TRIALS FOR PREPARATION AND EVALUATION OF PEROXIDASE AND FLUORESCENT ISOTHIOCYANATE CONJUGATED ANTISERA AGAINST EQUINE HERPESVIRUS-1

By

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SUMMARY

A preliminary study was conducted to prepare a polyclonal antibody against local strain of equine herpesvirus-1 (EHV-1), labeled with horseradish peroxidase and fluorescein isothiocyanate. The prepared antiserum in rabbit was conjugated by the most cheaper and easier method (periodate method). It was divided into two parts; the first one was conjugated with peroxidase which gave strong positive reaction at dilution of 1/2000. The second part was conjugated fluorescein isothiocyanate which give the strongest positive reaction at dilution of 1/4000. Both conjugates were succeeded to show its efficiency and liability for detecting EHV-1 when tested against field-samples within its test proper.

INTRODUCTION

Since the ancient centuries, Middle East specially Egypt was considered a centre of breeding, and exportation of pure Arabian horses allover the world. This constitutes additional support to the national income. For this reason, we must protect our valuable equine industry from dangerous diseases either endemic or epidemic ones.

Equine herpesvirus-1 (EHV-1) is the major viral infection in all ages of equines. EHV-1 is characterized by acute febrile respiratory manifestation in yearling horses, perinatal mortality, abortion in mares and myeloencephalitis in adult horses *(Allen and Bryans, 1986, OIE, 2000).* Abortion occurred spontaneously without any signs of illness and the featus is enclosed with its membranes.

The virus could be isolated from aborted faetus, placenta, nasal swabs and blood plasma *(Carrigan et al., 1991).* Under any stress, latent EHV may be reactivated in most cases resulting disease *(OIE, 2000).* From the above mentioned data, accurate and rapid diagnosis of both viral antigen and antibodies must be done to prevent the spread of the disease. Therefore, the aim of this work is to prepare peroxidase and fluorescein isothiocyanate labeled antibodies against EHV-1 to be used as in performing an easy and rapid method for detection of equine herpes virus antigen.

MATRIALS AND METHODS

I. Materials:

1. Virus:

Locally isolated viral strain of EHV-1 from cases of aborted fetuses (*Hassanein et al., 2002),* obtained from Equine Research Dept., Veterinary Serum and Vaccine Research Institute, Abbasia, Cairo.

2. EHV-1 antigen:

It was prepared according to *Safaa et al. (2005*). It was used for detection of IgG antibody using ELISA technique after making identity test using reference antisera.

3. Embryonated chicken eggs (ECE):

specific pathogen free (SPF), 11-13 day old ECE was inoculated via chorioallantoic membrane CAM with locally isolated strain of EHV-1. It was used for virus propagation and preparation of viral antigen *(Hassanein et al., 2002).*

4. Rabbits:

Three apparently healthy adult male New Zealand white rabbits of 4-6 months old were used for preparation of hyperimmune serum against EHV-1. One rabbit was served as control.

5. Biological materials:

Anti- EHV-1 rabbit serum was prepared in Equine Research Dept., Veterinary Serum and Vaccine Research Institute, Abbasia, Cairo.

*** Reference antisera against EHV-1:**

Freeze-dried rabbit anti EHV-1 serum was kindly supplied by **Dr. Jannet Wellington,** Res. Flow, Dept. of Bio. Sci., Macquarie Univ., NSW, Australia. It was used for identification of the locally isolated strain of EHV-1.

*** Patent anti- EHV-1 conjugated with both peroxidase and fluorescein isothiocyanate:**

It was obtained from R & D Systems (UK). It was used for comparative evaluation of locally prepared peroxidase and fluorescein conjugated serum.

II. Methods:

1. Preparation of polyclonal antibodies against EHV-1:

It was prepared according to the method described by **Dutta et al. (1983) and Safaa et al. (2005)**. The ultracentrifuged purified EHV-1 with infectivity titre (8.5 log10/0.2ml) was inactivated at 56°C for 30 minutes and mixed with DEAE-Dextran solution 100 mg/ml (v/v) as adjuvant. 2 ml of the mixture was injected subcutaneously at a multiple sits in each rabbit. They received three injections one week apart. Finally, they were bled from jugular vein 10 days after the last injection. The serum was obtained. Anti- EHV-1 immunoglobulins content were evaluated by using SNT and AGPT.

2. Precipitation of immunoglobulin against EHV-1:

Precipitation of globulin was applied according to the method described by *Brown and Pietz (1977)* by using 70% saturated ammonium sulphate solution. The sulphate was removed by dialysis against 0.15 M NaCl. The globulin concentration was determined by Biuret method *(Peter, 1969)*, using Beckman DU 7400 Spectrophotometer. The concentration was adjusted to 20 µg/ml in 0.01 M $NaCO_3$ for conjugation.

