

TRIAL FOR PRODUCTION OF DIAGNOSTIC KIT FOR DIAGNOSIS OF EQUINE VIRAL DISEASES

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Abstract

A trial for production of diagnostic kit for diagnosis of equine viral disease was done. The antibodies were raised in white New-Zealand rabbits. The conjugation was achieved through application of the most popular, easier and cheaper method (Periodate method). The optimal dilution of the conjugate was determined by using of known AHSV antigen together with negative one. The conjugate was used in solid phase ELISA on 50 horse sera.

INTRODUCTION

Egypt was well known as one of the most famous countries in breeding and exportation of pure generations of Arabian horses to United States of America and many European countries.

From economical point of view, this particular field of selecting, rearing and trading pure Arabian horses constitutes a remarkable additional support to the national income. For this reason, it becomes a matter of importance to discover the equine viral infectious diseases in Egypt as they play an important role on the efficiency of breeding process.

African horsesickness (AHS) is still an important viral disease of equine as it causes a high mortality in horses (OIE, 1989). In Egypt, the last reported outbreak was in 1971 in Edfu and Aswan where AHSV type (9) was isolated and identified (Farid *et al.*, 1981). Vaccination programmes have been stopped all over the governorates since 1993.

Equine Herpes Virus (EHV) is a common cause of respiratory illness in young horses. Moreover, it causes abortion in mares, consequently, causing a high economic

losses (Campbell and Studdert, 1983). In Egypt, the virus was isolated for the first time by Hassanein *et al.*, 1999).

Equine Influenza (EI), is a highly contagious acute respiratory illness caused by orthomyxovirus of RNA viruses. The disease has a wide geographic distribution (Kastner *et al.*, 1999). The virus was first isolated in Egypt in 1990 by Ayoub *et al.* (1990). Last isolation was conducted by Hamoda *et al.* (2001) from Kaleobia province.

From the aforementioned data about the equine viral diseases, it is clear that rapid diagnostic methods for detection of viral antigens and antibodies are so important. These diagnostic methods, including solid phase ELISA and immunoperoxidase techniques, require antihorse radish peroxidase conjugate which cost a lot and take a lot of time till any company offered it. This work aims to save money and time by preparing a locally antihorse conjugate with peroxidase.

MATERIALS AND METHODS

Materials

1. Animals

a. Horse

One apparently healthy horse of 3-5 years old was used for obtaining horse serum.

b. Rabbits

Six apparently healthy male New-Zealand white rabbits of 6-8 months of age were used for preparation of polyclonal antibodies against horse serum. The average weight of each was 3-5 kilograms. They were kept under observation in good hygienic measures and supplied with ration containing about 17% protein.

2. Virus

African horsesickness virus (type 9), (MO₁₀₃ MS₃VE₃) was kindly supplied from Plam Island. This strains was passaged for three times on Vero cells in AHS Unit at Veterinary Serum and Vaccine Research Institute, Abbasia.

3. Tissue culture

Monolayer of Green Monkey Kidney cell (Vero) cells were obtained from Rasi Institute, Iran in 1968. The Vero cells were grown and maintained as described by Ozawa (1967).

4. AHS antigen

It was prepared according to the method described by House *et al.* (1990) for IgG antibody detecting using ELISA technique.

5. Sera and antisera

Horse sera were subjected for ELISA technique using either the locally prepared conjugate (antihorse) and patent prepared one for comparative study.

Horse antisera against type (9).

6. Chemical and reagents

A. Chemical for ELISA

Chemical reagents required for ELISA were prepared as described by House *et al.* (1990).

