CHAPTER II

MATERIALS AND METHODS

A. DETERMINATION THE TYPES OF IMMUNOGLOBULINS ABSORBED ON FILTER PAPER STRIPS BY IMMUNOELECTROPHORESIS (IEP)

(Modification of direction given by Scheidegger (1955) and Wieme (1959) for Vokam IEP equipment)

Immunoelectrophoresis, a technique for the study of antigens and antibodies, is based on two of their properties: their ability to precipitate in agar gel with specific antibody and antigen, respectively (immunodiffusion), and their characteristic mobility in an electric field (electrophoresis). The technique has proved useful for detecting and identifying individual components in a multiple-component system; for determining the purity of a supposedly one-component system; for determining the efficacy of a fractionation procedure for the isolation of a given material; and for recognizing a typical proteins, among them certain pathological serum protein. (9, 33)

Filter Paper

The following study used the strip type filter paper (Toyo Roshi Co., Japan) which was provided by virus research institute, Department of Medical Science , Ministry of Public Health. The shape is more suitable than the disc type (antibiotic disc) which was usually used by other investigators because it has the holding area so the blood absorbing area won't be touched. (Fig. 1)

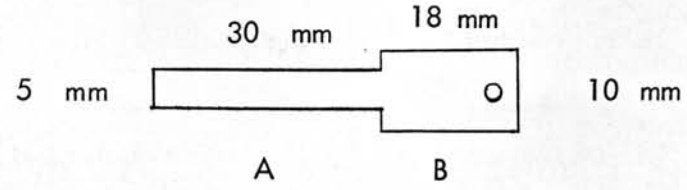


Fig. 1 Filter paper (strip type), A: Blood absorbing area
B: Blood diffusion area (holding area)

Serum

Paired sera obtained from Virus Research Institute, Department of Medical Science, Ministry of Public Health, which serologically confirmed dengue hemorrhagic fever by the HI-method were used. Each serum was absorbed on strip type filter paper, left overnight at room temperature (22°C) then kept in screw cap at room temperature for about 1 week. The serum dried filter paper was cut into 5 - 6 pieces, extracted with 0.5 ml phosphate buffer saline (PBS), centrifuged 2,000 rpm for 2 hours and left overnight at 4°C in refrigerator. The supernatant, containing eluted serum was diluted 1:5 and kept in freezer.

The non-absorbed serum was diluted with PBS to obtain 1:5 serum dilution, then was kept in freezer.

Agar electrophoresis or immunoelectrophoresis was carried out on a frame which can support 8 microscope slides of standard size (25 x 75 mm). The slide was washed in detergent, rinsed thoroughly with tap water and then with distilled water, and finally placed into 70 % alcohol until used. (9) Each slide was covered with 2 ml 1 % agar (Difco, special noble agar) in 0.05 % sodium diethyl-barbiturate buffer pH 8.6 and allowed to solidify at room temperature. The agar was cut with cutter into 2 wells and 1 trough. A simple device can be prepared by fastening with Scotch tape a double-edges razor blade to each side of a thin piece of metal about 6-cm long, 3-cm wide, and thick enough that the distance across the cutting edges

of the attached blades is 1 mm; the cutting edges should extend approximately 4 mm beyond the metal holder.

The agar-covered slide was placed over a pattern such as that shown in Fig. 2. The holes may be situated at any desired distance from the trough, 3 mm being a convenient space.

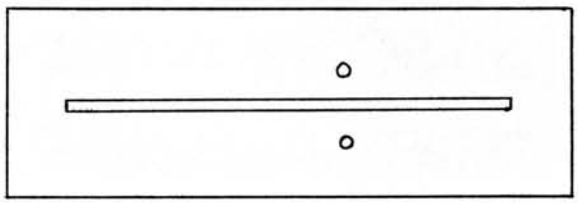


Fig. 2 A pattern of trough and wells

The agar in the wells was gently removed by capillary pipette. The samples (sera) were applied to the wells with microhematocrit tubes. Four slides were used for testing one case, in the first and second slide the IgG and IgM were compared between absorbed and non-absorbed acute serum, and in the third and fourth slide the IgG and IgM were compared between absorbed and non-absorbed convalescent serum.

