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PJBS

ISSN 1028-8880

Pakistan Journal of Biological Sciences

ANSInet
Asian Network for Scientific Information
308 Lasani Town, Sargodha Road, Faisalabad - Pakistan

Pakistan Journal of Biological Sciences 3 (10): 1716-1718, 2000
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Production of Rabbit-antibuffalo Antibodies Horseradish Peroxidase Conjugate and Standardization of ELISA for *Pasteurella multocida* Antibodies

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Abstract: To standardize the indirect ELISA under local conditions, antispecies-antibodies were produced in rabbits. The antibodies against *Pasteurella multocida* were produced in buffalo calves by injecting the formalin, heat-killed organism_ These antibodies were inoculated into rabbits to produce rabbit-antibuffalo antibodies. These were isolated and partially purified by ammonium sulfate precipitation technique. Protein contents were estimated using Biuret method and the presence of rabbit-antibuffalo antibodies was confirmed and measured through agar gel precipitation test (AGPT). These isolated anti-antibodies were conjugated with horseradish peroxidase by two-step glutaraidehyde method. Ten mg of horseradish peroxidase was applied for the Anti-antibody-Enzyme Conjugate formation and the best results were recorded at 1 :100 and 1.200 dilutions of above conjugate when tested through indirect ELISA for H.S. anti-antibodies.

Key words: Antispecies-antibodies, Horseradish peroxidase, Conjugation, Indirect ELISA, Haemorrhagic septicaemia

Introduction

Haemorrhagie septicaemia (HS) is an acute fatal disease of cattle and buffaloes in Asia and Africa caused by specific seratypes of *Pasteurella multocida* (De Alwis, 1992). The disease has been reported to occur in almost all countries of south and south-east Asia where it causes serious economic losses to livestock industry. Vaccination is accepted as the most effective method of controlling the disease (Horadagoda *et al*., 1993).

Many serological tests are being used for reflecting the immunity but in the early 1970's Enzyme Linked immunosorbent Assay (ELISA) has replaced many of the conventional serological tests as it has proven to be more sensitive and specific having the ability to detect both antigens and antibodies in a wide range of viral, bacterial, parasitic diseases may be in animals (Spencer, 1988; Horadagoda *et al*., 1993) as well as in human and plants (Kemeny and Challacombe, 1989; Carlson and Lindburg, 1978). ELISA has been successfully developed to access the immunity to *Pasteurella multocida.* It also can be used to measure the maternally derived HS-antibodies in new born buffalo calves (Horadagoda *et al*., 1993). ELISA kits which are used commonly for prompt and precise diagnosis of infectious diseases, antibody titer and hormonal levels, are being imported. The economy of our country can in no way afford to purchase high priced ELISA kits and enzyme conjugates for wider use in the country. So, the aim of the present work was to prepare such ELISA kits locally at an economical cost.

Materials and Methods

Preparation of Antigen and HS-Antibodies: Casein-Sucroseyeast (CSY) broth was prepared which was incubated at 37°C on stirrer for 6 hours after inoculating *Pasteurella multocida.* After incubation, it was centrifuged at 7000 rpm for 30 minutes and sediments were dissolved in 5 ml normal saline containing 0.3% formalin. It was kept in refrigerator overnight then again centrifuged at 7000 rpm for 30 minutes, Sediments were dissolved in normal saline and the concentration of organism was adjusted by spectrophotometer at 640 nm wavelength (Wijewardena, 1992; Mahmood, 1999).

Two buffalo calves were inoculated by subcutaneous route with above suspension. An amount of 1.5, 2.0, 2.5 and 3.0 ml of antigen was inoculated at an interval of 4 days. Seven days after last inoculation, 1.0 ml of live 6 hours broth-culture of *P. multocida* was injected. The blood samples were collected from jugular vein of calves after 14 days of injection of live culture are sera was separated (Hussain, 1979; Hamid, 1998).

Production of rabbit-Antibuffalo Antibodies: Ten rabbits were inoculated intravenously with buffalo immuoglobulins. An amount of 0.50, 0.75, 1.0, 1.25, 1.50 and 1.75 while 0.75,1.0,1.25,1.50,1.75 and 2.0 ml of buffalo antibodies to group A and B respectively at an interval of 3 days while group C was remained un-inoculated as control. Fourteen days after last inoculation, the blood was collected and serum was separated (Wijewardena, 1992).

Purification of Rabbit-antibuffalo antibodies: The rabbitantibuff ale antibodies were isolated and partially purified through ammonium sulfate precipitation technique (Hudson and Hay, 1980) and globulin fraction was measured by spectrophotometer at 280 nm wavelength.

