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Standardization of Direct ELISA for Determination of *Staphylococcus aureus*

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ABSTRACT

Heat killed *S. aureus* was inoculated in rabbits to produce the antibodies which were conjugated with peroxidase that was extracted from soybean. Antibodies and peroxidase from serum and soybean respectively were isolated by ammonium sulfate precipitation technique. Protein contents were estimated by biuret method. Peroxidase (10mg) containing the activity of 83.02 U/mL was conjugated with antibodies by two step glutaraldehyde method to prepare antibody enzyme conjugate. Best result was recorded at the dilution of 1: 100 when tested through direct ELISA.

Key Words: Soybean peroxidase; Antibodies; Conjugation; *Staphylococcus aureus*; Direct ELISA

INTRODUCTION

ELISA is an immunological technique used to diagnose various infectious diseases of human, animals as well as plants which is more than 99% sensitive and specific than any other serological test (Kemeny & Challacombe, 1989). ELISA is used for the detection of *Staphylococcus aureus* which is Gram +ve bacteria, exist in pairs, short chains, bunched or grape like clusters and of golden yellow in color. It is widely distributed in nature and most commonly present in wound infections resulting in the formation of pus (Malik *et al.*, 1996).

Staphylococcus aureus cause pathogenicity by two ways including invasion and toxin production causing a number of diseases like pneumonia, bone and joint infection, infection of heart valves, bacterimia, meningitis, food poisoning, toxic shock syndrome. but most important is mastitis As mastitis is a disease of economic importance because infection is usually spread from infected to non-infected susceptible animals during milking process (Guss *et al*., 1992).

Direct ELISA method has been proved to be very popular for diagnosing most infectious diseases like Hepatitis, HIV etc. in humans and in animals like mastitis, haemorrhagic septicemia etc (Nickerson *et al.,* 1989; Rehmant *et al*., 2002).

If sufficient quantities of purified antigen are not readily available, it has been much more practical to device direct ELISA that make use of enzyme labeled antibody rather than antigen (Kemeny & Challacombe, 1989). Direct ELISA is also capable of detecting antigens from haemophilus and other lower respiratory tract infections in clinical specimens where culture might prove difficult, especially those which have become -ve because of previous treatment with antibiotics (Spencer *et al*., 1988).

Presently ELISA reagents/ kits are being imported at the cost of high foreign exchange. The present research project was, therefore, designed to produce antibody peroxidase conjugate and standardization of direct ELISA for detection of *Staphylococcus aureus*. The results of this work will facilitate the local production of enzymes, enzyme conjugates and ELISA kits saving foreign exchange being spent on their import.

MATERIALS AND METHODS

Preparation of antigen. *Staphylococcus aureus* was cultured on Staph.110 media incubated at 37°C for 24 h. Culture was harvested with phosphate buffer saline (pH 6.8) containing 0.3% formalin and heated at 100°C for one hour (Heddleston *et al.*, 1972; Zia *et al*., 2000).

Production of antibodies. Three rabbits were inoculated subcutaneously by above suspension with an amount of 0.5, 1, 1.5 and 2 mL at an interval of four days. After seven days 1mL of live broth culture of *Staphylococcus aureus* was injected. Then 14 days of post inoculation of live culture, the blood was collected by slaughtering the rabbits and serum was separated (Rehman *et al.*, 2002).

Partial purification and protein estimation. Rabbit antibodies were isolated and partially purified through ammonium sulfate precipitation technique (Hudson & Hay, 1980) and protein contents were estimated by biuret method after the preparation of standard curve of bovine serum albumin (Zia *et al.*, 2001).

Conjugation. Soybean peroxidase (10 mg) was conjugated with partially purified rabbit-antibodies using two-step glutaraldehyde method (Zia *et al.*, 2000).

Coating of antibody. 100µL rabbit-antibodies (1/10 diluted in coating buffer) was poured in each well of round bottom, polystyrene, 96-wells microtitration plates. These were incubated at 4°C for 24 hours. Then Plates were washed five times with washing buffer. The plates were blocked with blocking PBS by pouring 100µL in each well of the plate and incubated at 37°C for 24 hours. After incubation, plates

were washed with washing buffer (Horadagoda *et al.*, 1993).