For electrophoresis, the frame was placed in the IEP apparatus (Vokam, Shandon) as a bridge between the anode and cathode reservoirs allowing the paper strips to dip down into the 0.05 % sodium diethyl barbiturate buffer solution of the reservoirs. A junction was made with a little molten agar in buffer. Electrophoresis was performed at room temperature at about 100 Volts and at constant current of 20 mA/frame of 8 slides for 2 1/2 hours.

After electrophoresis, the agar was removed from the troughs, and the troughs were filled with antiserum (IgG or IgM, Wellcome). Immunodiffusion took place in moist chamber (Shandon) for about 24 hours at room temperature.

After immunoelectrophoretic patterns were developed, made a permanent records by photographs or staining method. In staining method, the developed slides are flooded with 0.9 % saline, to wash away any unreacted protein, for 24 hours then washed with distilled water overnight. The agar was then dried down to a thin film by covering with several sheets of moist filter paper and left at room temperature preferably with a current of air being passed through.

For staining bands, 0.1 % solution of amido-black (Merck) was used for about 15 minutes. After staining, excess stain was washed out with 5 % acetic acid and tap water, thereafter the slides were air-dried in a vertical position and could be kept as a permanent record.

B. COMPARISON OF THE HI-ANTIBODY TITERS AGAINST DENGUE VIRUS IN BLOOD SAMPLES COLLECTED BY FILTER PAPER METHOD AND SYRINGE METHOD AT DIFFERENT CONDITIONS OF STORING

Absorption by Blood Sampling Papers (Filter Paper Strips)

37 clinically suspected cases of hemorrhagic fever (1 man, 40 yrs; and 16 boys and 20 girls, 4-14 yrs) and 16 normal control cases (1 woman and 2 men, 19-24 yrs; and 7 boys and 6 girls, 4-13 yrs) were subjected to this study. Each one was bled at both sites of cubitus vein and finger tip simultaneously. From the cubitus vein using syringe and needle, flowing blood from the syringe was fully absorbed in the absorbing area of 4 paper strips and the excess blood in the syringe was collected in the test tube to separate the serum.

Blood from finger tip was fully absorbed on 1 paper strip and the excess blood was diffused to the diffusion area. The absorbing area contains 0.1 ml of blood (serum 0.04 ml) according to Kenzo Nobuto, who has developed this filter paper method. After the absorption of blood, the filter paper strips were allowed to

dry overnight, at room temperature (≃24 °C) on the spongy holder as shown in Fig. 3, and kept in different conditions in the test tubes covered with rubber stoppers. The first strip was kept at 4 °C for about 2 weeks, the second strip was kept at 24 °C for about 2 weeks, the third strip was kept at 4 °C for about 2-15 weeks, and the fourth strip was kept at 24 °C for about 2-15 weeks, before extraction of serum.

