

# Detection of Dengue Virus Serotype-specific IgM by IgM Capture ELISA in the Presence of Sodium thiocyanate (NaSCN)

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## Abstract

Dengue virus serotype-specific IgM was detected by IgM-capture enzyme-linked immunosorbent assay (IgM-ELISA) in the presence of a chaotropic agent, sodium thiocyanate (NaSCN). NaSCN did not affect the reactions between anti-human IgM and patients' IgM, and between dengue viral antigens and detecting antibody, peroxidase-conjugated flavivirus-specific monoclonal antibody D1-4G2 IgG. Among 18 dengue-confirmed cases, highest IgM responses were detected to infecting serotypes in 14 cases in the presence of 0.5 M of NaSCN. The results indicate that: (i) the protein-denaturing agent, NaSCN, affects antigen-antibody reaction in IgM-ELISA, and enables the differentiation of serotype-specific IgM from cross-reactive IgM; and (ii) IgM responses against the infecting serotypes are higher than those against the other three serotypes in most primary dengue virus infection. In conclusion, the addition of NaSCN to IgM-capture ELISA is useful for highlighting serotype-specific IgM responses in primary dengue virus infections.

**Keywords:** Dengue, IgM-capture ELISA, serotype-specific IgM response, sodium thiocyanate, NaSCN.

## Introduction

Dengue is currently one of the most important arboviral disease in humans. Dengue viruses, belonging to the family *Flaviviridae*, are comprised of four antigenically cross-reactive serotypes and are responsible for epidemics in tropical and subtropical countries. Since the dengue outbreaks in Osaka, Kobe, Hiroshima and Nagasaki from 1942 to 1945, dengue has not occurred in an epidemic form in Japan<sup>[1]</sup>. However, imported dengue cases have been reported<sup>[2]</sup>. Approximately five million Japanese people annually visit countries in

tropical and subtropical areas and nearly two million people visit Japan from these areas. Therefore, dengue fever (DF) and dengue haemorrhagic fever (DHF) has become an infectious disease of significance and worthy of more attention from the medical community in Japan.

We have earlier reported the laboratory diagnosis of dengue by reverse transcription polymerase chain reaction (RT-PCR) and IgM-ELISA<sup>[3-5]</sup>. We demonstrated that IgM-ELISA was a reliable diagnostic method and that IgM responses were generally serotype cross-reactive but often highest against

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infecting virus serotype in most Japanese cases<sup>4</sup>. The serotype specificity of IgM responses in dengue patients has been controversial<sup>6-8</sup>. Burke had reported that serotype-specific IgM responses corresponding to the isolated virus type were detected in primary dengue virus infection<sup>6</sup>. Gubler had reported that in dengue infection, frequent monotypic IgM responses were not correlated with the virus serotype isolated from patients<sup>7</sup>. In 1984, Inouye et al.<sup>9</sup> demonstrated a new technique for the differentiation of antibody avidity after virus infection, i.e. rubella, rota and Japanese encephalitis viruses. They estimated the antibody avidity to viral antigen using a low concentration of a protein-denaturing agent, guanidine hydrochloride, in the diluent of antibody in the ELISA. They concluded that the "stringent immunosorption" technique was useful for investigating the antigenic relationship among closely-related viruses.

In the present study, we examined serotype-specific IgM responses under stringent conditions in the presence of a chaotropic agent, sodium thiocyanate (NaSCN), in the reaction mixture of dengue viral antigens and patients' sera. The development of a simple method to distinguish serotype-specific reaction from cross-reaction will be useful not only for laboratory diagnosis but also for seroepidemiological studies.

## Materials and methods

Twenty-eight serum specimens from 18 confirmed Japanese dengue cases were used in the study. Serum samples from 22 Japanese subjects with other illnesses, who had never been to areas where dengue was epidemic-prone or endemic, were used as the control. These sera were obtained for diagnostic purposes in clinics and hospitals in

Japan from 2000 to 2002 and sent to the Department of Virology 1, National Institute of Infectious Diseases, Tokyo, Japan.

