STORAGE STABILITY OF DENGUE IgM AND IgG ANTIBODIES IN WHOLE BLOOD AND SERUM DRIED ON FILTER PAPER STRIPS DETECTED BY ELISA

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Abstract. Blood specimens from 133 patients clinically diagnosed as dengue virus infection by physicians in Nakhon Phanom Hospital, Thailand, were examined to detect anti-dengue IgM and IgG antibodies by antibody capture ELISA. The blood specimens were divided into 3 types of storange; (1) frozen serum aliquots, (2) whole blood dried on filter paper strips, and (3) sera dried on filter paper strips. These specimens were stored for the periods of 1, 3, 4, and 5 months, at -20°C in the case of frozen serum aliquots, or at room temperature in the case of specimens dried on filter paper strips, before examined in paralled by the ELISA. Anti-dengue IgG antibodies were stable for at least 5 months of storage as dried whole blood or serum on filter paper strips. So were the anti-dengue IgM antibodies in the dried whole blood from secondary dengue cases. Anti-dengue IgM antibodies from primary dengue cases declined slowly in whole blood and more rapidly in serum, both dried on filter paper strips. In the serum dried on filter paper strips, even anti-dengue IgM antibodies from secondary cases decreased significantly on storage. We suggest that diagnosis on dengue infections by IgM-capture ELISA should be performed within 1 month after the test specimens are collected as whole blood, not as serum, when the filter paper method is used for sample collection.

INTRODUCTION

Dengue virus infections are the most serious viral diseases in tropical areas, with estimated number of 100 million cases annually occurring in the entire world (Halstead, 1988). In Thailand, more than 403, 405 cases (with 2,395 fatalities) of dengue hemorrhagic fever (DHF) were reported between 1982 and 1987 (WHO, 1988). Recent report in the year of 1991 alone showed 43,511 cases with 137 fatalities (Division of Epidemiology, 1991).

Although many investigations have been reported to develop more rapid, sensitive and specific methods for the diagnosis on dengue virus infection, until now most of the specimens have been diagnosed by serological tests, especially ELISA. In Thailand, the Virus Research Institute, Department of Medical Sciences is the center for laboratory diagnosis of dengue virus infection where the cases are confirmed by ELISA. Approximately 90% of the test specimens have been sent to us from regional hospitals by mail, as blood specimens dried on filter paper strips because of the convenience. Some of them had been kept for more than 4 months before laboratory diagnosis.

Cohen *et al* (1968) examined the effect of storing filter paper-dried blood specimens on the serological test results. They reported that yellow fever hemagglutination-inhibition (HI) antibody titers recovered from the filter paper declined rapidly on storage when the antibody activity existed primarily in the IgM fraction. However, the antibody titers remained reasonably stable over 4 months when the antibody activity was confined to the IgG fraction. A comparative HI test on the serum and whole blood dried on filter paper strips was carried out by Fukunaga *et al* (1974) using dengue and Japanese encephalitis (JE) virus antigens. They reported that both specimens gave comparable results in general, except some unsatisfactory results with those of low HI titers. Rojanasuphot (1982) determined the anti-dengue antibodies by microneutralization test and reported that there was no significant difference whether the test specimens were serum or eluate from dried blood on filter paper strips. However, the storage duration of blood dried on filter paper stirps was not longer than 1 month before testing. Burke *et al* (1985) compared antibody capture ELISA (MAC-ELISA) and the HI test for the assay of anti-JE IgM antibodies from dried blood on filter

paper. They indicated that a large decrease in the eluted IgM would be expected leading to a proportionate decrease in the HI titer response without appreciably affecting the results of MAC-ELISA. They also predicted that the MAC-ELISA would be well suited for the diagnosis of JE using this type of specimens. Nevertheless, the total interval between the blood collection and testing was not more than 1 month and the comparison between the results from serum and dried blood on filter paper was not reported.

We imagined that there would also be some effect of the storage of filter paper-dried blood or serum on the detection of anti-dengue antibodies by ELISA. This study was designed to evaluate the maximum duration and type of specimens for storage on the filter paper strips in order to obtain reasonable results in the laboratory diagnosis on dengue by ELISA.

