DEVELOPMENT OF AN ENZYME LINKED IMMUNOSORBENT ASSAY FOR DETECTION OF ANTIBODY AGAINST *PASTEURELLA MULTOCIDA* IN CHICKEN

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ABSTRACT

In order to obtain a simple, cheap and sensitive method for detecting an antibody response in vaccinated and non-vaccinated chickens an Elisa technique was developed using heat stable antigen from *Pasteurella multocida* P-1662 as a coating antigen.

The Elisa system in this study found to be sensitive however non-spesific reaction need to be avoided. Chickens with high antibody titer need to be diluted in 1/1000, while a dilution of 1/100 could be satisfactorily used for monitoring SPF chickens for laboratory purpose.

INTRODUCTION

The Enzyme Linked Immunosorbent Assay (ELISA) is now used to detect antibodies and antigens in a variety of test systems. The assay is specific, sensitive and relatively inexpensive to set up. The reagents involved are stable and much safer than techniques in which radio isotopes are used.

Indirect ELISA is almost exclusively used for the detection of antibodies. Serum antibodies formed in response to *P. multocida* can be detected with high sensitivity by ELISA (Avakian and Dick 1986, Avakian *et al.* 1986, Briggs *et al.* 1984, Hofacre *et al.* 1986, Choi *et al.* 1990).

Prior to the development of ELISA, methods used for the detection of antibody against fowl cholera included the tube agglutination test, microtitre agglutination test, and the indirect haemaglutination (IHA) test (Schlink and Olson 1979, Carter and Rappy 1973). Most agglutination assays, with the exception of IHA, have shown poor correlation between *P. multocida* antibody and protection against challenge (Alexander and Soltys 1973). The IHA is highly correlated with protection against challenge, however this test is less sensitive and less convenient compared to the ELISA (Solano *et al.* 1983).

Several researchers (Avakian and Dick 1986, Briggs and Skeeles 1984, Dick and Johnson 1985, Marshal *et al.* 1985, Solano *et al.* 1983) have reported successful detection of *P. multocida* antibodies in avian sera by ELISA either for chickens or turkeys, and all have used sonicated bacteria as the ELISA coating antigen and OPD as substrate. Moreover, in each of these reports, birds were vaccinated with a Clemson University (CU) Fowl cholera live vaccine.

Avakian and Dick 91985) reported that the best monovalent ELISA antigen for measuring antibody formed in response to commercial vaccines (Clemson University (CU) live and polyvalent bacterins) was prepared using the CU strain. They also found that an ELISA antigen prepared from strain P-1662 gave similar results but with slightly higher background binding.

In this study the ELISA antigen was prepared from strain P-1662, because the CU strain is not available in Indonesia. Furthermore, commercially available vaccines in Indonesia consist of 3 serotypes of *P. multocida,* serotype 1, 3, and 4, represented by strains X-73, P-1059 and P-1662 respectively.

It has been reported also that heat stable and formalinized extract ELISA antigens have higher specificity than sonicated cell lysate or whole cell antigens (Choi *et al.* 1990). There is also evidence to suggest that the heat stable antigen is a protective antigen (Al Lebban *et al.* 1988). Therefore, in order to obtain a simple, cheap, and sensitive method for detecting an antibody response in vaccinated chickens, an ELISA technique was developed using heat stable antigen from *P. multocida* P-1662 as the coating antigen.

MATERIALS AND METHODS

Preparation of Antigen

Antigen was prepared according to the method of Heddleston (1972) with slight modification. Briefly, bacteria *P. multocida* P-1662 was grown in brain heart infusion (BHI) broth, incubated at 37°C overnight. A 0.2 mL volume of this broth culture was transferred onto 5% blood agar plates, which were incubated at 37° C overnight. The overnight growth of each of heavily inoculated plate were harvested in 1 mL phosphate buffered saline (PBS) 0.02 M (pH 7.2) containing 0.3% formalin. The bacterial suspension was heated in a boiling water bath for 1 hour and the centrifuged at 12000 g for 30 minutes to sediment the bacterial cells. The supernatant fraction was then used as the antigen in the ELISA. The protein content of the antigen was measured by the BCA protein assay kit.

