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TRIALS FOR PREPARATION AND EVALUATION OF PEROXIDASE AND FLUORESCENT ISOTHIOCYANATE CONJUGATED ANTISERA AGAINST EQUINE INFLUENZA VIRUS

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SUMMARY

The present study was designed to produce anti-influenza antibodies conjugated by peroxidase and fluorescein isothiocyanate separately for rapid detection of equine influenza (EI) virus. Five rabbits were immunized with equine influenza (EI) virus (A/equi/2) and one was kept as control. Immunoglobulins were separated using concentrated ammonium sulphate, the globulin content was quantitated and it was 2.5 gm/dl. These globulins were divided into 2 portions, the 1st one was conjugated with peroxides and gave a strong positive result when diluted 1:2000. The 2nd portion was conjugated with fluorescein isothiocyanate and gave a strong positive result when diluted 1:20. Both conjugates were tested and compared with an imported one on allantoic fluids previously proved to be positive to equine influenza and gave a strong positive reaction. The two prepared labeled antibodies could be used successfully in detection of influenza antigen during the course of the disease.

INTRODUCTION

Equine influenza (EI) is a seasonally highly contagious acute respiratory viral disease of equidae that affected all ages and both sexes (Uppal et al., 1989).

EI virus is a member of family orthomyxoviridae, type A (WHO, 1959), which comprises two subtypes represented by reference strains A1/Equi/Prague/1/56 (Sovinova et al., 1958) and A/equi-2/Miami/1/63 (Waddell et al., 1963) H3N8 (WHO, 1979).

For isolation and identification of EI virus inoculation and propagation in 9-11 days embryonated

chicken eggs (ECE) through allantoic route was done (OIE, 2000). Enzyme linked immunosorbent assay (Elisa) (Vilaroza et al., 2000) and fluorescent antibody techniques (FA) (Anestad and Maagaard, 1990) were applied on allantoic fluid for identification of the isolate. Elisa always served as rapid turn around time and possibly lower costs for both detection of the virus and antibody (Tijssen and Kurstak, 1984).

FA techniques have gained wide acceptance in emergency diagnosis as the cellular localization of viral antigen could be determined at the high microscope level (Watson and Coons, 1954, Spendlove et al., 1963). Various methods are available for linking antibody molecules to enzymes. The enzymes which are used frequently as traces localization studies are horseradish peroxidase and alkaline phosphatase. Both the enzymes are sufficiently able and yield coloured products upon activity which can be visualized even under a light microscope. The following coupling methods are discussed with horseradish peroxidase. The prepared labelled antibody could be used successfully in detection of equine influenza antigen (Schuurs et al., 1977).

The present study was designed to prepare peroxidase and fluorescein isothiocyanate labeled antibodies against equine influenza virus to be used for rapid detection of equine influenza viral antigen.

MATERIAL AND METHODS

MATERIAL:

1. Rabbits:

Five apparently healthy adult male New Zealand white rabbits of 4-6 months old were used for preparation of hyperimmune serum against equine influenza virus type (A/equi/2), one rabbit served as control.

2. Preparation of virus fluid:

It was prepared as described by OIE (2000). Briefly, the freeze dried equine influenza virus (EP10) A/equi-2 which was locally isolated by Hamoda et al. (2001) was propagated for 3 successive passages on embryonated chicken eggs (ECE) 9-11 days old through amnioallantoic route. The virus fluid was harvested after 3 days and centrifuged at 1500 rpm for 15 minutes. The supernatant fluid was carefully collected and unacyn 1500 IU added (0.5ml/2ml virus fluid) stored in liquid nitrogen as seed virus.

3. Identity test:

The identity of EI virus fluid (EP13) A-equi-2 which was locally isolated by Hamoda et al. (2001) confirmed by HI test using reference antisera against equine influenza subtype-2, which was kindly supplied from Dept. of Virology, Faculty of Veterinary Medicine, Cairo University.

4. Patent anti-influenza virus conjugated with peroxidase:

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

5. Patent anti-influenza virus conjugated with fluorescein isothiocyanate:

It was obtained from Sigma Chemical Company (USA). It was used for comparative evaluation of the locally prepared fluorescein conjugated serum.