MATERIALS AND METHODS

Patient specimens

Acute and convalescent blood specimens were collected from 133 patients clinically diagnosed as having dengue virus infection by physicians in Nakhon Phanom Hospital, Thailand, during June to July 1993. All patients were between 2 months to 14 years old, and the interval between the acute and convalescent specimens was 4.1 ± 1.3 days.

Specimen collection and storage

Blood was obtained from the antecubital vein, and a portion of each blood specimen was immediately applied to a No. 1 filter paper strip (Advantec, Japan) and allowed to air dry at ambient temperature (RT). After blood clotting serum was separated by centrifugation and a portion of it was dried on filter paper strips as above and the remaining was divided into 40 µl aliquots which was kept frozen at -20°C. Filter paper strips carrying dried blood or serum specimens were put in plastic bag and stored at RT until elution and assay by the ELISA.

Antibody capture ELISA

After 1, 3, 4, and 5 months from the blood collection, an aliquot of frozen serum was diluted 1:100 in PBS, pH 7.4, for the ELISA. At the same time, a piece of each filter paper strip carrying dried blood or serum was cut out and eluted with PBS to prepare 1:100 final dilution. All samples were examined to detect anti-dengue IgM and IgG antibodies by antibody capture ELISA (Innis *et al,* 1989). Briefly, 96 well microplates (Nunc, Immunoplate, Maxisorp, Denmark) were sensitized with 1:800 dilution of goat anti-human IgM or IgG antibodies (Cappel, Organon Teknika Corp) overnight at 4°C. After washing each well was added with diluted test serum and incubated for 2 hours at RT. Then a mixture of 16 HA units of dengue type 1, 2, 3 and 8 HA units of dengue type 4 antigens was applied and the plates were incubated again at RT for 2 hours. The optimal dilution of horseradish peroxidase-conjugated human anti-flavivirus IgG was prepared which should yield an absorbance (OD) at 492 nm of 0.4 for the standard positive specimen. After washing the plates were reacted with the diluted enzyme-comjugate and incubated at 37°C for 1 hours. The plates were washed and incubated with substrate solution containing O-phenylene diamine and hydrogen peroxide. The reaction was stopped by adding 4M H_2SO_4 and the developed OD in each well was recorded. The results were expressed as P/N ratio (positive/negative ratio) which were calculated by the following formula :

$$\text{P/N ratio (in units)} = 100 \times \frac{\text{(OD test sample – OD negative control)}}{\text{(OD standard positive – OD negative control)}}$$

A cut-off level of P/N ratio was 40 units for IgM and IgG. A ratio of IgM to IgG units equal to or greater than 1.8 was considered as primary infection, while the ratio less than 1.8 as secondary infection, respectively.

RESULTS

Laboratory diagnosis of dengue virus infection using patient sera in the ELISA

The IgM and IgG antibodies were determined for aliquoted and frozen serum specimens from 133 patients. After 1 month of storage, the test samples showed 91 positive (68.4%), 40 negative (30.1%), and 2 uninterpretable (1.5%) cases, respectively. The uninterpretable cases were those whose convalescent serum specimens were not obtained. Among 91

positives, 6 cases (6.6%) were diagnosed as primary, and remaining 85 (93.4%) were secondary infection, respectively.

Reproducibility of IgM- and IgG-capture ELISA on aliquoted and frozen sera for laboratory diagnosis on dengue

Reproducibility of IgM- and IgG-capture ELISA was examined by repeated test on the aliquoted and frozen sera after 1, 3, 4, and 5 months from blood collection, and the results are summarized in Table 1. Among 119 serum specimens for which ELISA were repeated 4 times, 118 specimens (99.2%) gave identical results. A single specimen, for which ELISA were primary, not dengue, not dengue, and not dengue showed borderline IgM-ELISA units (42, 39, 31, and 32). ELISA was not repeated 4 times for the remaining 14 specimens because of insufficient amounts of serum. However, repeated ELISA on these specimens (3 times for 4 specimens, and twice for 7 specimens) showed identical results (Table 1).