Reactive Sera and non-reactive Sera

Hyperimmune serum used in this ELISA was obtained from chickens vaccinated with *P. multocida* antigen for several times. Non-reactive serum was prepared from several specific patogenic free (SPF) chicken of different ages.

Conjugate and Substrate

Rabbit anti-chicken IgG peroxidase conjugate (Sigma Chemical Company, St. Louis, USA. lot. no. 063H4807) was used in this study. ABTS (2,2'-azino-di[3 ethyl benthiazoline sulphonic acid]) was used as the substrate.

ELISA Procedure

The principle of indirect ELISA was used in this study.

Determination of Antigen Concentration

Optimum concentration of antigen was determined by checker board titration. The antigen was diluted to protein concentration of 19.2, 9.6, 4.8, 2.4, 1.2, 0.6, and 0.3 µg/ml in PBS 0.1 M (pH 7.2). Rabbit anti-chicken IgG conjugated to horseradish peroxidase was diluted 1:4000 time in Tween Buffer Saline Casein (TBSC).

Determination of Conjugate Dilution

To quantify antigen-antibody binding, an appropriate concentration of rabbit anti-chicken IgG peroxidase conjugate had to be determined. Reactive and non-reactive sera were diluted serially from 1/100 to 1/6400 and detected by conjugate dilutions of 1/1000, 1/2000, 1/4000 and 1/8000.

Test Antibody

In order to validate the assay, a variety of reactive and non-reactive serum samples were used. Reactive serum samples derived from chickens after injection with *P. multocida* bacterin, and sera from broiler breeder chickens vaccinated with a commercial bacterin. Non-reactive sera were collected from unvaccinated SPF chickens. The sera were diluted 1/100 in TBSC 0.2% and 100 µl/well wass added into the plate. The 1/100 serum dilution was arbitrarily chosen due to the convenience of sample preparation.

High Reactive Sera

Serum samples with high titres of antibody were evaluated at three different dilutions. These consisted of serum samples from chickens from breeding farms, chickens vaccinated twice with either the field isolate or the commercial vaccine. These samples were diluted 1/100, 1/1000 and 1/10.000 in TBSC 0.2% and 100 µl/well was added to the plates. The plates were incubated or 1 hour at room temperature.

Construction of a Standard Curve

In order to rank the chicken immune respones to *P. multocida,* hyperimmune chicken serum was diluted 2 fold in SPF (non-reactive) chicken serum. The SPF chicken serum was chosen as diluent in order to maintain an even background signal. The serum standards were diluted 1/100 in TBSC 0.2%, as were the test samples. One hundred microliters of each diluted serum standard was added in duplicate to the plate. Results were plotted as a graph.

RESULTS

Optimisation of Antigen Concentration

The effect of varying dilutions of the coating antigen is shown in figure 5.1. The sensitivity of the system. is similar over the protein concentration range from 9.6 µg/ml to 1.2 µg/ml. There is a decrease above 1.2 µg/ml such that at 0.15 µg/ml, the highest dilution tested, the level is less than 50 per cent of the maximum observed at a concentration of 2.4 µg/ml. The positive/negative (P/N) ratio, increased from 14.5 to above 20. This incrase is a reflection of the decrease in OD415-490 for the negative serum sample from 0.124 to 0.040 at antigen concentrations of 19.2 µg/ml and 0.15 µg/ml respectively. Maximum sensitivity is observed at the antigen coating concentration of 2.4 µg/ml and for this reason this dilution was chosen for further testing.

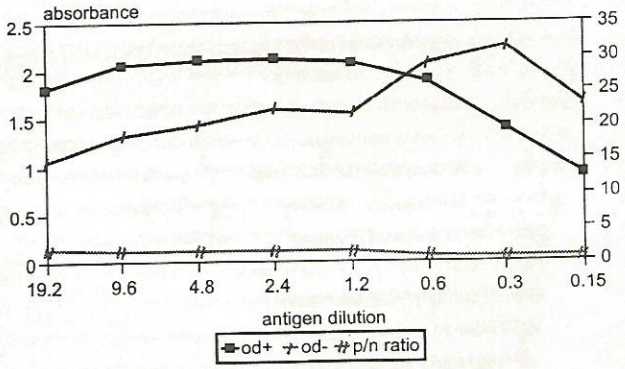

Figure 5.1. Optimisation of the antigen dilution using reactive and non-reactive serum samples