Prototype dengue viruses were propagated in the *Aedes albopictus* mosquito cell clone C6/36, and the infected cell culture supernatants were inactivated by incubation with beta-propiolactone at a final concentration of 0.2% for 30 minutes at 37 °C as previously reported<sup>10</sup>. Tetravalent and four monovalent dengue viral antigens were prepared by the method as previously reported<sup>4,5</sup>.

The IgM capture ELISA was carried out according to the method described previously<sup>3-5</sup>. Anti-human IgM ( $\mu$  chain specific) goat serum and peroxidase-conjugated anti-human IgM were purchased from Sigma-Aldrich, Inc, USA. The IgG fraction of the flavivirus-specific monoclonal antibody, D1-4G2, was prepared by the Protein G affinity chromatography kit (ImmunoPure G IgG Purification kit, PIERCE, USA), and then conjugated with horseradish peroxidase by a commercial kit (EZ-Link Plus Activated Peroxidase kit, PIERCE, USA).

In order to detect the serotype-specific reaction between dengue viral antigen and dengue virus-specific IgM antibody, patient serum was treated with the Protein G affinity chromatography kit described above. The IgG fraction in the serum specimen was removed by adding Protein G beads according to the manufacturer's instruction. Unless otherwise stated, data were presented as the mean of independent two-to-three assays.

## Results and discussion

One of the authors has previously reported that the addition of NaSCN to the reaction mixture of ELISA highlights serotype-specific

reaction between crude dengue viral antigen and anti-dengue hyperimmunized mouse sera<sup>[11]</sup>. The antigen-antibody reaction was affected not only by the concentration of NaSCN but also by the procedures of the NaSCN treatment. A concentration higher than 0.7 M inhibited the reaction non-specifically, while a concentration lower than 0.3 M had no effect on the discrimination of serotype-specific reaction from the cross-

reactive one. According to these results, we decided to use NaSCN in IgM-capture ELISA at the following conditions: (i) NaSCN was included at a final concentration of 0.5 M in 10% normal calf serum-PBS; (ii) peroxidase-conjugated flavivirus-specific monoclonal antibody D1-4G2 (D1-4G2) was diluted in 0.5 M NaSCN; and (iii) viral antigens captured by patients' IgM were detected by the detection antibody in 0.5 M NaSCN.