6. Chemicals:

A. Chemicals for peroxidase conjugation (Periodate method):

Horseradish peroxidase:

Horseradish peroxidase product Batch No. P39033 was obtained from BDH Chemicals LTD, Poole, England. Its activity was 60 sumner purpurogallin unit per mg or 320 EU per mg.

Sodium periodate ($NaOI_4$):

Sodium periodate GRG (Sodium meta periodate), obtained from Winlab Laboratory Chemicals Reagents (Fine Chemicals). Its molecular weight is 213-84.

Sodium borohydride ($NaBH_4$):

It was obtained from SD Fine Chemical LTD Scientific Company, Chemical Manufacturing Division Fair Lawn Jersey. Its molecular weight is 105,99.

Ammonium sulphate ($NaH_44)_2SO_4$:

Ammonium sulphate analar was obtained from Hopkin and Williams LTD Chadwell Health, ESSEX, England. Its molecular weight is 132.15.

B. Chemical reagents for fluorescein isothiocyanate (FITC) conjugate:

Fluorescein isothiocyanate:

Fluorescein isothiocyanate product Batch No. 44041 was obtained from BDH Biochemicals LTD, Poole, England. Its purity was not less than 80%.

C. Chemical reagents for Elisa test:

Chemical reagents for Elisa were prepared as described by Watanabe et al. (1980).

D. Chemical for fluorescein conjugation:

Chemical reagents for conjugation were prepared according to Hudson and Hay (1989).

METHODS:

1. Preparation of polyclonal antibodies against equine influenza virus:

Polyclonal antibodies against equine influenza virus type 2 (A equi 2) were prepared in rabbits according to the method described by Watanabe et al. (1980) and Mackenzie et al. (1975). Each rabbit was inoculated S/C by 1ml ECE fluid (log10 EID50/0.2ml) mixed well with complete Freund's adjuvant. Boostering of these rabbits was done weekly for further 3

weeks with emulsion contained incomplete adjuvant. Ten days after last injection the rabbits were bled from the jugular vein. The collected blood was allowed to clot and the serum was separated. The anti-influenza immunoglobulins content were evaluated using HI (Swenson, 1992).

2. Precipitation of immunoglobulin against influenza virus:

Precipitation of immunoglobulin was performed according to the method described by Peter (1969). The globulin content was determined by the method described by Henry (1974) using Bechman DU 7400 Spectrophotometer. The concentration was adjusted to 2.5 gm/ml in 0.01 M $NaCO_3$ for conjugation.

3. Conjugation of anti-influenza globulin with horseradish peroxidase:

The conjugation was conducted using the periodate method that described by Tijssen and Kurstak (1984).

Standardization of the prepared horseradish peroxidase conjugate:

It was done according to Hudson and Hay (1989).

a. Make 2-fold dilutions of the conjugate using phosphate buffered saline pH 7.6 with 0.05 tween 20.
b. Add 0.05 ml of each dilution to a series of wells in a microtitre plate (Linbro).
c. As a control, add 0.05 ml of PBS pH 7.6 with 0.05% tween 20 to a series of wells beside the wells containing each dilution of conjugate.
d. Add 0.01 ml of the substrate solution to each well containing the conjugate dilutions and PBS.
e. Observe well for color change.
f. The last dilution of conjugate which gave observable colour could be used in the test proper.

5. Direct Elisa technique:

The test was conducted according to the method of Denyer et al. (1984).

6. Conjugation of anti-influenza immunoglobulin with fluorescein isothiocyanate:

It was done according to Hudson and Hay (1989).

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 influenza virus. Then was incubated at 37°C for 1 hour. Wash the slides with PBS and then add drop of glycerin buffer and examine under the microscope to detect the last 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 isothiocynate conjugate:

It was done according to the method described by Johnson et al. (1978). In this procedure, the viral antibody was conjugated to FITC and

served as a highly specific stain that combined with antigens in virus infected fluids (allantoic fluids).

a. Fixation of infected allantoic fluids:

The cover glass was rinsed in tris-buffered saline, then drained and air-dried. Fixation was performed in acetone for 5 minutes at room temperature. After drying, the cover glass was dipped into tris-buffered saline. Excess fluid was wiped off the back with absorbent paper and the cells became ready for staining.

b. Staining:

The cover glass was placed in humid chambers and the cell sheet was flooded with a drop of fluorescent antibody. Staining was accomplished in as little as 10 minutes, but in some cases extensive staining period up to 24 hours has proved advantageous. Staining was carried out at room temperature or at 37°C. After staining, the cells were washed twice, 10 minutes each time, with PBS and mounted in buffered glycerol. Control samples were included using non-infected allantoic fluid.