Determination of Optimum Conjugate Dilution

Figure 5.2a shows the reaction of four dilutions of conjugate with different dilutions of reactive and non-reactive sera respectively. A 1/1000 dilution of conjugate produced the highest absorbance values with the reactive serum sample. However it also produced the highest non-specific background at the same dilution of conjugate with the non-reactive SPF chicken serum. A dilution of 1/200 of conjugate produced lower absorbance values with the test antibody compared to the 1/1000 dilution of conjugate, however at this dilution of conjugate there was slightly higher non-specific background binding. At conjugate

dilution of 1/4000 and 1/8000, the non-reactive serum had almost similar absorbance values. At a 1/8000 dilution however the conjugate produced lower absorbance values with the high reactor serum when compared to dthe dilution of 1/4000. Positive/negative (P/N) ratio of four dilutions of conjugate with different dilution of reactive and non-reactive sera are shown in figure 5.2b. At 1/1000 dilution of conjugate the P/N ratio ranging from 4.333 to 8.46.

A dilution of 1/2000 of conjugate produced higher P/N ratio compared to the 1/1000 dilution of conjugate. It was ranging from 8.2 to 13.65. The highest P/N ratio was observed at the dilution of conjugate of 1/4000. Therefore, 1 1/4000 dilution of conjugate was chosen, as it produced acceptable absorbance values with reactive sera and relatively low background with the non-reactive SPF chicken serum and the highest P/N.

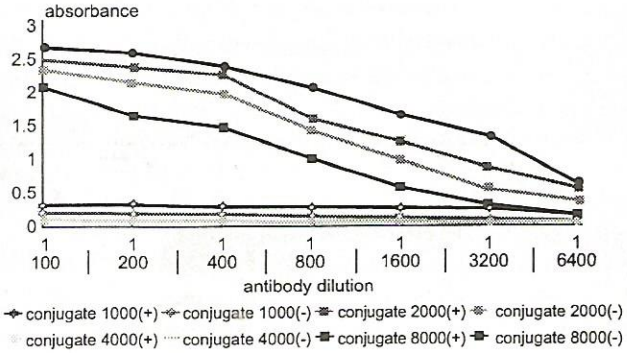

Figure 5.2a Optimisation of the conjugate dilution requred in ELISA using reactive and non-reactive serum sample .

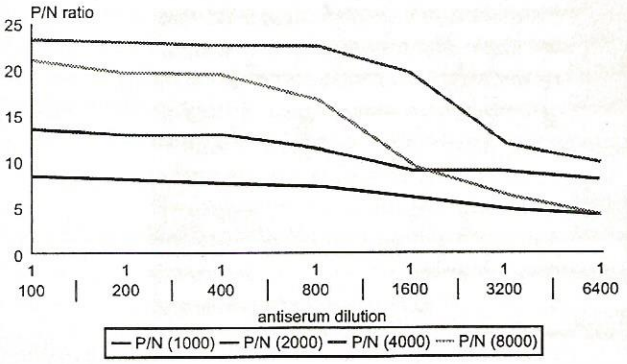

Figure 5.2b Positive - negative ratio of reactive and non-reactive serum sample using different dilution of conjugate

Standar Curve

Figure 5.3 shows the absorbance values for the standard curve. Twofold dilution of hyperimmune serum produce a curve with a constant drop in the absorbance values. Six standards were chosen to represent a range from non-reactive serum to high reactive reactions. Absorbance values of test samples could thus be compared with those of the standard curve.