Figure 1. The effects of NaSCN treatment on antigen-antibody reaction

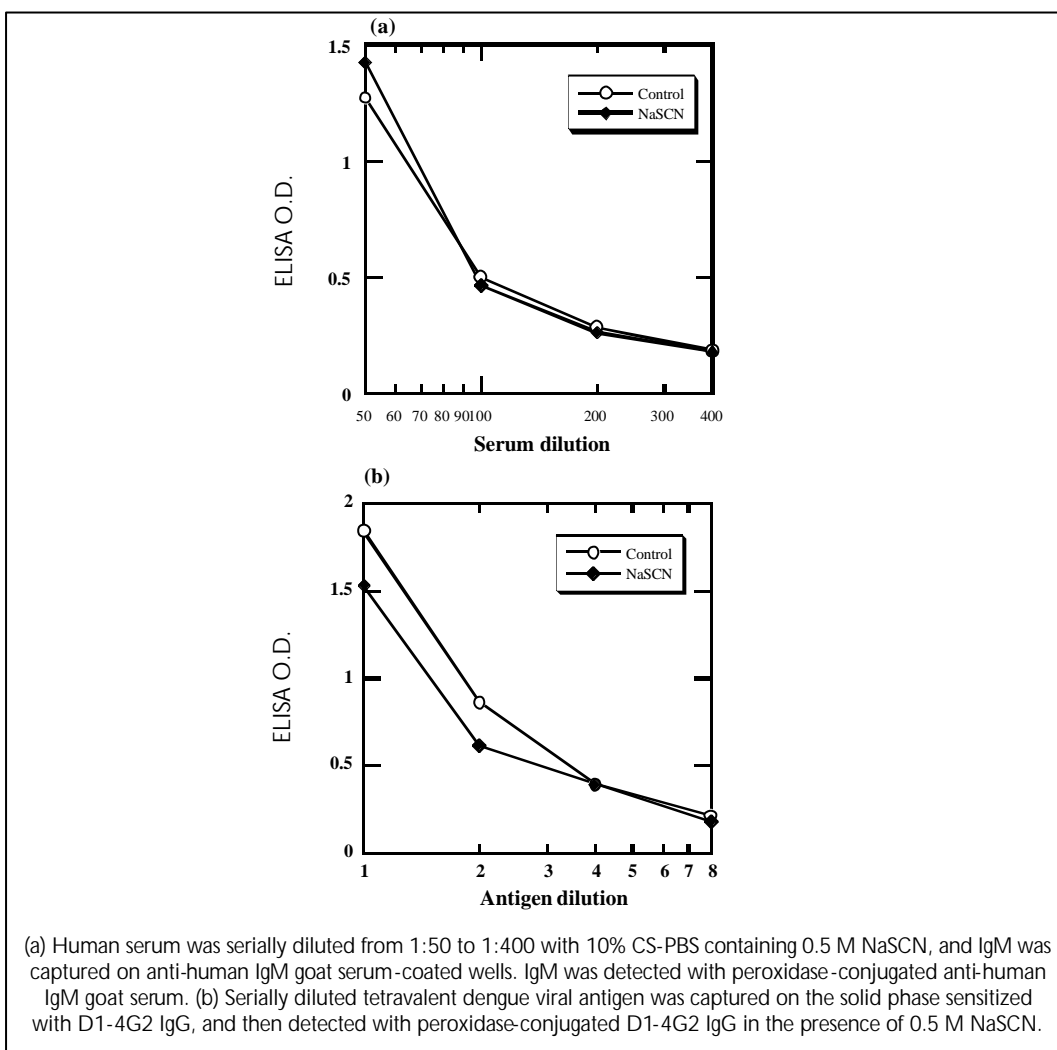
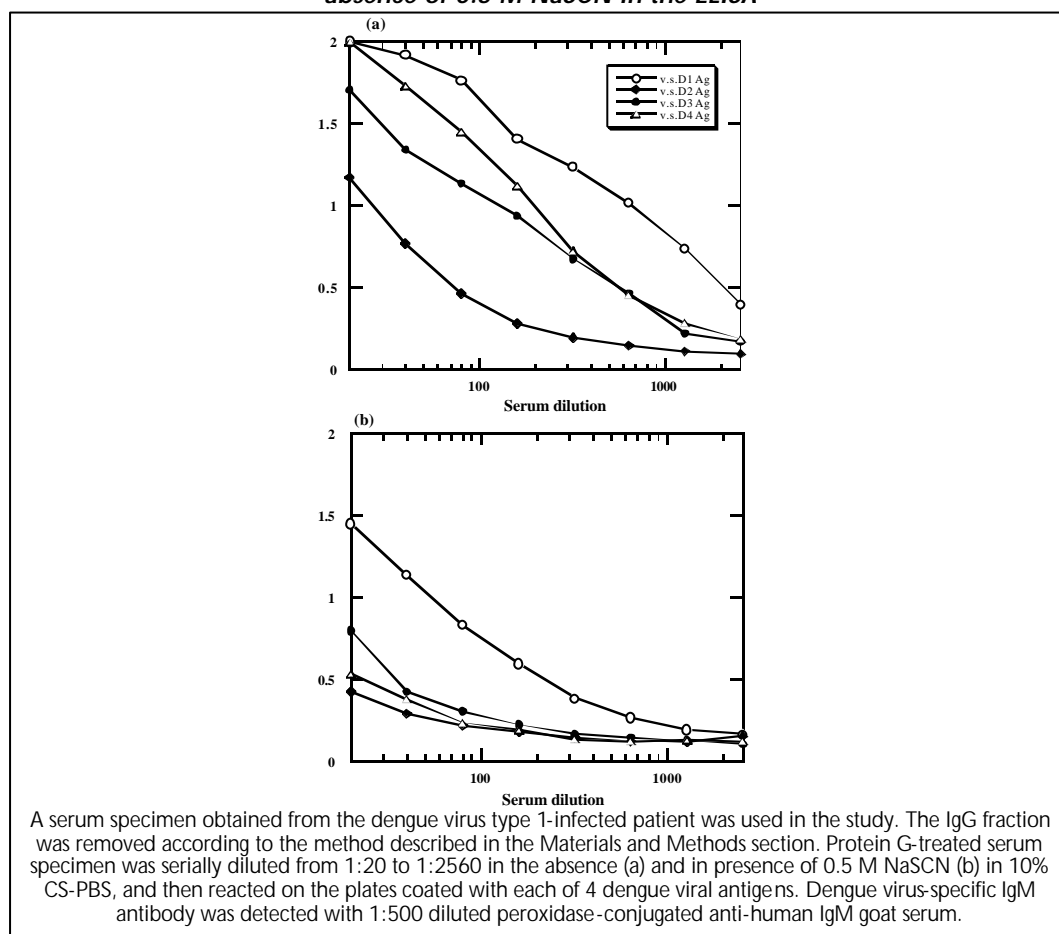