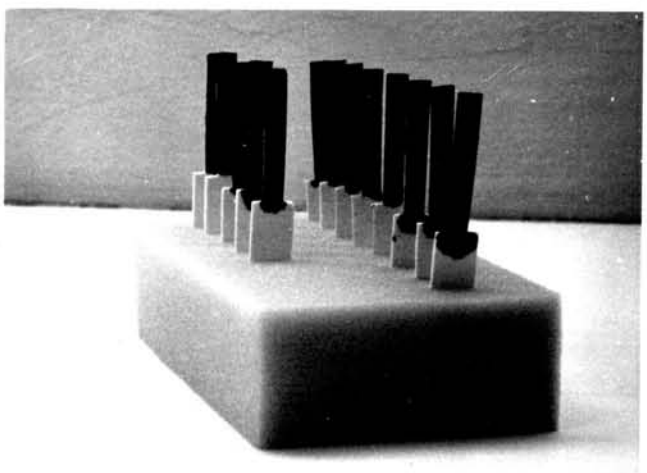


Fig. 3 Filter paper strip holder

Extraction and Treatment of Serum for Diagnostic Tests

The dried paper strip was cut into 5-6 pieces and then absorbed serum was eluted by soaking in 0.4 ml PBS pH 7.4 at 4 °C for 12 hours. Non-specific inhibitors was removed from eluted serum by adding 0.4 ml of 25 % acid washed Kaolin (Fisher Co.) and kept at room temperature for 20 minutes with frequently shaking. The filter paper and kaolin were removed by centrifugation at 3,000 rpm for 20 minutes at 5° C. The supernatant fluid (kaolin-treated 1:10 serum dilution) was incubated with 0.025 ml of packed goose red blood cell at 4 °C for 20 minutes

to remove non-specific hemagglutinins in serum. The serum was cleared by centrifuging at 1,500 rpm for 10 minutes at 5°C. The resulting supernate was the 1:20 serum dilution ready for testing.

On the other hand, serum from the cubitus vein was also treated with kaolin and goose red cells in the similar manner and HI antibodies were titrated by microtiter method against Dengue type 1 (Hawaiian strain) simultaneously with those eluted from the filter paper.

Red Cells for Arbovirus Hemagglutination

Red cells with highest agglutinability is goose red cells when test with arboviruses group A (Sindbis), B (Dengue and Japanese B), and Banyamwera (Chittoor). (3) So the adult white domestic goose (Anser cinereus) is the source of choice for arbovirus HA RBC. One and a half mililiter of ACD solution (see appendix) and 8.5 ml of blood from the jugular or wing vein were mixed at 4°C. The erythrocytes were washed four times in 4°C DGV solution. (see appendix). First washing used 2.5 volumes, second, third and fourth used 3 volumes of DGV solution. The cells were centrifuged at 1,500 rpm for 15 minutes and the supernate was decanted. After the final washing, the cells were resuspended in 3 volumes of DGV solution and kept overnight at 4°C before standardization. (17, 47)

Standardization of RBC Suspension

The most accurate method for preparing erythrocyte suspensions is by spectrophotometry. The use of accurate concentrations of red blood cell is necessary for standardization of HA and HI test. (24)

The 0.5 ml of the final DGV-cell suspension was diluted with 11.5 ml of 0.9 % NaCl.

The suspension (1:24) of red blood cell was subjected to a Coleman, Jr. spectrophotometer for O.D. reading at 490 mμ, using cuvettes with an internal diameter of 10 mm. The O.D. should be 0.750.

The total volume of cells was adjusted by adding or removing DGV according to the formula:

$$\text{Final volume} = \text{initial volume} \times \frac{\text{observed O.D.}}{\text{desired O.D.}}$$

For some purposes the cells can be used up to 3 weeks after collection, although their sensitivity continually diminished. It is safe, however, to use a stock which has been stored at 4°C for as long as one week when characterizing hemagglutinins. (47)

Detection and Titration of Hemagglutinin

In preparing the virus (Dengue, type 1) for HA test, the lyophilized antigen (Dengue type 1) was diluted with pH 9 bovine-albumin-borate solution (BABS) at 4°C to 1:10 dilution. For complete dissociation and exposure of antigenic determinant the solution of antigen was kept at 4°C for an hour before HA test.

Because arbovirus hemagglutination is pH-dependent and varies widely for different antigenic types and groups, it is necessary to test the hemagglutination capacity of a virus at different pH e.g. pH: 5.75, 6.0, 6.2, 6.4, 6.6, 6.8, 7.0, 7.2, and 7.4. (17, 47)

HA-Test

Microtiter® "V" plate was labeled as shown in Fig. 4. The virus was made a serial two fold dilution with 0.4 % BABS pH 9.0 by microdiluters of 0.025 ml capacity from dilution 1:10 to 1:5,120. A volume of packed goose red blood cell

was diluted to 1:24 (O.D. = 0.750) by VAD solution at different pH (see appendix) and 0.025 ml was added to each well with micropipette (0.025 ml capacity). Then the mixture was shaking carefully and the plate was covered and incubated at 37° C for 45 minutes.

