglobulin was precipitated using saturated ammonium sulphate where its value was found to be 2.5g/dl. The concentration of the globulin content was adjusted to be ,18mg/ml in normal saline then it was conjugated with horse radish peroxidase then evaluated through the application ELISA where it resulted in strong positive reactions up to a dilution of 1:2500 while the imported commercial conjugate reactions were up to 1:2000. Competitive ELISA was carried out on serum samples obtained from vaccinated cattle and revealed that the obtained values of optical density using the prepared and the imported conjugates were in approach.

INTRODUCTION

Bovine ephemeral fever (BEF) is an acute non-contagious arthropod born viral disease of cattle and water buffaloes *(Nandi and Negi, 1999)*. BEF is suddenly occurring epidemic, which sweeps through a herd causing fever, prostration, stiffness and lameness with occasional death and complication *(Hungerford, 1990)*. The causative agent is an arthropod born rhabdovirus, which has been classified as the type species of genus ephemerovirus *(Murphy, et. al.1999)*.

Attention was drawn to the situation of BEF in Egypt due to its economical losses focused on decreased milk and meat production and after the occurrence of several outbreaks, which were recorded by *Hassan, et al. (1991); Soheir (1994); Hassan (2000); Zaghawa, et al. (2000); Soad, et al. (2001); Daoud, et al. (2005) and Yonis, et al. (2005)*.

The diagnosis of BEF depends on isolation of the virus in culture of BHK-21 or Vero cells *(St.George, 1988)* or by the intracerebral inoculation of mice less than two day old *(Van Der Westhuizen, 1967)* and identification by a neutralization tests with known antiserum *(Tzipori, 1975)*.

With the world rapid changes toward GATT and Intellectual Property Rights; developing countries; like Egypt; must have their own tools and skills for rapid and accurate diagnosis of dangerous diseases which could destruct their national wealth like BEF.

Recently labeled reagent assays have come to play a major role in the diagnostic laboratory purposes. The enzyme linked immunosorbent assay (ELISA) always served as rapid turn around time and possibly lower costs for both detection of the virus and antibody *(Tijssen and Kurstak, 1984)*.

So the present study was designed to prepare peroxidase labeled antibodies against BEF virus to be used for rabid detection of BEF viral antigen by using the double antibody sandwich ELISA or its antibody by applying the competitive ELISA, as a local product saving the needed long time and high cost for importation of a native one.

MATERIALAND METHODES

1-Animal:

1.1. Rabbit:

Nine apparently healthy adult boscat rabbits of 3 Kg body weight were used for preparation of polyclonal antibody against BEF virus.

1.2. Cattle:

Seven crossbreed susceptible cattle of 1-1.5 year were screened using SNT and found to be free from BEF neutralizing antibodies. Five of them were vaccinated with the local inactivated BEF vaccine in a dose of 2ml S/C and a booster dose after two weeks, others two animals were kept as unvaccinated control. Serum samples were obtained weekly from these animals to monitor the level of BEF antibodies. All animals were kept under hygienic measures receiving balanced rations and adequate water.

2. BEF virus:

A local strain of bovine ephemeral fever virus "BEF-AVS 2001"*(Soad et al., 2001)* of a titer of $10^7 TCID_{50}$/ml. The Dept. of Pet. Animal Vaccine Research supplied it. It was used for preparation of BEF antigen in different cell cultures and in the brain of suckling baby mice as well as in serum neutralization test.

3.Cell culture:

Monolayer of baby hamster kidney (BHK) cell culture *(Macpherson and stocker, 1962)* and Vervet Monkey kidney (Vero) cells *(Yasumara and Kawatika, 1963)* were used for propagation of BEF virus.

4. Preparation of BEF hyper immune serum:

It was carried out according to *Edries, et al. (1999).*

5. Preparation of BEF antigen:

It was carried out according to *Brian and Hiller (1996)* from infected BHK cells.

6. Patent anti-BEF serum conjugated with peroxidase:

It was obtained from Sigma chemical company (USA) and used for comparative evaluation of the locally prepared peroxidase conjugated serum.

7.Chemicals and reagents:

Chemicals and reagents required for antibody peroxidase conjugation (Periodate method) were prepared according to the method described by *Tijssen and Kurstak (1984)* included the following:

7.1. Horseradish peroxidase:

Horseradish peroxidase product No.p-8375 type VI, lot number 25C-9510 was supplied by Sigma Chemical Company, Its activity was 365-purogallin unit/mg.

7.2. Ammonium sulphate $(NH_4)_2So_4$:

Hopkin and Williams LTD, Chad Well Health, Essex, England supplied it. It has a molecular weight 132.15 and used for precipitation of serum immunoglobulin in the prepared BEF hyper immune serum.