Table 1

Reproducibility of IgM- and IgG-capture ELISA on sera for degue infection diagnosis.

Diagnosis by antibody capture ELISA				No. of specimens
1	2	3	4	
1°	1°	1°	1°	5
2°	2°	2°	2°	75
not	not	not	not	37
uni	uni	uni	uni	1
1°	not	not	not	1
2°	2°	nt	2°	2
2°	nt	nt	2°	6
2°	nt	nt	nt	2
not	not	nt	nt	1
not	not	nt	not	2
uni	nt	nt	nt	1

Diagnosis: 1^0: primary dengue infection, 2^0: secondary dengue infection, uni: uninterpretable, not: not dengue, nt:not tested

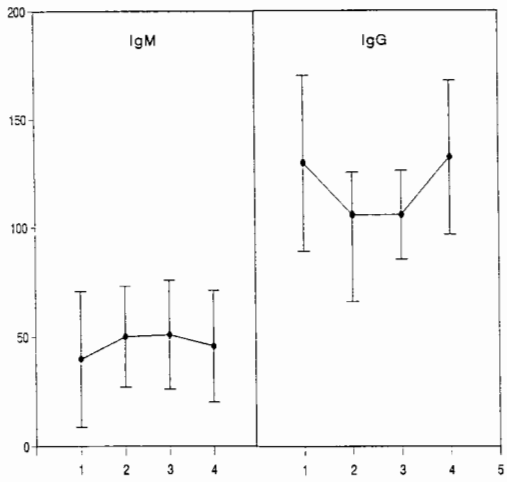

Fig 1–Reproducibility of IgM- and IgG-capture ELISA on serum specimens aliquoted and stored at -20°C.

The average and standard deviation of the P/N ratio obtained in 4 repeated ELISA were calculated and presented in Table 2 and Fig 1. The data showed that these values did not show significant difference among 4 repeated assays.

Table 2

Reproducibility of IgM- and IgG-capture ELISA on serum specimens aliquoted and stored at -20°C.

ELISA assay	P/N ratio:average (standard deviation)	
	IgM (n = 87)	IgG (n = 83)
1	39.75 (31.00)	129.48 (40.61)
2	50.03 (23.09)	105.51 (19.50)
3	50.97 (24.99)	105.53 (20.35)
4	45.79 (25.53)	132.12 (35.54)

Data on the specimens showing P/N ratio above the cut-off values (IgM:40 units, IgG: 100 units) in any of the 4 assays were used for calculation.

Effect of specimen storage on filter paper strips on serodiagnosis

The specimens dried on filter paper strips were kept at RT for varying period before examined by ELISA as described in the Materials and Methods. The results of serodiagnosis on these specimens were summarized in Table 3, in which the percent agreement with the results on frozen serum aliquots, percentage of positive specimens, and the number of primary and secondary dengue cases were shown.

In The case of blood specimens dried on filter

Table 3

Serodiagnosis on whole blood and serum specimens dried on filter paper strips:Effect of storage at room temperature.

	ELISA			
	1	2	3	4
Whole blood dried on filter paper				
: Agreement with stored serum (%)	96.1	93.1	94.5	92.9
: Positive dengue cases (%)	65.1	61.1	60.9	59.8
: No. of primary dengue	6	3	1	0
: No. of secondary dengue	78	77	77	76
Serum dried on filter paper				
: Agreement with stored serum (%)	97.8	94.7	95.4	95.4
: Positive dengue cases (%)	64.1	60.6	61.1	61.1
: No. of primary dengue	4	0	0	0
: No. of secondary dengue	80	80	80	80

paper strips, the percent agreement was 96.1%, 93.1%, 94.5%, and 92.9%, while the percent positive cases was 65.1%, 61.1%, 60.9%, and 59.8%, after 1, 3, 4, and 5 months of storage, respectively. In the case of serum specimens dried on filter paper strips, the percentage agreement was 97.8%, 94.7%, 95.4%, and 95.4%, and the percent of positive specimens was 64.1%, 60.6%, 61.1%, and 61.1%, after 1, 3, 4, and 5 months of storage, respectively. The data indicated slight but definite decrease in the percentage of agreement as well as percent positive cases along with the period of sample storage.