B. Chemicals and reagents for peroxidase conjugation (periodate method)

i. Horseradish peroxidase

Product No. P-8375 peroxidase (Horseradish), type VI Lot No. 25C-9570 was obtained from Sigma Chemical Company. Its activity was 365 purpurogallin unit per mg solid.

ii. Sodium periodate (NaIO_4)

Sodium periodate GRG (Sodium meta periodate), obtained from Winlab Laboratory Chemical Reagents Fine Chemicals. Its molecular weight is 213.84.

iii. Sodium borohydride (NaBH_4)

It was obtained from S.D. Fine Chemical Ltd. Scientific Company, Chemical manufacturing Division Fair Lawn, New Jersey. Its molecular weight is 105.99.

iv. Ammonium sulphate $(\text{NH}_4)_2\text{SO}_4$

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

Methods

1. Preparation of polyclonal antibodies against AHSV

Polyclonal AHS hyperimmune sera were prepared according to the method described by Salama *et al.* (1979), through inoculation of each rabbit with 1 ml of AHSV with a titre of 10^7 TCID₅₀/0.1 ml mixed with 1 ml of complete Freund adjuvant. The mixture was thick and creamy and the injection was done deeply in the muscle of different legs.

Boostering of these animals was done weekly for further 4 weeks with emulsion contained incomplete adjuvant. Ten days after last injection, the rabbits were bled from the marginal ear vein. The collected blood was allowed to clot and the serum was separated. The antiserum was tested by indirect ELISA techniques. If the antibody has a high titre, three further bleeds were collected on successive days. If it was unsatisfactory, 1 ml was injected and serum was tested 10 days later.

2. Precipitation of gamma globulin

Precipitation of globulin was performed according to the method described by Peter and Vagot (1969), by using saturated ammonium sulphate solution. Finally, remaining sulphate was removed by dialysis against 0.15 M NaCl. The globulin concentration was determined as the method described by Henry (1974), by using of Bechman DU 7400 Spectrophotometer. The concentration was adjusted to be 18 mg/ml in 0.01 M Na_2CO_3 .

3. Conjugate of immunoglobulin with horseradish peroxidase

The conjugation was conducted according to the method described by Tijssen and Kurstak (1984) as follows

- i. Ten mg of horseradish peroxidase (HRPO) were dissolved in 2 ml distilled water followed by the addition of 0.4 ml of freshly prepared sodium periodate solution with gently stirring for 20 minutes at room temperature. The mixture should turn greenish brown.
- ii. The pH of the solution was raised to 9.5 by adding of 50ml of 0.2 M Na_2CO_3 .
- iii. Two ml of rabbit antihorse immunoglobulin (containing 18 mg/ml in 0.01 M Na_2CO_3) were added to the mixture. The pH should be 9.5. The mixture was then put on an end to end shaker for 2 hours at room temperature.
- iv. 0.2 ml of 4 mg/ml sodium borohydride was added and the reaction was allowed to continue for 2 hours at 4°C.
- v. The prepared solution was dialyzed against phosphate buffer saline solution and stored after the addition of equal volume of glycerol at -20°C.

4. Preparation of AHS antigen

It was prepared and concentrated as the method described by House *et al.* (1990).

5. Checkboard ELISA

It was used for titration of locally prepared antihorse peroxidase conjugated sera as well as for determination of optimal antigen coating level for ELISA technique. The experiment was conducted as described by House *et al.* (1990).

6. Solid phase ELISA

It was done according to House *et al.* (1990).

RESULTS

The cytopathic effects (CPE) of AHSV in the inoculated cell culture (Vero) was begun 48 hours post-inoculation (PI) and completely degenerated (70-80%) was obtained on 72 hours (PI). Titre of propagated virus free cells, before concentration was 10^7 TCID₅₀/0.1ml.

Measuring the optimal dilution of prepared AHSV antigen was conducted by checkboard ELISA and the results are represented in Table 1, which revealed that the highest antigen dilution was of 1/20 as it gave an absorbance value of 0.94 with known serum with a titre of 1/128 and 0.12 with negative one.

Series of precipitation with concentrated ammonium sulphate was applied on the prepared hyperimmune sera (globulin) with removal of its sulphate content. The quantity of both total protein and albumin were measured by the use of Bechman DU 7400 Spectrophotometer as the method described by Henry (1974). The globulin was 5.004gm/dl with only traces of albumin (0.77gm/dl).