Direct ELISA. Direct ELISA was performed for the detection of antibody enzyme conjugate. The conjugate was diluted in PBS as 1:100, 1:200, 1:400 and 1:800, 100 µL of PBS was added in each well of microtitration plate. Now the rabbit serum was diluted in PBS in ratio of 1:10. It was 2 fold serially diluted from 2nd upto 11th well, as 100 µL was added. The 12th column was used as control. These plates were incubated at 37^{o}C for 2 hours. After incubation plates were washed five times with washing buffer. Then a volume of 100 µL of each dilution in each duplicate rows at Ist dilution in A+B, 2nd dilution in C+D, 3rd dilution in E+F, 4th dilution in G+H rows of microtitration plates. The plates were incubated at 37^{o}C for 2 hours. After incubation plates were washed five times with washing buffer. Then 100 µL guaiacol (guaiacol + H_2O_2 substrate) was added in each well and incubated at 37^{o}C for 20 minutes. Then 50 µL of 1M added in each well and incubated at 37^{o}C for 20 minutes. Then 50 µL of 1M H_2SO_4 (stop solution) was added in each well of plate. O.D. was recorded immediately in micro well plate reader/ELISA reader at 450 nm wavelength (Kemney & Challacombe, 1989).

Statistical analysis. The data was analyzed through Duncan's Multiple Range (DMR) test under completely randomized design (CRD) (Steel & Torrie, 1984).

RESULTS AND DISCUSSION

Different serological tests have been used for antigen detection such as rapid slide agglutination test, agar gel precipitation test but ELISA is best method for detection of antigen (Rehman *et al*., 2000). ELISA system based on color change, has advantage over other tests such as agglutination, fluorescent or radioactivity in that antigen-antibody reaction can be measured objectively in simple colorimeters (Engvall & Perlmann, 1971). In addition, the use of ELISA microtitre plates enable a large number of reactions to be read in short period of time (Dawkins *et al*., 1990).

Different enzymes are used in ELISA but peroxidase is preferred over others because of its purity, specific activity, sensitivity of substrate detection, ease of conjugation, efficacy when conjugated and the stability of conjugate (Kemeny & Challacombe, 1989). Peroxidase is an important biological oxidant and wide spread in plant material. In plants it is present in tomato (Zia *et al*., 2001) horseradish, soybean (Ambreen *et al*., 2001) potato, turnip, carrot, wheat, pears, bananas (Reed *et al*., 1975).

Peroxidase was extracted from soybean due to high activity of peroxidase and easy availability of source. The activity of crude peroxidase was 146.12U/mL which proved that soybean is a rich source of peroxidase as also reported by Ambreen *et al*. (2000).

Then peroxidase was partially purified by using ammonium sulfate precipitation technique. This technique was used because it is most commonly used reagent for salting out of proteins due to high solubility which permits the achievement of solution with high ionic strength (Voet *et al*., 1999).

Antibodies were produced by injecting the *Staphylococcus aureus* antigen to rabbits and these were also partially purified by using ammonium sulfate precipitation technique (Hudson & Hay, 1980). The protein contents were estimated by using biuret method as well as U.V. method and results of both methods were close which are given in Table I.

There are different methods for conjugation like meleimide method, preiodate oxidation method, one step glutaraldehyde method and two step glutaraldehyde method but two step glutaraldehyde is preferred over other because the results of conjugate adopting two step glutaraldehyde method is more reliable and more efficient (Baker, 1989).

Antibodies were conjugated with 10mg of peroxidase and conjugation was tested through direct ELISA. The mean O.D. of various conjugates dilutions are shown in Fig. 1, 2 & 3.

The findings indicated that in dilution of 1:100 of sample A OD was ranged from 1.18 to 0.972. In 1:200 dilution OD ranged from 0.97 to 0.93. In 1:400 OD values ranged from 0.714 to 0.72. In 1:800 dilution the values of absorbance were from 0.5 to 0.6.