RESULTS

Developed antibodies specific to equine influenza virus (A/equi-2/Cairo) in immunized rabbits were evaluated by haemagglutination inhibition test (HI) and the result is represented in table (1). It revealed that the highest antibody titre was 1024. After 10 days from injection, the hyperimmune sera subjected to a series of precipitation with concentrated ammonium sulphate till complete removal of albumin content as the globulin reaching the highest value 2.5 gm/dl. The hyperimmune sera were divided into two portions, the first one was conjugated with peroxidase and the 2nd portion was conjugated with fluorescence isothiocyanate.

The optimal dilution of peroxidase labeled antibodies was determined using checkerboard Elisa method and the results are represented in table (2).

It was clear that the optimal dilution to be used is 1:2000. In order to evaluate the efficacy of the locally prepared peroxidase labeled antibodies, it was applied on infected allantoic fluids with influenza virus and the results were tabulated in table (3).

Concerning to fluoresceince isothiocyanate conjugate it was found that the highest positive dilution of the prepared conjugate was 1/20, 1/30, but at dilution of 1/20, this dilution gave strong positive when used with allantoic fluid infected by influenza virus (Photo 1 and Table 4).

Table (1): Preparation of rabbit immune serum against locally identified equine influenza virus (A/equi1/Cairo-2/2000)

Animal No.	Isolate	Passage	Infectivity titre	Volume/ dose	Immuno-stimulant	Route of inoculation	HI titre	Time of sample collection
1 2 3 4 5 6	A.equi2/Cairo/2000	EP10	9.5	1	Freund's (complete and incomplete	S/C	1024 Control	10 days after last inoculation

EP: Egg Passage.

- Infectivity titre $\log_{10}$ EID_{50}/0.2 ml
- HI titre expressed by HI unit.

Table (2): Optimal dilution of peroxidase labeled influenza antibodies as tested by Elisa

Conjugate dilution	Absorbance value with known influenza antigen		
	Positive		Negative
	1/50	1/100	
1:500	0.950 *	0.912 *	0.110
1:750	0.911	0.864	0.134
1:1000	0.898	0.812	0.150
1:1250	0.805	0.796	0.144
1:1500	0.794	0.766	0.134
1:1750	0.794	0.750	0.113
1:2000	0.794	0.741	0.121
1:2250	0.711	0.688	0.121
1:2500	0.671	0.650	0.111
1:2750	0.598	0.566	0.121
1:3000	0.512	0.494	0.120

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

Table (3): Results of enzyme linked immunosorbent assay for detection of equine influenza antigen on allantoic fluids using locally prepared peroxidase conjugated antibodies (A/equi2/Cairo-2/2000)

Samples	Patent peroxidase labeled antibody	Local peroxidase labeled antibody
1	1.04 *	0.99 *
2	1.22	1.50
3	1.10	1.00
4	1.99	1.61
5	1.80	1.29

* The reading was conducted at 495 nm.

Table (4): Evaluation of fluorescene isothiocyanate conjugate labeled antibodies against influenza virus (A/equi2/Cairo-2/2000) in comparison with patent one

Samples	Conjugate dilution											
	Patent						Locally prepared					
	1/10	1/20	1/30	1/40	1/50	1/60	1/10	1/20	1/30	1/40	1/50	1/60
* Infected allantoic fluid by A/equi2/Cairo 2/2000	+++	+++	++	+	-	-	+++	+++	++	+	±	
* Control samples	-	-	-	-	-	-	-	-	-	-	-	-

