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Conjugation of Peroxidase with Antibodies against Haemorrhagic Septicaemia

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ABSTRACT

Formalin, heat-killed antigen of *Pasteurella multocida* was prepared by growing organism on casein-sucrose-yeast agar. The antigen at dose rate of 1.5, 2.0, 2.5 and 3.0 mL was inoculated in buffalo calves at an interval of four days through subcutaneous route. The animals were inoculated by live culture of the organism seven days after last inoculation. Blood was collected through jugular vein puncture of the experimental animals 10 days after exposure to live culture. Serum was separated and antibodies were partially purified by ammonium sulphate precipitation technique. The antibodies were conjugated with horseradish peroxidase using two-step glutaraldehyde method. The enzyme concentrations of 5.0, 7.5, 10.0 and 12.5 mg were applied for the formation of Antibody-Enzyme conjugate. The best results were recorded with enzyme concentration of 10 mg when the conjugate was tested through ELISA.

Key Words: Peroxidase; Antibodies; Conjugate; Haemorrhagic septicaemia

INTRODUCTION

Haemorrhagic septicaemia (HS) is an acute disease of cattle and buffalo in Asia and Africa, caused by specific serotypes of *Pasteurella multocida* (De Alwis, 1992; Wijewardena, 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).

ELISA has been successfully developed to assess the immunity to *P. multocida*. In addition, ELISA has been reported as one of the advanced, sensitive and rapid tests for the qualitative and quantitative examination of an antigen or antibody (Kuby, 1994; Hyde, 1995). It is 10 to 10,000 fold more sensitive than other serological assays (Spencer, 1988). ELISA kits are used commonly for prompt and precise diagnosis of human and animal infections. These kits are very expensive and mostly imported at the cost of high foreign exchange. The most important component of the assay is antibody-enzyme conjugate. The aim of the present work was to prepare antibody (HS) enzyme (Peroxidase) conjugate and finally the ELISA kit.

MATERIALS AND METHODS

Preparation of antigen and HS-antibodies. *P. multocida* was inoculated on the sterile solid medium of Casein-Sucrose-Yeast (CSY) agar and incubated at 37°C for 24 h. The growth was harvested by 5 mL normal saline containing 0.3% formalin and heated at 100°C for 1 h to prepare the inoculum of antigen.

Two buffalo calves were inoculated using multiple short regimens with an amount of 1.5, 2.0, 2.5 and 3.0 mL of antigen at an interval of four days. Seven days after last inoculation, 1.0 mL of live broth-culture of *P. multocida* by subcutaneous route was injected. Ten days after inoculation of live culture, the blood was collected by bleeding the jugular vein of the calves and serum was obtained (Zia *et al.,* 2000).

Purification of HS-antibodies. The buffalo antibodies from collected serum were isolated and partially purified by ammonium sulfate precipitation technique (Hudson & Hay, 1980).

Conjugation of antibodies with peroxidase The partially purified antibodies were conjugated with horseradish peroxidase using four concentrations of the enzyme i.e. 5.0, 7.5, 10.0 and 12.5 mg per mL by two step glutaraldehyde method (Avrameas & Ternynck, 1971; Zia *et al.,* 2000).

Coating of antigen. Antigen of 1:10 dilution was prepared in carbonate coating buffer and 100 µL was poured in each well of round bottom polystyrene 96-welled microtitration plates. The plates were incubated at 4ºC for 24 h, then washed five times with washing buffer by immunowasher. Finally, 100 µL of blocking PBS was added in each well and incubated at 4ºC for 24 h. Then plates were washed five times with washing buffer (Horadagoda *et al.*, 1993; Zia *et al.,* 2000).

Confirmation of conjugation (Direct ELISA). Direct ELISA was performed for detection of antibody-enzyme conjugation. The conjugate was 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 1st dilution in A+B rows, 2nd dilution in C+D rows, 3rd dilution in E+F rows and 4th dilution in G+H rows of micro-titration plate which already had 100 µL of PBS in each well and two fold serial dilutions were made. The plates were incubated at 37°C for 1 h and washed five times with PBS or washing buffer. Then 100 µL orthophenylene diamine (OPD) and H_2O_2 substrate were added in each well and again incubated at

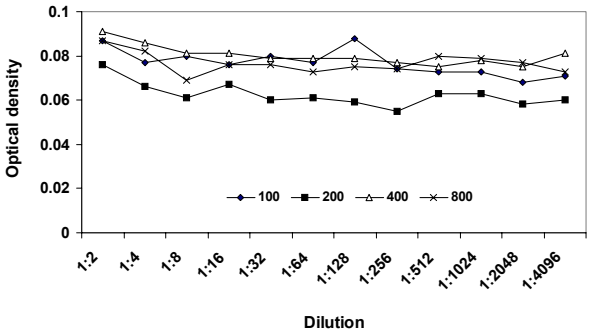
37°C for 30 min. After that 50 µL of 1 M HCl (stop solution) was added and OD was recorded at 490 nm at ELISA reader (Kemeny & Challacombe, 1989; Tortora *et al*., 1995).

