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STANDARDIZATION OF COMPETITIVE ELISA FOR MEASUREMENT OF ANTIBODIES PRODUCED AGAINST *STAPHYLOCOCCUS AUREUS*

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Abstract: Formalin, heat killed *Staphylococcus aureus* was prepared by growing on staph-110 agar medium. Different doses of antigen were injected in rabbits and after 14 days collected the serum. Antibodies isolated by ammonium sulfate precipitation, protein contents were estimated through Biuret as well as UV methods. The antibodies were conjugated with 10 mg soybean peroxidase having activity 83.02 U mL^{-1} using two-step glutaraldehyde method. Conjugation was detected by competitive ELISA and best results were recorded at 1:200 dilution of the conjugates.

Keywords: Antibodies, ELISA, rabbits, *Staphylococcus aureus*.

INTRODUCTION

Staphylococcus aureus is a Gram +ive bacteria which is spherical in shape, exists in pairs, having short chains or bunched grape like clusters. *S. aureus* is present in air, dust, sewage water, foods and in the nasal passage, throat and on the hair as reported [Malik 1996]. *S. aureus* causes a wide variety of diseases in human and animals. Major virulence factor of this organism include entertoxins that cause both food poisoning and toxic shock syndrome as reported [Orwin *et al*. 2003].

ELISA is a diagnostic technique, which is more than 99% sensitive and specific, commonly used for detection in a wide range of human, animal and plant diseases. It is also being used for residue determination of different classes of pesticides like organochlorines, organophosphates etc. indicated by Kemeny and Challacombe [1989].

Present research was designed to produce "rabbit antibodies peroxidase conjugates" and to develop the competitive ELISA for detection of *S. aureus* antibodies. The results of study will help to facilitate local production of enzyme antibody conjugates and ELISA kits to save the national economy.

MATERIALS AND METHODS

PREPARATION OF ANTIGEN

Staphylococcus aureus was cultured on Staph. 110 agar media incubated at 37°C for 24 hours. 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].

PRODUCTION OF ANTIBODIES

Two 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. Seven days after last injection 1 mL of live broth culture of *Staphylococcus aureus* was injected to the experimental animals. After 14 days of it 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 [Zia *et al*. 2000]. Protein contents were estimated by UV method as absorption at 280 nm wavelength and Biuret method after the preparation of standard curve of bovine serum albumin [Gornall *et al*. 1949, Zia *et al.* 2000].

CONJUGATION OF ANTIBODIES WITH PEROXIDASE

Soybean peroxidase (10mg) having activity of 83.02 U mL^{-1} was conjugated with partially purified rabbit-antibodies using two-step glutaraldehyde method [Avrameas and Ternynck 1971, Zia *et al*. 2000].

ENZYME ASSAY

Buffered substrate as 46 mL sodium phosphate buffer (0.2 M; pH 6.5) was prepared containing H_2O_2 (0.32 mL) as substrate and guaiacol chromogen (2 mL). Then 0.06 mL of enzyme sample was added to 3 mL buffered substrate and optical density of reaction was noted at 470 nm wavelength after 3 min. [Civello *et al*. 1995].

ANTIGEN COATING IN MICROTITRATION PLATES

Antigen of 1:10 dilution was prepared in carbonate coating buffer and 100 μL was poured in each well of flat bottomed, polystyrene, 96 welled microtitration plates. These were incubated at 4ºC for 24 hours, then washed five times with washing buffer. Then, 100 μL of blocking phosphate buffer saline was added in each well incubated at 4ºC for 24 hours and again washed five times with washing buffer [Horadagoda *et al*. 1990].

COMPETITIVE ELISA

For detection of conjugation competitive ELISA was performed. 100 μL of 1:10 dilution of rabbit serum was added in each well of antigen coated microtitration plates which had already 100 μL of phosphate buffer saline (PBS). This was incubated at 37ºC 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 1^{st} dilution in A + B rows, 2^{nd} in C + D rows, 3^{rd} in E + F rows and 4^{th} dilution in G + H rows. These were

incubated at 37ºC for 2 hours and then washed five times with washing buffer by immunowasher. Now 100 μL of substrate buffer solution (o-phenylene diamine) was added in each well of plate and incubated at 37ºC for 20 minutes. After it 50 μL of 1M H_2SO_4 was added and OD was noted at 450 nm wavelength in ELISA reader [Kemeny and Challacombe 1989].

STATISTICAL ANALYSIS

The data was analyzed by Analysis of Variance (ANOVA) under Completely Randomized Design (CRD) [Steel and Torrie 1984].

RESULTS AND DISCUSSION

Different serological tests have been used for antibody detection such as rapid slide agglutination test, agar gel precipitation test but ELISA is best method for antibody detection as indicated by Rehman *et al*. [2002]. ELISA has a number of advantages over other techniques. The reaction can be read visually without the need of expensive apparatus. The labeled reagents are used and easily stored for longer periods of time without the loss of activity [Montoya and Castell 1987]. Microwell microtitre plates are used as the solid phase in place of tubes as easily handled and washed when a number of samples to be assayed as reported [Voller *et al*. 1974].