Protein estimation and confirmation of Anti-antibodies: Protein contents were estimated by Biuret method after the preparation of standard curve of bovine serum albumin (Gornall *et al*., 1949). Then these anti-antibodies were confirmedkletermined through agar gel precipitation test (Hudson and Hay, 1980).

Conjugation of Anti-antibodies to Horseradish peroxidase: Ten mg of horseradish peroxidase was conjugated with partially purified anti-antibodies by two-step glutaraldehyde method (Avrameas and Ternynck, 1971).

Preparation of Antigen and Coating of Microtitration Plates: *Pasteurella multocida* was inoculated and spreaded on CSY agar media. After 24 hours incubation at 37°C, growth was washed with 2 ml normal saline containing 0.3% formalin and kept for 24 hours. Then it was heated at 100°C for 1 hour in water bath and centrifuged at 6000 rpm for 30 minutes as supernatants containing the antigen (Wijewardena, 1992; Horadagoda *et al*., 1993).

Table 1: Protein contents of rabbit-antibufialo antibodies

Samples	Protein contents (mg/ml)	
	Ammonium sulfate precipitation technique	Biuret method
A_1	1.191	0.967
A_2	0.899	0.716
A_3	4.506	3.419
A_4	1.260	0.691
A_5	0.741	0.989
B_1	1.694	0.139
B_2	3.290	2.957
B_3	1.650	1.736
B_4	1.123	0.780
B_5	5.328	5.452
C_1	0.222	0.537
C_2	0.173	0.072
C_3	1.157	0.909

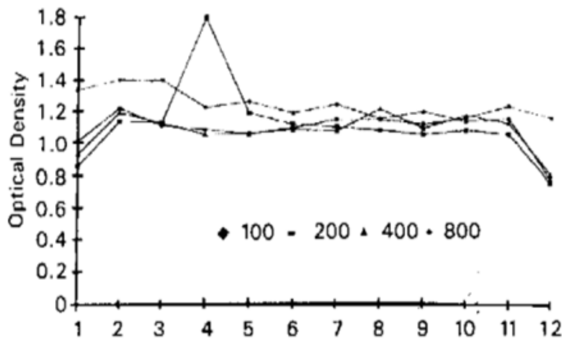

Fig. 1: Mean optical density of various dilutions of conjugates of Group A

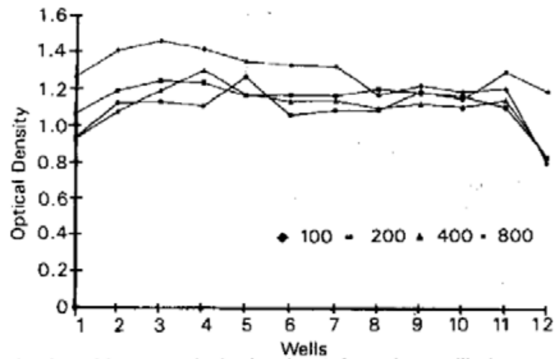

Fig. 2: Mean optical density of varius dilutions of conjugates of Group B

Antigen of 110 dilution was prepared in coating buffer and 100 μl was poured in each well of flat-bottomed, polystyrene, 96-welled microtitration plates. These plates were incubated at 4°C for 24 hours, then washed five times with washing buffer by immunowasher. Now 1000 of blocking PBS was added in each well and plates were incubated at 4°C for 24 hours. Then plates were washed five times with washing buffer (Horadagoda *et al.*, 1993).

Indirect ELISA: For detection of conjugation indirect ELISA was performed. 100 μl of 1:10 dilution of buffalo serum was

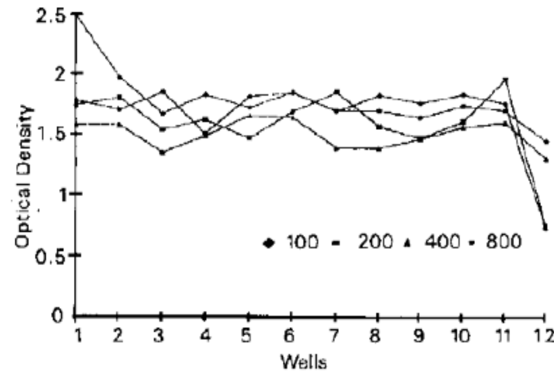

Fig. 3: Mean optical density of various dilutions of conjugates of Group C

added in each well of antigen coated microtitration plate which had already 100 μl of PBS. Then it was two-fold serially diluted from 2nd to 12th well as 1:2 to 1:2048 diluted and this was incubated at 37°C for 2 hours then washed five times with washing buffer, The enzyme-conjugates were diluted in PBS as 1:100, 1:200, 1:400 and 1:800. A volume of 100 μl of each of the dilution was added in duplicate rows as Pt dilution in A+B rows, 2nd in C D rows, 3rd in E+F rows and 4th in G+H rows. These were incubated at 37°C for 2 hours then washed with washing buffer by immunowasher five times. Now 100 μl of OPD (orthophenylene diarnine) substrate was added in each well of plate then incubated at 37°C for 20 minutes. After it 50 μl of IM H_2SO_4 was added and OD was noted at 490 nm wavelength in ELISA reader (Kemeny end Challacombe, 1989).