3. Conjugation of anti- EHV-1 globulins with horse radish peroxidase:

The conjugation was performed according to *Tijssen and Kurstak (1984) and Wisdom (2002).*

Purification was made by gel filtration on a column of sepharose CL-6B in PBS. Highly purified peroxidase has a RZ (absorbance ratio 403 and fl 280) ranged from 0.4 to 3.

4. Standardization of the prepared horse radish peroxidase conjugate:

It was done according to *Hudson and Hay (1989).* The highest dilution of conjugate which give observable colour could be used in the test proper.

5. Direct ELISA test:

It was done according to the method of *Dutta et al. (1983).*

6. Conjugation of anti- EHV-1 immunoglobulins with fluorescein isothiocyanate:

It was conducted according to *Hudson and Hay (1989)*.The conjugated protein was separated from the free fluorescein by passing the mixture in G-25 sephadex column equilibrated with PBS or dialyzed against PBS at 4°C for 3 days with changing twice daily.

7. Standardization of the prepared conjugate:

It was done according to *Peter (1969).* Two fold dilutions of the conjugate with PBS were made. Then from each dilution, one drop of the conjugate was added to each fixed slide with locally isolated strain of EHV-1 then were incubated at 37°C for 1 hour. Wash the slides with PBS 3 cycles with 10 minutes for each one and then add drop of glycerin buffer and examine under the microscope to detect the highest dilution of conjugate which gave suitable reading could be used in the test proper (i.e. dilution which gave a high specific fluorescence with low background).

8. Application of the prepared fluorescence isothiocyanate conjugate:

It was done according to *Weiland and DiniTroides (1970) and Gunn (1992).* Infected chorioallantoic membrane (CAM) section, liver-lung of both mice and aborted feotus suspensions smears were examined.

Fixation and staining of infected samples:

Infected CAM sections and smears with locally isolated strain of EHV-1 were fixed on slides. After air dryness, fixation was performed in acetone for 20 minutes at 4°C. After dryness, the slides were dipped into phosphate-buffered saline, dried with filter paper. Then it flooded with the proper dilution of fluorescein conjugated antibodies against EHV-1.

Staining took a time varied from 10 minutes up to 24 hours at room temperature. After staining the CAM was washed twice with PBS, 10 minutes each time then mounted in glycerol buffer. Uninfected negative control CAM was subjected to the same procedure.

RESULTS

Hyperimmune sera of rabbits against locally isolated strain of EHV-1 were evaluated by passive haemagglutination, serum neutralization test and agar gel precipitation test as shown in table (1). The precipitated globulin of the prepared hyperimmune serum after complete removal of albumin was 7.05 gm/dl.

DISCUSSION

Serological diagnosis of equine herpesvirus is important specially in case of the presence of paired serum samples in acute and convalescent stage. Traditional serological methods are complement fixation (CF), neutralization test (SNT) *(Stokes et al., 1989)*, passive haemagglutination (PHA) *(Frymus, 1978)*, enzyme linked immunosorbent assay (ELISA) *(Dutta et al., 1983)* and fluorescent antibody technique (FA) *(Magda et al., 2004)* are used for EHV diagnosis.

Direct FA technique is sensitive and rapid test for detection of EHV. It could be detected at 8 hours post infection (PI) and gives the peak at 18 hours *(Weiland and Dini-Troides, 1970, Magda et al., 2004)*.

Coupling of fluorescent dyes with antibodies occurred without impairment their activity. It can be served as specific and sensitive method for detection of both cell surface and intracellular antigens *(Luciana, 1979)*.

Labeled antibodies provided one of the most in dispensable tools for localization of antigen in blood smears, tissue sections, microbial and paracytological preparation *(Nowotny, 1979)*.

ELISA test expressed itself as rapid sensitive and specific which has been widely used to detect viral antigen and antibodies in clinically or subclinically infected equines *(Dutta et al., 1983 and Drummer et al., 1995)*.

In the present work, peroxidase as well as fluorescein labeled EHV-1 antibodies were successfully prepared. The peroxidase conjugate was evaluated for their effectiveness that could be diluted 1/2000 to produce positive results (Table 2). The conjugate was compared with an imported one (Table 3), that previously proved to be positive to EHV-1 by SNT and ELISA technique *(Dutta et al., 1983, Mumford and Bates, 1984)*.