Antibodies were produced by injecting the formalin, heat killed antigen of *S. aureus* in rabbits. Rabbit antibodies were isolated and partially purified through ammonium sulfate precipitation technique [Zia *et al*. 2000]. It is preffered as a commonly used salt for salting out for derived protein and its high solubility permits the achievement of solution with high ionic strength as well as easy to perform, rapid and economical [Zia *et al*. 2000]. Protein contents were measured by taking optical density at 280 nm of partially purified rabbit antibodies and also by Biuret method after preparing 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.

Table 1: Protein contents of Rabbit antibodies samples.

Sample	Protein Contents (mg mL^{-1})	
	Burette Method	Direct Method
A	1.1227	1.090
B	1.0791	0.998

Partially purified antibodies were conjugated with 10 mg soybean peroxides by two step glutaraldehyde method as followed [Avrameas and Ternynck 1971, Zia *et al*. 2000]. Peroxidase, alkaline phosphatase and β-D-glactosidase have been extensively used in ELISA but peroxidase is more important for conjugation [Kemeny and Challacombe 1989] and wide spread in plant material. In plants it is present in radish, soybean

[Ambreen *et al*. 2000] tomato, potato, turnip, current as reported by Zia *et al*. [2001]. Peroxidase is better than other enzymes having high turn over rate, pure, readily available, cheap and it can readily coupled to proteins [Kemeny and challacombe 1989]. On well documented review we selected peroxidase for conjugation. As Barker [1989] concluded that two-step glutaraldehyde method is preferable over other methods like one-

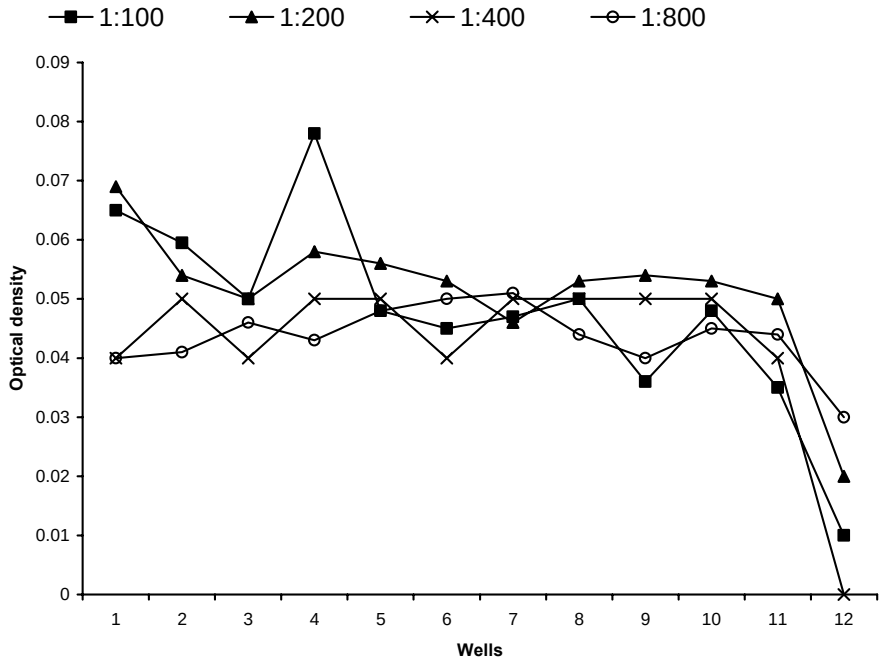

Fig. 1: Mean optical density of various dilutions of conjugates of Sample A.

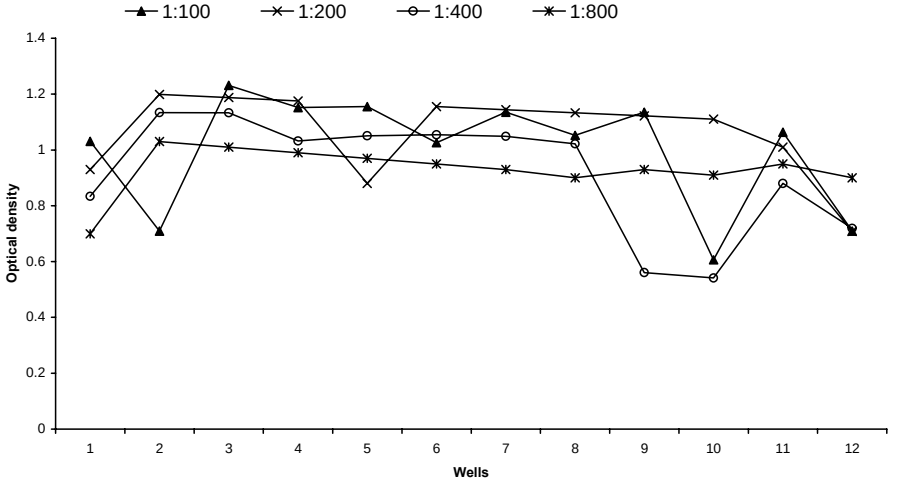

Fig. 2: Mean optical density of various dilutions of conjugates of sample B.

step glutaraldehyde method [Avrameas 1969] and periodate oxidation method [Nakane and Kawaoi 1974]. For the detection of conjugation competitive ELISA was performed. The mean OD values of various conjugate dilutions are shown in Figs. 1 and 2. A dilution of 1:200 showed good results so it is concluded and recommended that 1:200 dilution of conjugate is best for the detection of antibodies against *Staph. aureus*. Same conclusion was drawn when the results were statistically analyzed.

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