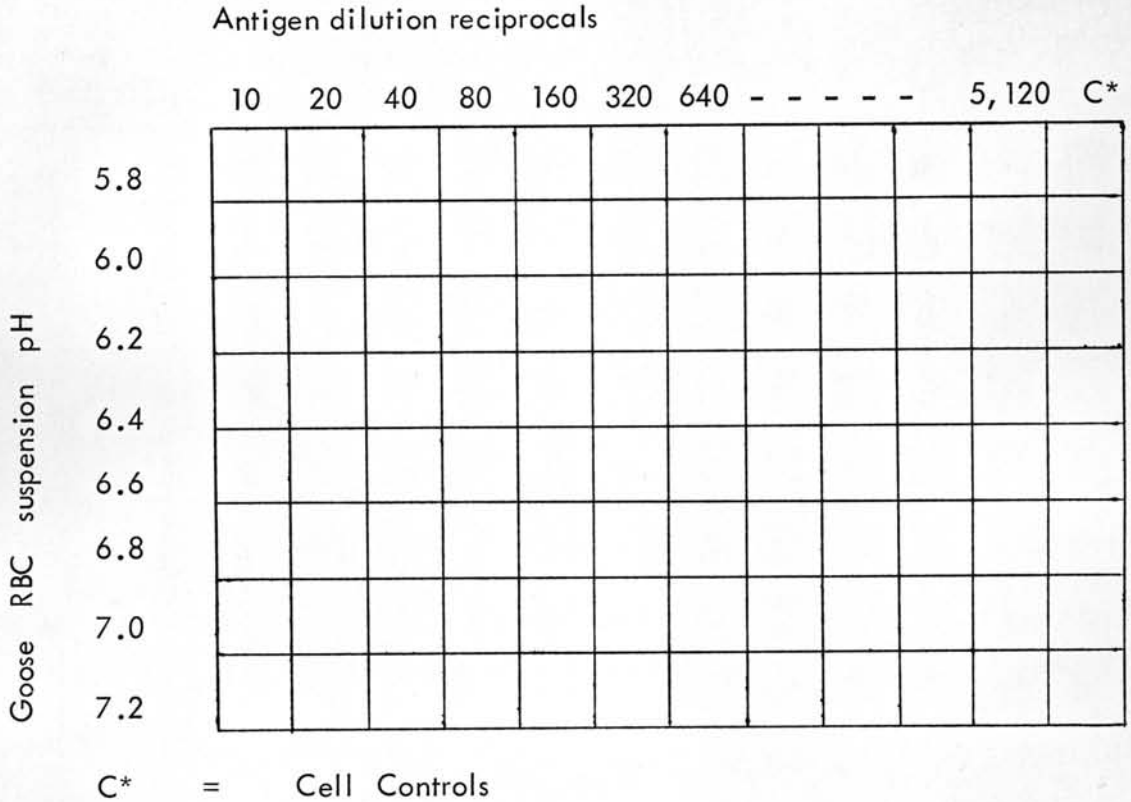


Fig. 4 Labeled microtiter® V plate for HA-test

Reading

+ 4	=	Complete agglutination
+ 3, + 2	=	Partial agglutination
+ 1	=	Slightly agglutination
±	=	Trace of agglutination
0	=	No agglutination

The highest dilution of virus causing complete or partial agglutination is defined as one HA unit. The reciprocal of this dilution is considered the HA titer. The optimal pH is that yielding the highest HA titer and clearest HA pattern.

Determination and Preparation of Antigen Dilution

To determine the dilution of virus which contains four HA units per 0.025 ml, the HA titer as determined in 0.025 ml volumes was devided by 4. The antigen was prepared at this dilution.

Example: titer = 128, $\frac{128}{4}$ = 32

The 1:32 dilution contains 4 HA units per 0.025 ml

Control for Hemagglutination-Inhibition Tests

Hemagglutination-inhibition tests for all viral agents must include the following controls:

(i) The serum control indicates whether the test serum alone agglutinates the test erythrocytes.