7.3. Sodium periodate ($NaIO_4$):

It was obtained as Sodium Meta periodate from Win Lab laboratory reagents for fine chemicals, its molecular weight is 213.84.

7.4.Soduim borohydrate ($NaBH_4$):

It was obtained from SD Fine Chemical LTD Scientific Company, Chemical Manufacturing Division Fair Lawn Jersey. Its molecular weight is 105.99.

8.Precipitation of immunoglobulin:

Globulins were precipitated according to the method described by *Peter (1969).* The globulin content was determined by the method described by *(Henry, 1974)* using Beckman DU7400 spectrophotometer. The conc. was adjusted to be 18mg/ml in normal saline.

9. Conjugation of anti-BEF globulin with Horseradish peroxidase:

The obtained globulins were labeled with peroxidase as described by *Tijssen and Kurstak (1984)* as follow:

1- 5mg of Horseradish peroxidase (HRPO) were dissolved in 1ml double distilled water followed by addition of 0.4ml of freshly prepared sodium

periodate solution with gently steering for 20 minutes at room temperature till a greenish color was obtained.

2-The pH was adjusted to 9.5 by adding 0.25ml of0.2Mol. $NaCo_3$.

3-18mg of BEF immunoglobulin in 1ml normal saline were added to such solution and placed on a shaker for 2 hours at room temperature.

4-0.1 ml of 4mg/ml Sodium borohydrate was added and the reaction was allowed to continue for 2hours at 4°c.

5- the prepared conjugate was dialyzed against phosphate buffer solution and an equal volume of glycerol was added to it then stored at –20°c till used.

10. Check board ELISA:

It was done for titration of the prepared immuneperoxidase conjugated BEF immune globulin and to detect the optimal antigen, antibody dilution for plate coating according to *Rose, et al. (1986).*

11. Serum neutralization test:

The quantitative method was carried out using the microtitre technique according to *Young and Spradbrow, (1990).*

12. Enzyme Linked Immunosorbant Assay:

According to *Voller, et al. (1982)*

a- The competitive method was applied to estimate the antibody in the sera of vaccinated animals.

b- The double antibody sandwich ELISA was applied to detect and estimate of the virus antigen.

RESULTS AND DISCUSION

BEF is a disease of economic importance and its rapid diagnosis is the first step to plan a suitable control program *(Nandi and Negi, 1999).* The serological diagnosis of BEF is based on the detection of a rise in neutralizing antibody titer between the acute phase and convalescent phase *(St.George, 1988).*

The traditional methods for serological diagnosis include neutralization test, complement fixation test, Fluorescent antibody technique and the ELISA, which is reported to be simple, and the preferred test *(Zakrzewski, et al. 1992). Schuurs and Van Weemer (1977)* mentioned that peroxidase labeled antibodies is designed to provide maximal assay signal and minimal background in immunoassays.

So the present work was aimed to prepare and evaluate an antibovine ephemeral fever polyclonal serum conjugated with horseradish peroxidase to be used in ELISA either to detect the presence of BEF virus or its antibodies.

The obtained results indicated that the developed antibodies specific to BEF virus in immunized rabbits had a titer of neutralizing antibodies of 1024, which could be considered a great value. The prepared hyperimmune serum

subjected to a series of precipitation with concentrated ammonium sulphate till complete removal of albumin content and the globulin reached its highest value 2.5 g/dl.

It was known that the conjugation with horseradish peroxidase using the periodate method did not result in an interference with the enzymatic or antibody activity of the serum as stated by *Tijssen and Kurstak (1984) and Zeidan, et al. (2000).*

The optimal dilution of the prepared peroxidase labeled antibodies was determined using check board ELISA and found to be 1:2500 as shown in the table (1).

In order to evaluate the efficacy of the prepared peroxidase labeled BEF antibodies, both the competitive method and the double antibody sandwich ELISA were carried out on the sera of vaccinated cattle to measure the antibody level or on the infected fluid obtained from infected Vero, BHK cells and baby mice brain to detect and measure the infectivity titer of the virus.

Table (2) showed that the differential effectiveness of the patent and local peroxidase labeled antibody on the detection of the BEF antigen in mice brain and VERO cells.

Table (3) & (4) showed the results of the neutralizing antibody test and the absorbance values of the double antibody sandwich ELISA applied on the sera of vaccinated and unvaccinated calves with the BEF vaccines .The results of these assays come to be appearing in a harmony with them and these results were similar to *Zakrewski, et al. (1992) and Azab, et al. (2003).*

As regard to this conjugate, it could be concluded that the local prepared peroxidase labeled BEF antibodies was available for use on request having the advantage of low cost for huge amount in addition to its high quality.

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Table (1): The optimal dilution of peroxidase labeled BEF antibodies.