In case of sample B, at 1:100 dilution OD was ranged from 1.406 to 1.205 while in 1:200 dilution OD ranged from 1.185 to 1.103. In 1:400 OD values ranged from 0.714 to 0.72. In 1:800 dilution the values of absorbance were from 0.99 to 0.93.

Table I. Protein estimation of rabbit samples

Samples	Biuret Method (mg/mL)	UV Method (mg/mL)
A	1.6	1.544
B	1.024	0.917
C	1.0791	0.932

Fig. 1. Mean of O.D. of various dilutions of conjugates of Sample A

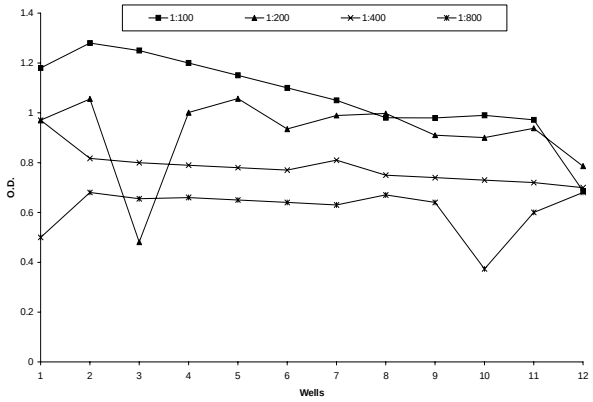


Fig. 2. Mean of O.D. of various dilutions of conjugates of Sample B

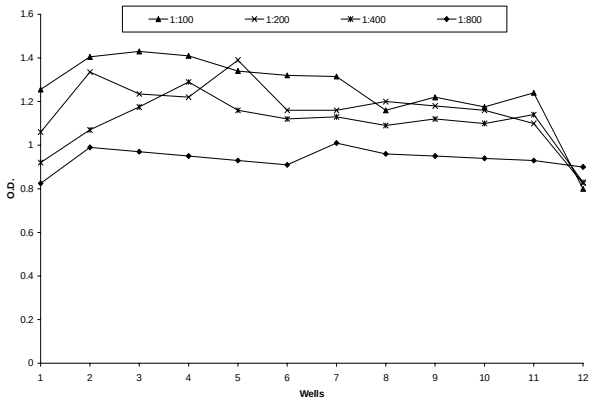
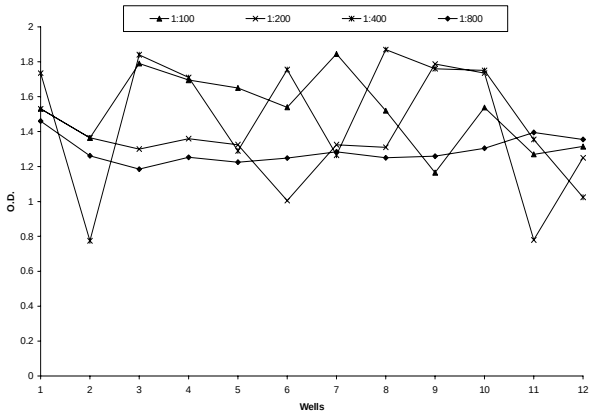


Fig. 3. Mean of O.D. of various dilutions of conjugates of Sample C



In dilution 1:100 of conjugate of sample C, OD was ranged from 1.74 to 1.61 In 1:200 dilution OD ranged from 1.36 to 1.23. In 1:400 OD values ranged from 1.21 to 1.40. In 1:800 dilution the values of absorbance were from 1.26 to 1.39.

There was a good decreasing pattern in O.D. values of 1:100 dilution compared to 1:200, 1:400 and 1:800 dilutions as 1:100 dilution gave the best results. So, it is concluded and recommended that 1:100 is the best for direct ELISA against *Staphylococcus aureus*. Same conclusion was drawn when the result were statistically analyzed through DRM test under completely randomized design.

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