(ii) The antigen control is a check on the concentration of antigen, if the test antigen dilution is prepared to contain 4 hemagglutinating units, the antigen diluted to contain 4, 2, 1 and 1/2 antigen units is added to wells containing diluent rather than serum, the cell suspension is added, and the titration is incubated under test conditions. For a satisfactory test, the wells containing 4, 2 and 1 units of antigen should show complete agglutination, while the well with 1/2 unit should show little or no agglutination.

(iii) The third control, the cell control, is included to demonstrate that cells in the suspension settle properly under the test conditions and consists of wells containing diluent and the test volume of the erythrocyte suspension. These wells should show complete settling of the cells after incubation of the test. (39)

HI-Test Procedure

The microtiter plate was scored and labeled as shown in Fig. 5. Two fold dilutions of the 1:20 dilution-treated sera in 0.4 % BABS were prepared using the 0.025 ml microdiluters. Several microdiluters (loops) can be handled simultaneously. Control wells must be provided for serum hemagglutinin, antigen, and diluent controls, the serum controls for nonspecific hemagglutinin being run in 1:20 dilution. Appropriate volumes (0.025 ml) of 0.4 % BABS were substituted where necessary. The 0.025 ml (0.025 ml micropipette) of 4 units of antigen was added to the serum dilutions in the plates. The plates were covered, shaken gently but thoroughly, and held overnight at 4° C.

The goose cell suspension in VAD (optimal pH previously determined) was prepared by making a 1:24 dilution (O.D. = 0.750) of the pretested (8 % suspension) suspension and was added in 0.05 ml volumes in each well. This dilution of cells was completed just before their addition to the serum-antigen combinations. After careful but thorough shaking, the plates were covered and placed in a 37° C incubator for 45 minutes.

To determine the actual reactive units of antigen which went into the test, a separate control titration of the antigen was carried out using 0.025 ml of antigen, with 0.025 ml of 0.4 % BABS to make a serial two-fold dilutions of the antigen.

The wells in the antigen back-titration row now contain 4, 2, 1 and 0.5 HA units per 0.025 ml. Equivalent quantities of goose red cells (0.025 ml) were added to the antigen back-titration while were added 0.50 ml to the serum-antigen reactants.

Reading

The antigen back-titration was examined and the number of HA units actually used in the tests were determined. The erythrocyte and the serum controls should show no agglutination. If agglutination occurs in the serum controls, it is non-specific and that test serum should be retested. If the serum control is satisfactory, read the serum dilution and record results as indicated in HA-test. The reciprocal of the highest dilution of serum which completely inhibits agglutination is considered the HI-titer.

