CHAPTER IV

STUDIES OF OPTIMAL CONDITIONS FOR THE PROPOSED METHOD

1. Titration of anti immunoglobulin-peroxidase conjugate

The anti IgG-peroxidase conjugate had to be tested for sensitivity and optimal dilution before use

1.1 Optimal dilution of conjugate and sensitivity.

Coated polystyrene surface with 0.2 ml of serial dilution of IgG (10^6, 10^5, 10^4, 10^3, 10^2, 10, 9, 8, 7, 6, 5, 4, 3, 2, 1, 0 ng/ml) incubated for 1 hour at 37 C, washed the excess IgG using albumin buffer. To each row of microtiter plate coated with all dilution of IgG, add 0.2 ml of various different dilutions of conjugate 1:50, 1:100, 1:200, 1:400. Incubated at 37 C for 1 hour, and washed 3 times with albumin buffer, then added 0.3 ml of substrate. The dilution of conjugate which can detect the smallest amount of IgG clearly is the optimal dilution to be used in the proposed method.

For the following tests positive and negative sera were made two-fold serial dilution from 1:8 to 1:1024. Add 0.2 ml of each dilution to DNA coated plate each well. Finally 0.2 ml of optimal dilution of conjugate and 0.3 ml of substrate were added respectively, and the end point titer was read. In

order to perform the test properly, the titration for the optimal point of each of various conditions was necessary.

Results showed that the optimal dilution of conjugate usually fell in the range of 1:100 to 1:200 and it could detect as small as 7 ng/ml of IgG.

1.2 Optimal condition for incubating the conjugate

Polystyrene microtiter plate were coated with DNA (4 mg/100 ml) at 37 C for one hour, then 0.2 ml of 1:100 diluted normal rabbit serum was added and incubated at 37 C for one hour and test with positive and negative sera as described.

The conjugate was added and incubated as follows

- $\frac{1}{2}$, 1, 2, 3 hours at 37 C

- 3, 4, 18 hours at room temperature and

- 18 hours at 4 C

Substrate was added and read for the optimal condition that gave the best discrimination between positive and negative with minimal backgroud reaction.

Showing in Table 1, there was no difference in incubating at 4 C for 18 hours, from temperature for 3-4 hours and 37 C for $\frac{1}{2}$-2 hours. The chosen condition was 1 hour at 37 C for convenience in routine laboratory work.

2. The optimal time for incubating substrate

Optimal condition found in 1.2 was followed. The diluted substrate was then added and results were read at 1, 5, 10, 15, 20 and 30 minutes. Read for the optimal time that gave the sharp difference between positive and negative with minimal back ground staining.

Table 2 shows the results of the optimal time at 10 minutes after adding substrate. The color of the reaction should be read at 10 minutes and the reaction was stopped in 10 minutes. The longer the incubation, the darker color will be. This would make more difficult to differentiate between negative and positive results.

3. Dertermination of optimal condition for coating plate with DNA

Polystyrene surface were coated with 0.2 ml of DNA working solution at the following concentrations: 1, 2, 3, 4, 5, 6, mg DNA/100 ml and incubated for

- ½, 1, 1½, 2, 3 hours at 37 C

- 18 hours at 4 C and

- 3, 4, 24 hours at room temperature

Added 1:200 diluted normal rabbit serum and incubate at 37 C for one hour. The coated plate then test with positive

and negative sera and followed other steps in 1.2, 2, 3.

The optimal DNA concentration was 4 mg/100 ml and the optimal time for coating was 1-2 hours at 37 C, or 1 hour at 37 C followed by 18 hours at 4 C in which the positive and negative results could be clearly differentiated.

4. Determination of optimal condition for coating plate with normal rabbit serum.

Diluted normal rabbit serum 1:50, 1:100, 1:200, 1:400 with phosphate albumin buffer pH 7.0. Added 0.2 ml of the diluted rabbit serum to the DNA coated plate. Incubated as follows:

- ½, 1, 1½, 2, 3 hours at 37 C
- 3, 4, 18 hours at room temperature and
- 18 hours at 4C

Tested with positive and negative sera and followed the method described earlier in 1.2, 2.

The optimal dilution of normal rabbit serum was at 1:200 and the optimal condition for coating normal rabbit serum was 1 hour.

5. The optimal condition for incubating the diluted unknown serum

Coated the plate with DNA and diluted normal rabbit serum, added 0.2 ml of each of two-fold serial dilution of unknown sera starting from 1:8, 1:16 to 1:1024, and incubated in the following fashions:

- $\frac{1}{4}$, 1, $1\frac{1}{2}$, 2, 3 hours at 37 C
- 3, 4, 18 hours at room temperature and
- 18 hours at 4 C

The tests were proceeded to finish and the optimal condition recorded.

Results were shown in Table 3, the optimal time for incubating diluted unknown sera that provided a sharp difference between positive and negative sera was at 1 hour at 37 C. Incubation for $\frac{1}{2}$ hour at 37 C found to be insufficient to differentiatic positive and negative sera and at 4 C negative control sera gave false positive results.

6. Buffer used in washing plate

6.1 pH

Potassium phosphate buffer pH 6.0, 6.5, 7.0, 7.5. 8.0 was used to wash the tested plate 3 times in each washing step.

Results in Table 4 0.1 M Potassium phosphate buffer
pH 7.0 was found to be most suitable.

6.2 Protein used in the washing buffer.

0.1 M Potassium phosphate buffer pH 7.0 was used
as washing buffer. Bovine serum albumin at 0.5%, 1%, 2% and
3% (W/V) were added to the buffer used for washing. Gelatin
at 0.5%, 1% 2% and 3% (W/V) were also used. Most suitable
protein and its optimal concentration was selected.

1% bovine serum albumin or 1% gelatin added to
Potassium phosphate buffer was found to be most suitable
(Table 5).

6.3 Water for washing

Distilled water was used to replace washing buffer
in the third washing at every washing steps during the test
method mentioned, in order to see the difference from the control
which use buffer in all three washing.

The best results were obtained by washing 3 times
with 0.1 M potassium phosphate buffer containing 1% BSA or 1%
gelatin. Distilled water could replace the third washing with
buffer. The running tap water was found unsuitable because it
developed a dense background color (Table 6).

7.   The stability of DNA coated plate

The plate coated with optimal DNA concentration and then tested at monthly intervals for 6 months, using 5 positive and 5 negative sera.

The DNA coated plate was found stable for 3 months when stored at 4 C (Table 7).

8.   The stability of the conjugate

The anti IgG-peroxidase conjugate was stored at 4 C. The conjugate were tested monthly intervals with positive and negative sera.

The conjugate was found to retain original reactivity for 3 months when stored at 4 C. The activity began to decrease after 4 months of storage.

9.   The inhibition test for specificity of the method

Positive sera was incubated with an equal volume of DNA (4 mg/100 ml) working solution for 1 hour at 37 C and 18 hours at 4 C then tested for anti-DNA as described. The results were compared with that of unabsorbed control sera.

It was found that DNA could reduce antibody titer in sera which was found to have anti-DNA antibody. Sera with titer in the negative range was not absorbed by DNA.