Fluorescein isothiocyanate labeled antibodies against EHV-1 was titrated for its efficacy in different samples and stained sections of CAM that give clear green fluorescence in dilution 1/4000. Moreover, this conjugate was compared with patent one that previously proved to be positive to EHV-1 (Table 4) *(Magda et al., 2004)*.

FA test is highly sensitive and rapid test that could be used to detect several viruses *(Gamal El-Din et al., 1999, Abd El-Aty et al., 2001, El-Kabbany and Zaki, 2004)*.

In conclusion, the locally prepared peroxidase labeled antibodies against EHV-1 could be successfully for detection of EHV-1 infection during epidemics. Also, the locally prepared fluorescein conjugated antiserum against EHV-1 is a rapid, easy, economic and accurate diagnostic reagent that could be used in direct FA test.

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Table (1): Mean neutralizing, passive haemagglutinating (PHA) and precipitated antibodies titre against EHV-1 in rabbit

Serological investigation	Weeks post immunization			
	3	4	5	Control negative
Neutralization test (log_{10})	0.9	1.6	1.8	-
PHA test *	64	128	512	-
AGPT **	+	++	+++	-

* Reciprocal of serum dilution of passive haemagglutinating antibodies.
** AGPT: Agar Gel Precipitation Test.

Table (2): Titration of the prepared peroxidase conjugated EHV-1 antibodies

Conjugate dilution	Results	
	Positive	Negative
1:400	0.980	0.066
1:800	0.960	0.065
1:1000	0.780	0.067
1:1200	0.690	0.068
1:1400	0.550	0.069
1:1600	0.500	0.065
1:1800	0.450	0.066
1:2000	0.330	0.068
1:2300	0.250	0.066
1:2500	0.199	0.065

- The reading was conducted at 495 nm. This reading represents the single OD without any interference

Table (3): Detection of equine herpesvirus using locally prepared peroxidase conjugated antibodies (against local isolate 2002)

Samples	Locally peroxidase labeled antibody	Patent peroxidase labeled antibody
Lung and liver of aborted foetus	1.065 *	0.99
Lung and liver of infected mice	1.024	1.50
Infected chorrioallantoic membrane (CAM)	1.080	1.11
Infected tissue culture	0.95	0.99
Known positive sample	1.070	1.08
Negative sample	0.066	0.067

* The reading was conducted at 495 nm and represents the single OD without any interference.

Table (4): Evaluation of fluorescein isothiocyanate conjugate labeled antibodies against locally isolated strain of EHV-1 in comparison with patent one

Samples	Conjugate dilution													
	Locally							Patent						
	1/500	1/1000	1/2000	1/4000	1/8000	1/10000	1/120000	1/500	1/1000	1/2000	1/4000	1/8000	1/10000	1/12000
Infected chorioallantoic membrane suspension	++++	++++	+++	+++	++	++	-	++++	++++	++++	+++	+	-	
Infected mouse lung and liver suspension	+++	+++	+++	++	++	++	-	+++	+++	+++	+++	+++	-	
Fixed section of infected CAM	+++	+++	+++	+++	++	+	-	+++	+++	+++	++	++	+	-
Negative control samples	-	-	-	-	-	-	-	-	-	-	-	-	-	-

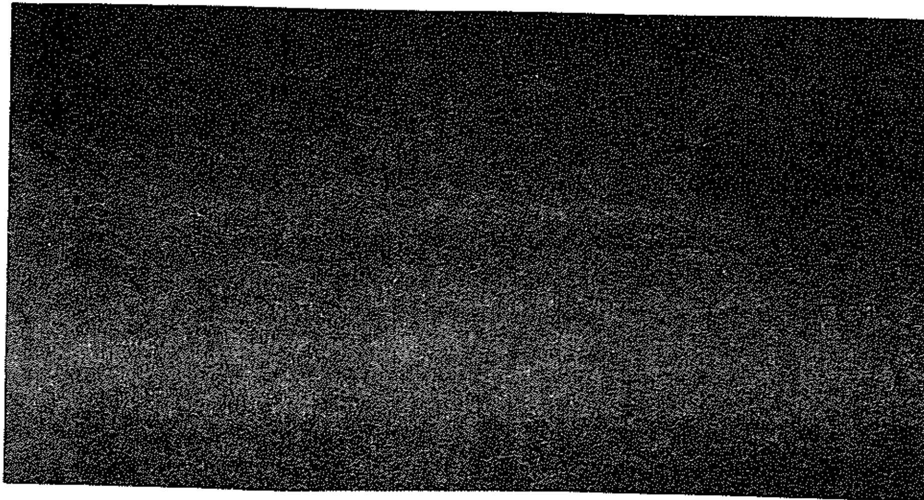

Photo (1): Direct FA technique for detecting EHV-1 in chorioallantoic membrane (CAM) using the prepared fluorescein conjugate in a dilution 1/1000 (40x)