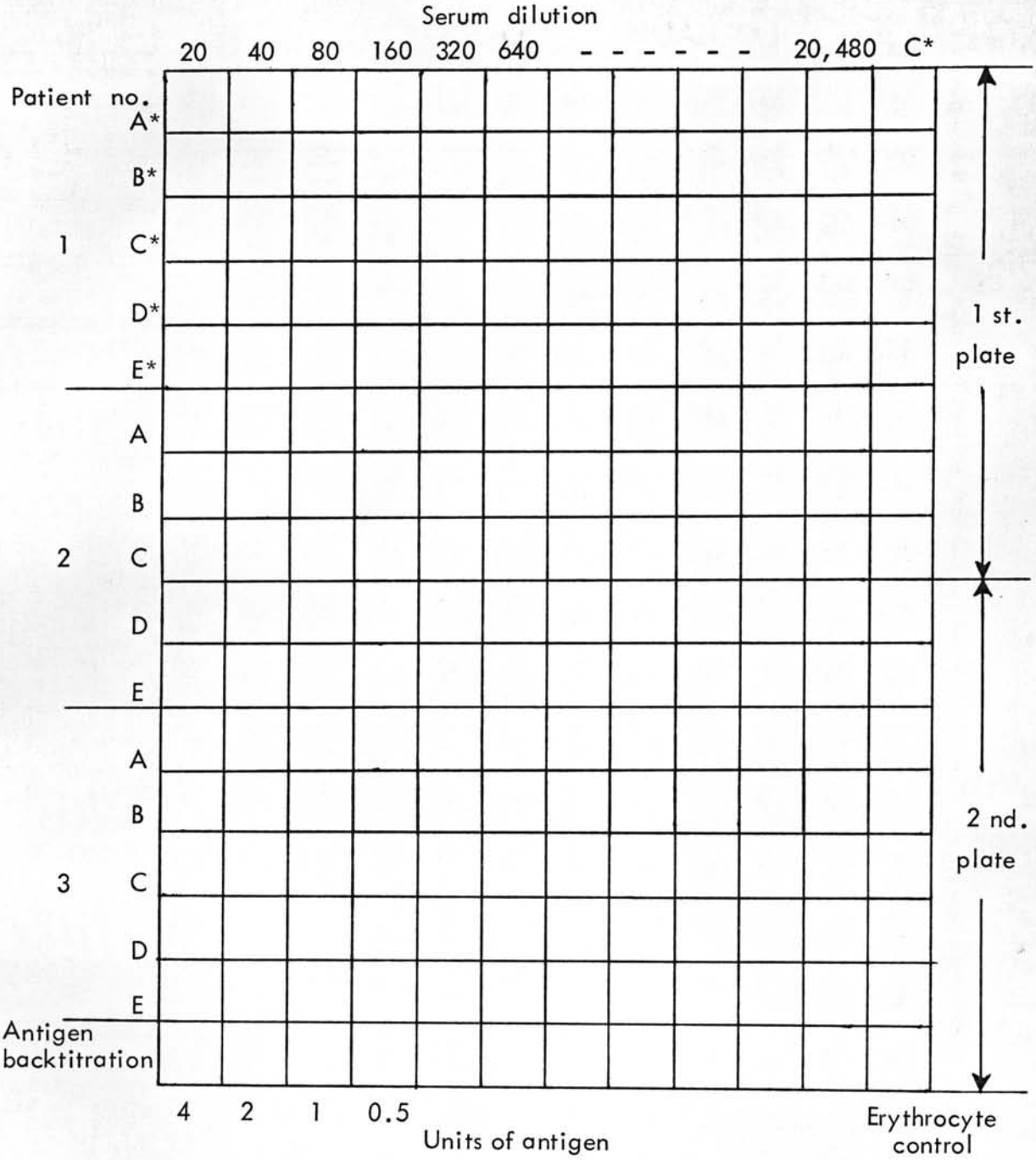


Fig. 5 Labeled microtiter® V plate for HI-test

*C = Serum controls

A = Nonabsorbed sera (Syringe method)

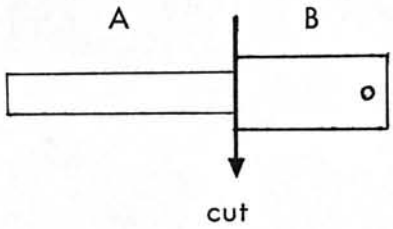
B = Absorbed serum (Filter paper method) keep at 4°C for 2 weeks.

C = " " " 24°C for 2 weeks.

D = " " " 4°C for 4 weeks.

E = " " " 24°C for 4 weeks.

C. CORRECTION OF ERROR ON BLOOD VOLUMES ABSORBED BY FILTER PAPER STRIPS



A = Blood absorbing area

B = Blood diffusion area

The blood absorbing area of 100 filter paper strips were cut and weighed by Mettler analytical balance (H 20 T). Another 100 filter paper strips were taken to absorb blood obtained by venapuncture using 20 ml syringe and needle. After absorbing, each paper strip was put on paper holder in the upward position which caused the excess blood to spread out to the diffusion area and to have the absorbed volume uniformly, then it was immediately put into the moist chamber (Shandon) to prevent evaporation.