Conjugate dilution	ELISA absorbance value with known BEF virus antigen	
	Positive	Negative
1:500	1.94	0.37
1:1000	1.81	0.36
1:1500	1.76	0.36
1:2000	1.69	0.34
1:2500	1.61	0.36
1:3000	1.54	0.35
1:3500	1.47	0.34
1:4000	1.42	0.36
PBS	0.35	0.34

Table (2): the double antibody sandwich ELISA applied on the infected fluid of Vero and BHK cells and in infected baby mice brain with the BEF virus.

The used samples	The absorbance value in the double antibody sandwich ELISA	
	Patent peroxidase labeled antibody	Local peroxidase labeled antibody
Infected Vero cell	3.36	3.1
Normal Vero cell	0.05	0.03
Infected BHK cell	3.9	3.64
Normal BHK cell	0.03	0.01
Infected baby mice brain	2.84	2.68
Normal baby mice brain	0.05	0.03
PBS	0.04	0.02

The reading was conducted at 495 nm

Table (3): The BEF neutralizing antibody titers in vaccinated calves.

Animal number	Neutralizing antibody titers*				
	1WPV**	2WPV	1WPB***	2WPB	3WPB
1	8	32	64	128	128
2	4	8	32	64	128
3	4	16	32	64	128
4	2	8	32	64	128
5	2	8	16	32	64
Unvaccinated control	0	0	0	0	0

*Antibody titer= the reciprocal of serum dilution which neutralized 100-200 $TCID_{50}$ of BEF virus.

**WPV= Week post vaccination. ***WPB= Week post booster vaccination

Table (4): Absorbance values of the competitive ELISA applied on the sera of the vaccinated calves.

Animal number	Absorbance values of the competitive ELISA									
	Patent peroxidase labeled antibody					Local peroxidase labeled antibody				
	1WPV*	2WPV	1WPB**	2WPB	3WPB	1WPV	2WPV	1WPB	2WPB	3WPB
1	1.77	1.42	1.14	0.61	0.54	1.32	1.03	0.76	0.34	0.21
2	1.83	1.79	1.36	1.09	0.79	1.44	1.32	0.99	0.81	0.35
3	1.81	1.63	1.45	1.11	0.65	1.47	1.21	1.08	0.89	0.39
4	1.86	1.77	1.39	1.03	0.71	1.51	1.29	1.02	0.74	0.28
5	1.94	1.74	1.56	1.34	0.92	1.54	1.26	1.18	0.95	0.68
Unvaccinated control	1.92	1.95	1.93	1.93	1.94	1.61	1.63	1.61	1.61	1.62

*WPV= Week post vaccination. **WPB= Week post booster vaccination

الملخص العربى

تحضير مصل مناعى ضد فيروس حمى الثلاثة أيام لاستخدامة فى اختبار الاليزا

نجلاء إبراهيم على

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تم تحضير مصل مناعى عالى العيارية ضد فيروس حمى الثلاثة أيام فى الأرانب البوسكات وقد أوضح أختبار المصل المتعادل أن هذا المصل ذو معيار 1024 وقد تم ترسيب الجلوبيولين المناعى فى هذا المصل باستخدام محلول سلفات الأمونيوم المشبع حيث وجد أن مقدار الجلوبيولين المترسب هو 2ر5 جم/دل فتم ضبط تركيزه ليكون 18 مجم/مل حيث تم إقرانه بالبيروكسيديز طبقا لطريقة بريوديت المعروفة بسهولتها ودقتها كما تم تحصين خمسة عجول خليط بجرعتين من لقاح حمى الثلاثة أيام المثبط المنتج محليا بفترة زمنية بينية اسبوعين مع ترك حيوانين كضوابط للتجربة0 أجرى أختبار المصل المتعادل على عينات أمصال جمعت من هذهالعجول أسبوعيا بعد التحصين (الجرعة الأولى والثانية) حيث أوضح الاختبار أن هذه الحيوانات قد أكتسبت أجساما مناعية نوعية لحمى الثلاثة أيام بمعيار يصل إلى 128 بالأسبوع الثالث بعد الجرعة الثانية وعند إجراء أختبارالإليزا باستخدام المقترن المحلى ومثيل له مستورد، تبين أن أنسب تخفيف للمقترن المستورد هو 1: 2000 بينما كان 1: 2500 للمقترن المحلى المحضر خلال هذا العمل وبإجراء أختبار الإليزا التنافسية على نفس عينات أمصال العجول المحصنة وجد أن متوسط قيم الأمتصاص الضوئى باستخدام المقترن المستورد مقاربة لمثيلاتها عند استخدام المقترن المحلى وعلى ذلك يمكن القول بأن لدينا الآن مستحضر محلى جيد يمكن استخدامه بفاعلية عالية لإجراء الفحوص السيرولوجية لحمى الثلاثة أيام.