The number of secondary dengue cases did not change at all for the serum specimens up to 5 months of storage, although it showed slight decreased for blood specimens dried on filter paper strips. On the other hand, significant decrease was noticed in the number of primary dengue cases, and this decrease was more pronounced for sera than for whole blood, both of which were dried on filter paper strips and stored in parallel. The number of primary dengue cases decreased to 0 after storage for 5 months in the case of whole blood or after 3 months in the case of serum, respectively.

The average and standard deviation were calculated for the P/N ratio which were obtained by the IgM- and IgG- ELISA on whole blood or serum dried on filter paper strips. The specimens were divided into 2 groups, diagnosed as primary and secondary dengue cases, and the results were shown in Fig 2. In

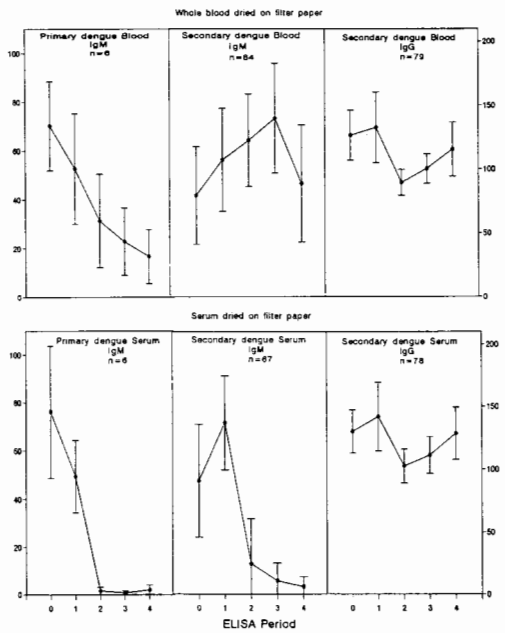

Fig 2–Reproducibility of IgM- and IgG-capture ELISA on specimens stored on filter paper strips.

the case of whole blood dried on filter paper strips, the averaged P/N ratio of IgM- as well as IgG-ELISA in the secondary dengue cases was stable during the storage period up to 5 months. However, the averaged P/N ratio of IgM-ELISA in the primary

dengue cases showed gradual but definite decrease over the storage period. In the case of serum dried on filter paper strips, this decrease in the averaged P/N ratio of IgM-ELISA was more pronounced than in whole blood, and remarkable even in the case of secondary dengue cases.

DISCUSSION

One of the most important processes in laboratory diagnosis is sample collection. The use of blood dried on filter paper strips offered various advantages in sample collection, shipment and storage of large number of specimens for serological investigations on infectious disease (Cohen *et al,* 1968). In this study, we demonstrated that anti-dengue IgM antibodies as detected by the ELISA decreased during the storage period, particularly those from primary cases, and when serum was stored instead of whole blood. This result should seriously be considered in the serodiagnosis of dengue by IgM-ELISA using filter paper strips for sample collection. We have to postulate that the whole blood, not serum, should be used to dry on filter paper strips and the specimens should be examined in an appropriate labolatory before 1 month of storage. Otherwise, there will be false negative results in the primary dengue cases, and such false negative cases will increase in number along with the storage period.

At this moment, we have no idea whether the anti-dengue IgM antibodies were destroyed on the filter paper strips, or they were irreversibly bound by the filter paper support. One of the possible mechanisms for the degradation of IgM antibodies may be their large molecular size with J chains which possess very low extinction coefficients (Rosen *et al,* 1989). Another possibility is the degradation by micro-organisms which eat up IgM but not IgG molecules on the filter paper strips. One of the intriguing results obtained in this study is the difference between primary and secondary dengue cases in terms of the stability of anti-dengue IgM antibodies. The result may indicate that IgM antibodies produced in primary dengue cases possess different structures from those in the secon-dary cases, or there may be some factors in the latter specimens which stabilize the IgM antibodies.

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