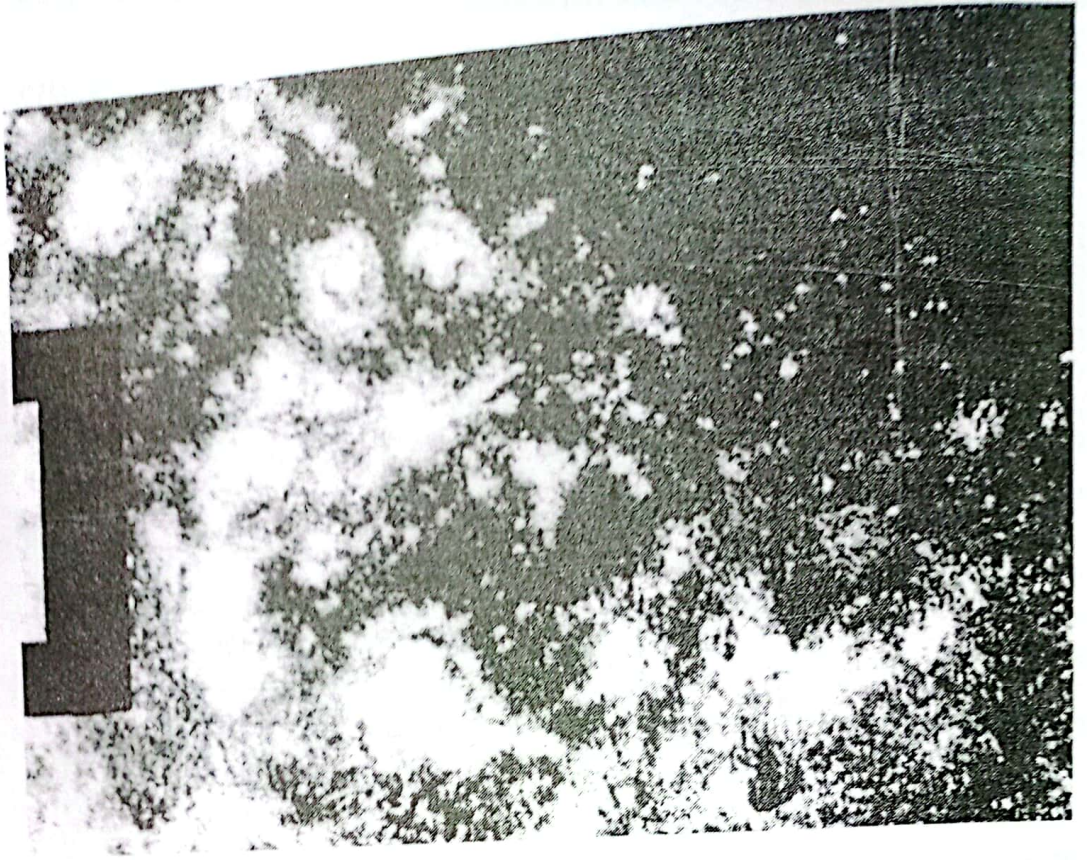

Photo (1): Direct fluorescent staining technique for detecting influenza virus in allantoic fluid using prepared fluoroscein conjugate of dilution end point 1/20 (40X)