Figure 1 shows the reactions between anti-human IgM and patients' IgM (a), and dengue viral antigen and the detection antibody (b), in the presence of 0.5 M NaSCN in the ELISA. These two reactions were not affected by 0.5 M NaSCN, suggesting that IgM capture and detection of dengue viral antigens were not affected in IgM-capture ELISA.

Figure 2 shows anti-dengue IgM titration curves in the presence or absence of 0.5 M NaSCN in the ELISA. The IgG fraction in the serum specimen was removed by adsorption

with the Protein G beads (UltraLink Immobilized Protein G Plus, capacity: ~25 mg IgG/ml of the gel, PIERCE). The levels of cross-reaction between dengue viral antigens and patients' IgM antibody (a) were decreased after the treatment with 0.5 M NaSCN in the ELISA (b): The level of reaction with homologous dengue-1 antigen was less affected. The results suggested that the addition of 0.5 M NaSCN to the reaction mixture of ELISA may highlight a serotype-specific reaction between crude dengue viral antigen and anti-dengue IgM antibody.

Figure 2. Titration curves of dengue virus specific IgM antibody in the presence or absence of 0.5 M NaSCN in the ELISA



We examined the IgM levels by ELISA with the antigen of 4 dengue virus serotypes. The table below shows the results of 28 serum samples from 18 Japanese dengue patients. The infecting dengue virus serotypes were determined by RT-PCR. The data were presented as the index value according to the method described previously<sup>[4]</sup>. We defined index values of 2.28 or greater and 26.60 or greater as positive, respectively, in the absence and presence of 0.5 M NaSCN in the ELISA. In

all the tested cases, the serotype-specific IgM levels were the highest against the infecting dengue virus serotype than against three other serotypes in the presence of NaSCN. The serotype-specific IgM responses were more highlighted in the presence of NaSCN than in its absence. These data suggest that IgM responses to the infected dengue virus serotype determined by RT-PCR in primary dengue infection were the highest. These results agreed with the report by Burke<sup>[6]</sup>.

**Table.** Serodiagnosis of dengue by IgM-ELISA with and without 0.5M NaSCN

Patient	RT-PCR	Disease day	Index values*							
			Without NaSCN (cut off = 2.28)				With 0.5M NaSCN (cut off = 26.60)			
			D1	D2	D3	D4	D1	D2	D3	D4
1	D3	4	1.20	1.47	2.29	1.42	5.33	3.67	45.67	5.67
		9	8.58	7.73	16.40	10.46	110.30	47.67	471.67	273.00
2	D2	7	4.89	52.18	7.96	4.49	128.00	1,246.00	670.00	239.00
		14	5.27	37.93	8.87	5.42	68.00	395.00	243.70	179.00
3	D3	6	5.34	3.34	9.60	2.35	50.25	15.00	283.00	39.50
		17	6.79	7.07	12.38	4.00	78.00	46.25	413.25	108.50
4	D3	8	5.61	2.84	9.20	2.63	67.25	12.75	305.50	77.00
		10	4.77	3.16	9.10	3.45	54.00	14.00	274.20	97.20
5	D2	6	1.13	4.77	1.71	0.81	2.78	9.36	8.00	0.00
		13	2.65	12.31	4.49	2.15	10.27	46.27	28.73	14.45
6	D3	6	3.07	1.75	6.55	0.89	11.83	4.00	140.33	2.50
		8	4.06	2.40	9.23	1.34	21.00	6.83	198.33	11.83
7	D1	5	2.76	1.46	6.33	1.39	7.86	0.29	20.71	0.00
		7	13.51	6.65	11.49	3.67	127.14	72.86	11.43	25.71
		8	16.91	9.53	13.28	4.43	143.37	100.00	20.37	42.00
		14	24.51	11.49	9.86	3.63	214.50	68.25	34.00	51.13