After that, each paper strip was removed and cut the blood-filled absorbing area and immediately weighed again. Now, we got the weight of fresh blood absorbed by 100 paper strips. Then the cut paper strips were dried under room temperature overnight, after drying the paper strips were again weighed on analytical balance. And now we got the weight of dried blood absorbed by 100 filter paper strips.

For extraction of the serum the filter papers with dried blood were placed in a physiological salt saline solution buffered with phosphate (PBS), pH 7.4 for 24 hours at 4° C. The quantity PBS per each paper strip was chosen in such a way that a serum dilution 1:20 was obtained. From the direction of the blood sampling papers (Nobuto's) given by Toyo Roshi Kaisha Ltd., it said that blood volume of the absorbing area corresponds to 0.1 ml (serum 0.04 ml). If we used PBS 0.8 ml we

would get the serum dilution about 1:20. This can possible be checked because the quantity of serum per separate paper strip was known accurately by weighing. Moreover, the microhaematocrite value (H) was taken into account.

In order to obtain a serum dilution 1:20 of the paper strip we applied the following method.

From fresh blood weight:

Quantity PBS in ml = Quantity fresh blood in gm x S_B x (100-H)

(*S_B = Specific gravity of blood)

From the weight of dried blood:

The blood dried into about 20 % of the fresh weight. Therefore, it was assumed that the weight of the freshly sucked up blood in the filter paper was five times the weight of the dried blood. (32) The culculation was as follow.

Quantity PBS in ml = Quantity dried blood in gm x 5 x S_B x (100-H)

Hematocrit (Packed Cell Volume) Determination (Micromethod)

(Modified of Direction from 18, 31)

A heparinized microcapillary for hematocrit determination (Clay-Adams, Inc.) about 7.5 cm long with a bore of about 1.1-1.2 mm was used. International microcapillary centrifuge, Model MB (International Equipment Comp., IEC) was available, producing about 12,000 G centrifugal force. This permits shortening of centrifugation to 5 minutes and gives a constant packed cell volume.

Collection of Sample

The finger tip was cleaned with alcohol-iodine solution and left dried before punctured with microlance. The puncture was deep enough to permit free flow of blood. The first two drops of blood was wiped away and the capillary filled

by capillary attraction to within not less than 1 cm from the end. The empty end was sealed in a small flame of a microburner or plugged with modeling clay. The filled tubes were placed in the radial grooves of the microhematocrit centrifuge head with the sealed end away from the center.

After 5 minutes centrifugation, the tubes were removed to read with International micro-capillary reader (IEC).

Normal Hematocrit Values (31)

Sex and Age	Range of PCV, %	Average PCV, %
Men	40-54	47
Women	37-47	42
Newborn	44-64	54
1 year	-	35
10 years	-	37.5

D. THE HI-ANTIBODY TITERS OF THE DENGUE INFECTION BLOOD COLLECTED ON FILTER PAPER STRIPS SENT FROM DIFFERENT PARTS OF THE COUNTRY

This part concerns about whether the lapse of time between blood sampling and serological examination in the laboratory or the time of transportation (from regions where laboratory facilities are few) influences the stability of the antibodies in the blood samples dried on filter paper, which proceeding as follow:

1. The high titer of antibodies against dengue infection in serum from sick children admitted at Chulalongkorn Hospital was found by HI-test. Sick child, 9-year-old, was found to have the titer 10, 240.

2. About 5 ml of blood was obtained by venapuncture using 5 ml syringe and needle, absorbed on 42 filter papers, and left dried overnight at room temperature.

3. After drying, the blood filled paper strips were 5 put separately in 8 letter-envelopes; took them to 8 different parts of the country and sent them back to the laboratory in order to imitate to climatic conditions (temperature, humidity, time etc) in practical uses. The other 2 filter papers were used as the controls to compare with the former sent.

4. 2 controlled filter papers were extracted, treated with kaolin, absorbed with packed goose cells then frozen storage.

5. When the letter came back, each paper in each envelope was immediately extracted and done in the same manner as above. Proceeding from the serum eluates 1:20, twofold dilutions were prepared in microplates, a HI-test in duplicate was carried out with each dilution.