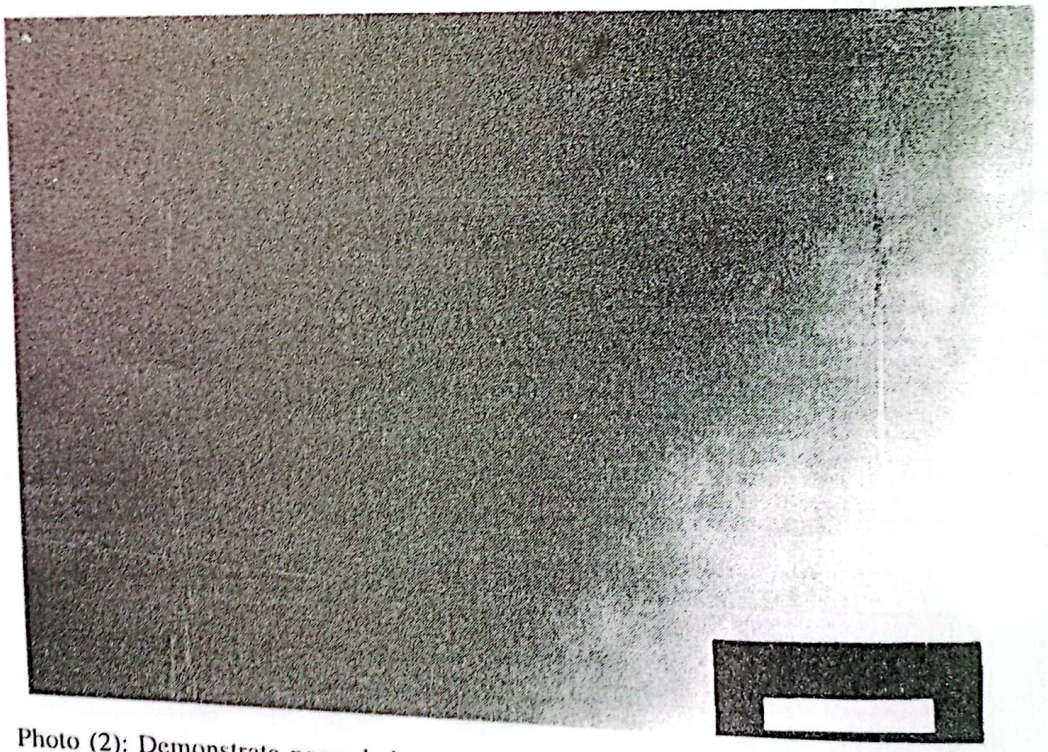

Photo (2): Demonstrate normal sheet of non-infected allantoic fluid, with very faint greenish illumination (40X).

DISCUSSION

Serological diagnosis of EI is based on demonstrating a four fold or greater increase in antibody between acute phase and convalescent phase. Thus, it requires collection of a pair of serum samples. The traditional methods for serological diagnosis are HI, complement fixation (CF) and neutralization.

The most recent addition to serological tests for EI diagnosis is the enzyme immunoassay and FA techniques (Denyer et al., 1984).

Elisa is more sensitive than CF and as sensitive as HI tests (Marphy et al., 1981). Direct FA technique could be used for rapid detection of EI virus as early as 24 hours post inoculation or infection (Kumanomido et al., 1972).

In the present work, a local peroxidase labeled influenza antibodies as well as a locally fluorescein labeled influenza antibodies were successfully prepared for the 1st time in African Horse Sickness (AHS) Department. The two conjugates were evaluated for their effectiveness as the peroxidase conjugate could be diluted into 1:2000 to give positive results. Moreover, this conjugate was comparable to an imported one on allantoic fluids that previously proved to be positive for influenza virus by HI technique and gave good results.

The haemagglutinin of influenza A particles, which is responsible for virus attachment to susceptible cells and for the ability of the virus to agglutinate erythrocytes, contains multiple antigenic determinants (Gerhard and Webster, 1978). Some of which are common to all viruses of the same antigenic drift series (HA-CM). Whereas others are specific to each individual strain (HA-ST) (Laver et al., 1974). However, Elisa was reported that 100-fold more sensitive than either HI or neutralization for measuring antibodies (Watanabe et al., 1979). Furthermore, anti-HA-ST antibodies were not detected by HI at the antibody concentrations employed, but they were readily assayed by Elisa.

Immunofluorescence entered cell biology as early as 1940 (Luciana, 1979), described the possibility of coupling fluorescent dyes to immunoglobulins molecules without impairment of antibody activity. He also proved that the use of such fluorescent reagents was sensitive and specific for detection of both cell surface and intracellular antigens, as well as soluble antigens in animal tissues.

Labeled antibodies provided one of the most indispensable tools for the localization of antigen in blood smears, in tissue sections, in microbial or paracytological preparations (Nowotmy, 1979).

Fluorescein isothiocyanate labeled antibodies against EI virus was also tested and titrated for its

effectiveness as it can be diluted into 1/20 to give clear positive plateau. Also this conjugate was compared with patent one on allantoic fluid previously proved to be positive to influenza and give good results. It is clear from our results decided in table (4) and Photo (1) that direct FA test is highly sensitive and rapid test that could detect influenza antigen in infected tissue as early as 6 hours of inoculation of allantoic sac. These results are in agreement with results obtained by (Tizard, 1996, Gamal El-Din et al., 1999 and Abdel Aty et al., 2001).

In conclusion, the locally prepared peroxidase labeled influenza antibodies could be successfully used for detection of influenza infection during epidemics. Also, the locally prepared fluorescein conjugated antiserum against equine influenza virus (A/equi-2/Cairo 2000) is rapid, easy, accurate and economic diagnostic reagent that can be used in direct FA technique for identification and detection of influenza antigen in samples collected from infected equines. Finally, both locally prepared conjugates may be used as rapid field diagnostic reagents for influenza antigen detection without using indirect FA, and Elisa and other complicated serological techniques.

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