The optimal dilution of peroxidase labeled antibodies was determined using checkboard ELISA method and the results are represented in Table 2. It was clear that the optimal dilution to be used is 1:2000.

The locally and the patent peroxidase labeled antihorse was applied on 50 horse sera from previously vaccinated horses with AHS inactivated oil adjuvant vaccine for detection of AHS antibodies using solid phase ELISA (Table 3).

DISCUSSION

The diagnosis of equine viral diseases is usually made on the basis of characteristic clinical, pathological and serological findings. For detection of antibodies against any kind of equine viral disease, we need rapid and sensitive technique for this purpose. ELISA technique is one of those technique.

In this study, we aimed to prepare anti-horse polyclonal antibodies conjugated with peroxidase to be used in solid ELISA as a cost benefits and reaching the optimum titre and spare time for calling any company.

After accurate precipitation of the immunoglobulin with removal of its sulphate content, it was measured 5.004gm/dl. The immunoglobulin was diluted by 0.01 M of sodium carbonate to be contained 18 mg/ml to be used for antibody-peroxidase conjugation as done by Tijssen and Kurstak (1984).

We preferred periodate conjugation method, whereas, many authors (Nakane and Kawaoi, 1974 and Tijssen and Kerstak, 1984), proved that the periodate treatment of the enzyme does not interfere with its enzymatic or antibody activities. The method depend on oxidation of the carbohydrate of the enzyme with sodium periodate forming aldehyde groups which react readily with primary amino groups in the antibodies. The addition of sodium borohydride is needed to stabilize the complex but will not interfere with the enzymatic or antibodies activity (Nakane and Kawaoi, 1974). Moreover, high percentage of the oxidized enzyme (70%) can be bound to antibodies and 99% of the immunoglobulin is labeled.

Results of checkboard ELISA for determination of the optimal dilution of the prepared antihorse conjugate indicated that it could be used as a dilution of 1:2000.

The obtained locally prepared antihorse conjugate was compared with that imported one on 50 horse serum (Table 3).

These results throw the light on the effectiveness of the locally prepared as it gave 5 times in effectiveness than the imported one, moreover the former is more cheaper.

Also, these results give an encouragement towards the locally preparation of peroxidase conjugate antihorse (anti-species) to be used in solid phase ELISA for rapid diagnosis of any equine viral disease.

Table 1. Checkboard ELISA method for determination of the optimal AHSV antigen coating level.

Known AHS serum	Absorbance value at antigen dilution of:				
	1/10	1/20	1/30	1/40	1/50
256	0.94	0.94	0.86	0.63	0.45
Negative	0.12	0.12	0.12	0.12	0.12

Table 2. Checkboard for determination of locally prepared antihorse IgG conjugated with peroxidase.

Conjugate dilution	Absorbance value of known AHS antigen	
	Positive antigen 1/20	Negative antigen
1/500	1.981	0.253
1/1000	0.998	0.263
1/1500	0.885	0.304
1/2000	0.834	0.225
1/2500	0.705	0.221
1/3000	0.672	0.204

Table 3. Detection of AHS antibodies in horse sera using solid phase ELISA.

Total sample No.	No. of reactors	
50	3	

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محاولة لإنتاج مادة مشخصة لتشخيص أمراض الخيول الفيروسية

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تمت محاولة لإنتاج أجسام مضادة للخيول مقترنة بأنزيم البيروكسيديز فتم تخليق الأجسام المناعية فى أرناب نيوزيلاندية بيضاء، وتم اقترانها بأنزيم البيروكسيديز من خلال تطبيق طريقة البير-أيودايت وهى الأكثر شهرة وسهولة وأقل تكلفة. تم تحديد التخفيف الأمثل للأجسام المناعية المقترنة بالبيروكسيديز باستخدام أنتيجينات معروفة موجبة وأخرى سالبة. وقد تم استخدام الأجسام المناعية المقترنة البيروكسيديز فى اختبار الاليزا وذلك على عدد ٥٠ عينة مصل خيول. وتلك العينات سبق لها التحصين بلقاح طاعون الخيل الميت ثنائى العترة.