RESULTS AND DISCUSSION

Antibodies in buffalo calves were produced by injecting the formalinized, heat-killed antigen of *P. multocida.* Production of such heat-killed antigen is easy to prepare and can be stored at 4ºC for long time (Voller & De Savigny, 1989).

Purification of HS-antibodies. Ammonium sulfate was used to isolate buffalo antibodies, because of its better efficacy to salt out the required proteins (Voet *et al.,* 1999). Moreover, the technique has some advantages over others as it is commonly used, easy to perform, rapid and quite economical (Zia *et al.,* 2000).

Fig. 1. Optical density of various dilutions of Horse radish peroxidase with 5 mg concentration



Conjugation of antibodies with peroxidase. Conjugation is the tough technical job to develop the ELISA kit. Different enzymes including peroxidase, alkaline phosphatase, β-D-galactosidase and urease are used for conjugation with antibodies but peroxidase was preferred because of properties like high turn over rate, rapid availability, pure, cheap, availability to readily couple to proteins and easy availability of substrates (Kemeny & Challcambe, 1989; Zia *et al.,* 2000). In the present research project, partially purified antibodies were conjugated with different concentrations of horseradish peroxidase by two-step glutaraldehyde method (Avrameas & Ternynck, 1971; Zia *et al.,* 2000) which is more efficient and reliable than others as reported by Barker (1989), and Shchipakin and Evtushenko (1990). The enzyme concentration of 10 mg in conjugate proved to be better than other concentrations (i.e. 5, 7.5, 12.5 mg/mL). The optical density (OD) values of such conjugate concentration were obtained by employing the direct ELISA.

Fig. 2. Optical density of various dilutions of Horse radish peroxidase with 7.5 mg concentration

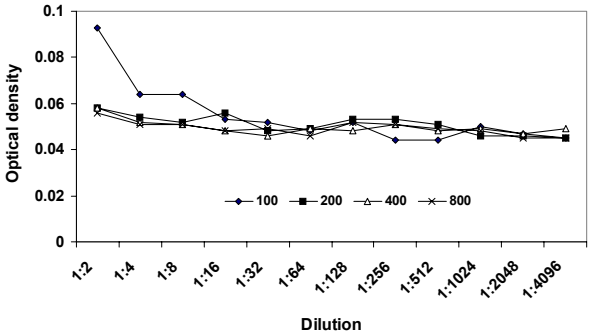


Fig. 3. Optical density of various dilutions of Horse radish peroxidase with 10 mg concentration

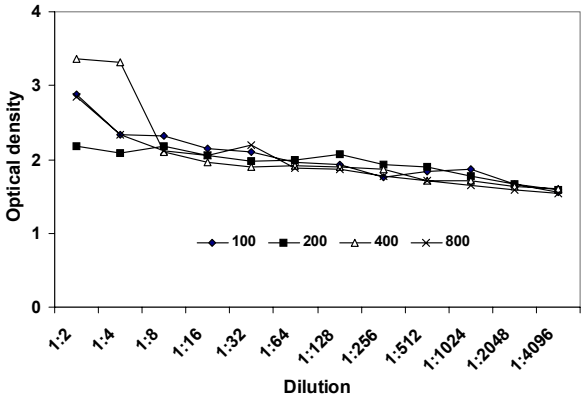
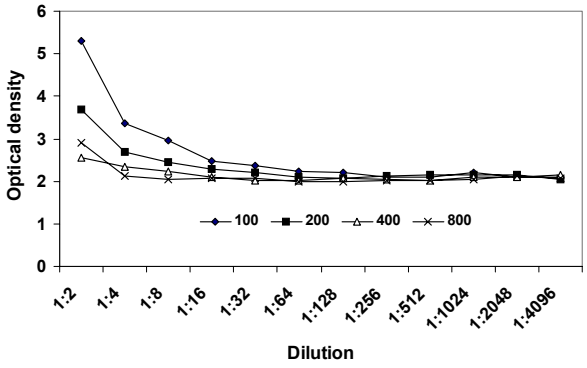


Fig. 4. Optical density of various dilutions of Horse radish peroxidase with 12.50 mg concentration



Direct ELISA. At 5 mg concentration of enzyme with dilution of 1:400, the conjugate showed the enzyme results (Fig. 1). In 7.5 mg concentration 1:400 dilution again proved to be better than other dilutions of conjugate in PBS (i.e. 1:100, 1:200, 1:400 and 1:800). There was a slight variation in the decreasing pattern of values of other dilutions except 1:400 in both of the conjugates, which may be due to pipetting errors or light fluctuations (Fig. 2). All the four dilutions of 10 mg concentration of enzyme gave the best

results (Fig. 3) as there was no deviation in results and the OD values were in smooth and gradual decreasing pattern according to dilutions. These results were also better than that of 12.5 mg concentration of enzyme in conjugate (Fig. 4). Our such findings are in agreement with those of Avrameas and Ternynck (1989) and Zia *et al.,* (2000) who also concluded and recommended that 10 mg of horseradish peroxidase enzyme is better for conjugation with HS-antibodies.

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