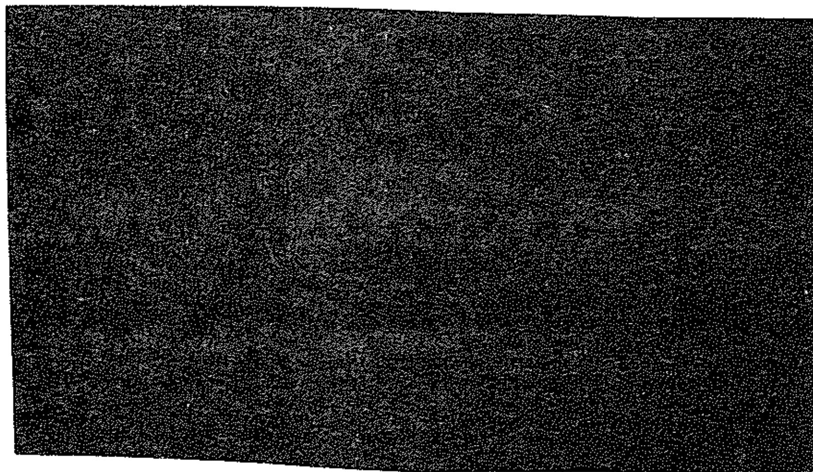

Photo (2): Detection of EHV-1 in liver-lung suspension of infected mice using the prepared fluorescein conjugate (40x)

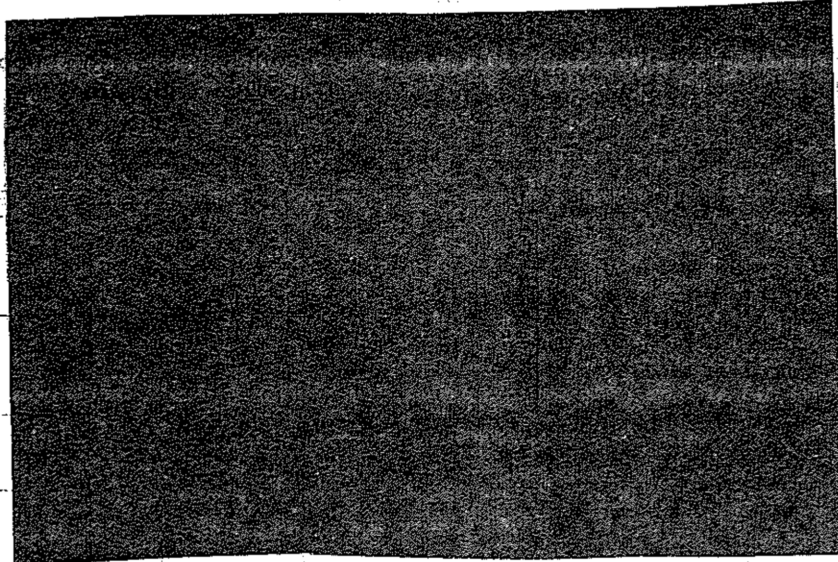

Photo (3): Normal sheet of non-infected CAM with faint greenish illumination (40x)

الملخص العربى

محاولات لتحضير وتقييم مصل كشاف بالبيوكسيديز وصبغة الفلورسنت لفيروس الهربس

صفاء عبد المنعم و نشوى كمال وإبراهيم سليمان

معهد بحوث الأمصال واللقاحات الحيوية البيطرية – القاهرة

أجريت دراسة مبدئية لتحضير سيرم مناعى فى الأرانب ضد العترة المحلية لفيروس الإجهاض المعدى للخيول المقترن بالبيروكسيداز والفلورسين بأسهل وأرخص الطرق (البيرايوديت). وقد تم ترسيب الجلوبيولين باستخدام كبريتات الأمونيوم ووجد أنه 7.05 جم/سم سيرم، قسم الى قسمين، الول أقترن بالبيروكسيداز وأعطى أفضل قوة عيارية عند تخفيف 2000/1 بينما أقترن القسم الثانى بالفلورسين وكانت أفضل قوة عيارية عند 1000/1. وقد استخدمت هذه القوة العيارية لكل منهما لاختبار عينات مختلفة مصابة بالفيروس باختبار الاليزا والفلورسين واثبتت النتائج حساسية وخصوصية المنتج لتشخيص فيروس الإجهاض المعدى فى الخيول.