Statistical analysis: The data was analyzed through Duncan's Multiple Range (DIV1R1 test under Completely Randomized Design (CRD) (Steel and Torrie, 1984).

Results and Discussion

Enzyme Linked Immunosorbent Assay (ELISA) is the most advanced, sensitive and specific test, based upon the antigen-antibody reaction. It is both quantitative and qualitative that gives test positive on color change. ELISA offers a number of advantages over all other techniques. The reaction can be read visually, without the need of expensive fluorescent microscope as needed in fluorescent antibody technique (Durrani, 1988). The labeled reagents are used and are easily stored for long periods of time without the loss of activity (Montoya and Castell, 1987). ELISA has been reported to be 10 to 10,000 folds more sensitive than other serological tests. It also needs a less reagents, labor and cost than in CFT (Spencer, 1988). Johnson (1993) concluded that ELISA is a simple, accurate and inexpensive assay to detect haemorrhagic septicaemia. Moreover, Asi and Lyisan (1992); Natalia *et al*. (1992) and Alonso *et al*. (1995) reported and recommended that ELISA is far superior than quantitative agar gel precipitation test and passive mouse protection test, There are many application of ELISA in diagnostic technology. Human pregnancy, malaria, typhoid, AIDS, hepatitis and many other diseases; hormones like lactogens; tumor marker like alphafetoproteins and carcinoembryonic antigens are being diagnosed currently (Kuby, 1994; Hyde, 1995).

Antibodies were produced by injecting the formalin, heat-killed

antigen of *Pasteureila multocida* in buffalo calves. Production of heat-stable antigen is easy to prepare and can be stored at 4°C for long time (Voller and de Sevigny, 1980). Rabbitantibuffalo antibodies were raised by multiple shot regimen which were isolated and partially purified through ammonium sulfate precipitation technique (Hudson and Hay, 1980). This technique has some advantages as it is commonly used, easy to perform, rapid and quite economical compared to other methods (Ehsan, 1997). Protein contents were obtained as optical density of above partially purified anti-antibodies was taken at 280 nm wavelength. Speotrophotometeric methods are widely used due to accuracy and rapidity for this purpose. Pliuret method of protein estimation was also employed after preparing the standard curve of bovine serum albumin (Gornall *et al.,* 1949). The results of both methods are close to each other which are given in Table 1.

The anti-antibodies were confirmed by agar gel precipitation test (Hudson and Hay, 1980). The appearance of fine precipitation lines around the adjacent wells indicated a positive reaction. The good results were obtained at 4°C not at 57°C or room temperature and at a distance of 2 cm between central to peripheral wells.

Conjugation is the most important step to develop the ELISA kit. Different enzymes including horseradish peroxidase, alkaline phosphatase, urease and 3-D-galctosidase are used for conjugation but peroxidase was preferred because of its properties like high turnover rate, rapid availability, cheap, pure, availability to readily couple to proteins and easy availability of substrates (Kemeny and Challacombe, 1989). Common vegetables like horseradish, turnip, radish, tomato and soybean are the cheap and rich sources of this enzyme (Reed, 1975; Ambreen *et al*., 2000). In the present project, partially purified anti-antibodies were conjugated with 10 mg horseradish peroxidase by two-step glutaraldehyde method (Avrameas and Ternynck, 1971). Although there are other methods of conjugation like One-step gluteraledehyde method (Avrameas, 1969) e Periodate-Oxidation method lelakane and Kawaoi, 1974) and Maleirnide method (Kato *et al*., 1976) for various enzymes but the method adopted here is more efficient than these (Barker, 1989; Shchipakin and Evtushenko, 1989). The mean OD values of various conjugate dilutions are shown graphically in Fig. 1-3. There was a good decreasing pattern in OD values of 1:100 and 1;200 dilution compared to 1:400 and 1:800 dilutions so it is concluded and recommended that 1:100 and 1:200 conjugate dilution are best for indirect ELISA using 10 mg concentration of horseradih peroxidase for conjugation. The conclusion is same when it was statistically analyzed through ANOVA and DIM test udner CRD.

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