Patient	RT-PCR	Disease day	Index values*							
			Without NaSCN (cut off = 2.28)				With 0.5M NaSCN (cut off = 26.60)			
			D1	D2	D3	D4	D1	D2	D3	D4
8	D1	7	1.73	3.89	1.66	1.45	5.54	6.38	6.00	1.62
		11	6.95	10.19	2.27	1.76	26.42	20.25	19.00	10.17
9	D2	5	1.93	3.02	1.28	0.72	1.80	4.70	3.90	0.00
10	D2	ND	0.75	2.98	0.92	0.28	2.13	4.63	3.75	0.00
11	D1	5	1.25	1.28	1.32	0.69	4.00	1.57	10.71	0.00
12	D1	ND	20.35	15.44	15.02	8.00	161.28	36.71	39.90	97.71
13	D1	19	18.37	10.69	10.02	12.75	244.43	22.86	138.14	135.00
14	D1	8	7.55	6.78	6.41	3.37	50.25	11.67	27.92	19.92
15	D4	19	1.12	1.44	1.19	2.41	2.56	3.44	7.22	35.44
16	D1	5	1.36	1.13	1.27	0.27	6.27	1.45	2.45	0.00
17	D1	6	4.74	2.01	5.20	0.98	58.67	3.71	17.58	0.00
18	D1	15	14.18	8.39	8.52	3.58	137.38	33.27	31.00	49.88

D1 to D4, dengue virus type 1 to 4, respectively. ND, not determined.

\* index values were calculated by the formula  $A_{492}$  with the viral antigen/ $A_{492}$  with uninfected control antigen.

The chaotropic agent, NaSCN, is known to denature protein structure and inhibit the formation of immune complexes as was exploited for immunoaffinity chromatography<sup>[12,13]</sup>. There are multiple factors which affect NaSCN's ability to highlight serotype-specific reaction in comparison with cross-reaction in the ELISA. One of the factors is a characteristic of dengue viral antigens, which are prepared from the infected mosquito cell culture. It was reported that a rapidly sedimenting haemagglutinin (RHA) was cross-reactive and labile, but the soluble complement-fixing antigen (SCF) was serotype-specific and relatively stable against the NaSCN treatment in the ELISA<sup>[11,14]</sup>. The predominant polypeptides in RHA and SCF fractions

represent the envelope glycoprotein and NS1, respectively<sup>[11]</sup>. Trent et al.<sup>[15,16]</sup> observed three antigenic determinants on the envelope glycoprotein: (i) flavivirus group-reactive; (ii) complex-specific; and (iii) serotype-specific. Henchal et al.<sup>[17]</sup> characterized four antigenic determinants on the envelope glycoprotein: (i) flavivirus group-reactive; (ii) dengue complex-specific; (iii) dengue subcomplex-specific; and (iv) dengue serotype-specific.

Brandt et al.<sup>[14]</sup> reported that the DEN-2 SCF antigen extracted from infected mouse brains was resistant to the treatment with protein denaturing agents. Falconar and Young<sup>[18]</sup> reported serotype-specific epitopes on NS1. These results suggest that

heterologous antigenic determinants on dengue viral antigens contribute to the cross-reactivity among the four dengue serotypes, and that the presence of a chaotropic agent in the reaction mixture induces changes of viral antigens and may decrease the cross-reactive antigenicity.

In conclusion, we demonstrated that the addition of NaSCN to IgM-capture ELISA highlighted the detection of serotype-specific IgM in comparison with serotype cross-

reactive IgM. This procedure may be useful for determining infecting dengue virus serotypes, especially in primary dengue virus infection.

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