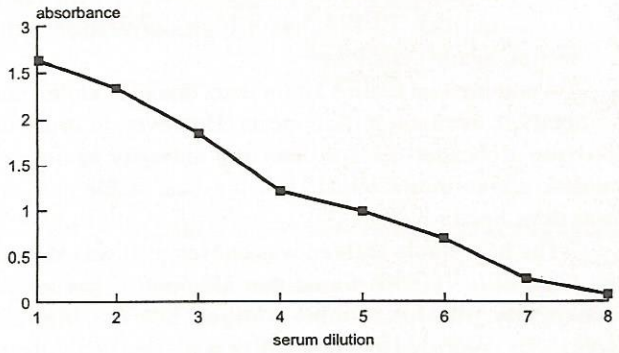

Figure 5.3 Standard curve constructed using dilutions hyperimmune serum Serum with High Antibody Titre

Figure 5.4 shows the result of three different dilutions of high titre reactive samples. At dilutions of 1/100, samples produced similar results, although 2 samples which had lower titres could be differentiated. At 1/1000 dilution of test serum, all samples could be differentiated from each other. Therefore a dilution of 1/1000 of high antibody titre was considered the optimum for diluting test sera to obtain discrimination between samples.

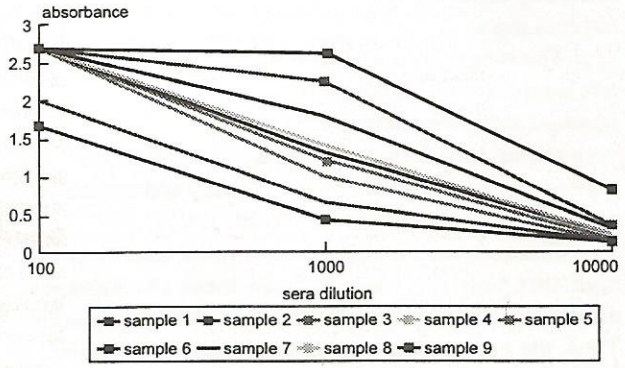

Figure 5.4 Evaluation of three dilutions (1/100, 1/1000, and 1/10000) of high titre antibodies in ELISA

DISCUSSION

One way of investigating the problem of occasonal vaccination failure is to determine the antibody titre following immunisation. Previous serological methods for antibody determination were based upon agglutination or precipitation tests. These tests were unable to correlate the immune response with protection, except for the indirect haemagglutination test (IHA). The IHA however is time consuming, especially in the preparation fo fixed red blood cells.

ELISA has been found to be superior over the IHA (Avakian *et al.* 1985) is at least twice as sensitive and large numbers of samples can be processed in one run. They also reported that ELISA has a high correlation with protection against challenge.

A commercial ELISA kit for detection of *P. multocida* antibody is available in Indonesia. However, in order to provide a cheaper test for detecting antibody against *P. multocida,* an indirect ELISA using heat stable antigen was developed.

The heat stable antigen was chosen as it was shown by Choi *et al* (1990) found that although it has lower sensitivity, the heat stable antigen has the highest specificity compared to whole cell or sonicated cell antigen and is more simple to produce. Studies from other laboratories (Klaasen *et al.* 1985 and Manning 1984) led to a report that the heat stable antigen extract contained lipopolysaccharide as the major antigens and proteins.

The ELISA system in this study was found to be sensitive, however, non specific reactions should be avoided. The non specific background binding was reduced with the addition of blocking solution (0.5% casein in PBS), after coating the microplates with antigen following the washing step. Higher background binding which was reported by Avakian (1985) in using P-1662 antigen was not found in this study. This difference result, probably caused by different in antigen preparation and the addition of blocking solution.

The standard curve was used in this study to rank the test samples according to absorbance value. The use of a standard curve for interpreting ELISA results is considered very important to eliminate variation between individual plastes, day to day variation in the assay and also between different laboratories (Kreider *et al.* 1991).

Chickens vaccinated twice or more usually produce high titre antibody. Serum from these chickens when diluted at 1/100, would possibly couse antigen saturation. Thus, the excess unbound antibody would be washed off before being measured. Thus, the ELISA could not differentiate between extremely high, very high and high antibody titres. To solve this problem samples with high titre antibody (in this study seum from breeding farm chickens and serum from the vaccine efficacy tests) were diluted further to allowed the samples to be ranked.

As shown in figure 5.4, at a dilution of 1/100 the sera with high antibody titre could not be differentiated. However, at a dilution of 1/1000, seven of nine serum samples could be well differentiated. Only two samples at this dilution could not be differentiated. These serum samples may have had the same titre. This dilution was considered to be the best dilution for discriminating between chicken sera with high antibody titre, while a dilution of 1/100 could be satisfactorily used for monitoring SPF chickens for laboratory purposes.

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