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Dengue-type Viruses Isolated in Singapore*

K. A. LIM,[1] Y. C. CHAN,[2] W. O. PHOON[3] & E. HANAM[4]

A dengue-like illness with marked haemorrhagic manifestations appeared in Singapore in 1960. Its similarity in many respects to the haemorrhagic fevers of Thailand and the Philippines led to its being described as "Singapore haemorrhagic fever".

This paper describes the isolation and identification of dengue-type viruses from patients in Singapore between 1960 and 1962. In addition to the conventional complement-fixation and neutralization tests, a new test, called the "sensitized erythrocyte agglutination test", was employed; this test method is described.

Altogether 21 dengue-type viruses were isolated, including dengue types 1, 2 and 4. Chikungunya virus, prominent in the Thailand disease, was not detected.

The author suggests that study of the epidemiology of haemorrhagic fevers in South-East Asia would cast further light on the transmission of arboviruses.

In 1960 there was an outbreak in Singapore of a dengue-like illness with a marked incidence of haemorrhages into the skin, mainly petechiae. The disease appeared in the middle of the year with over 200 patients admitted into hospital, and continued sporadically through 1961 to 1963. There was another increase in incidence in the middle of 1962, subsiding towards the end of that year.

The disease was soon recognized as being due to infection by a dengue type of virus because antibody rises for dengue-type antigens were detected in patients. Although many patients had antibody rises for the Japanese encephalitis virus also, this was not unexpected in view of the cross-relationships existing between these group B arboviruses.

In 1960, the disease affected mainly young adults and the principal clinical features have been described by Chew et al. (1961) and Lim et al. (1961). The main symptoms included headache, muscles and bone pains, nausea and vomiting. The main signs included rashes (including petechiae), thrombocytopenia and enlarged lymph-nodes. The spleen and liver were enlarged in a minority of patients. No deaths were attributed to the disease.

The Singapore disease was milder and more dengue-like than its counterparts in Thailand and the Philippines, in which dengue-type viruses have also been isolated. In those countries young children were mainly affected. There was frank bleeding such as epistaxis and melaena. The mortality was about 10%. To distinguish it, the Singapore disease has been referred to as "Singapore haemorrhagic fever". It must be pointed out that petechial haemorrhages are not generally regarded as an important feature in the classical type of dengue fever although mild rashes are common and pathognomonic. In 1954 Smith (1957) isolated dengue type 1 viruses from an outbreak in Malaya but he did not report any haemorrhagic lesions.

In 1962 the disease appeared to have changed somewhat in pattern. Dengue-type viruses were again isolated from the patients. The children, however, were the most numerous victims. The disease was more severe and attended with profuse and frank bleeding in the form of haemoptysis, haematemesis, or epistaxis as well as petechiae with an associated thrombocytopenia. The clinical picture was reminiscent of the forms encountered in Thailand and the Philippines. Hepatomegaly and splenomegaly were infrequent. Some patients presented with signs of meningeal irritation such as coma, neck rigidity and Kernig's signs, but none had an abnormal cerebrospinal fluid. A few patients

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[1] Professor of Bacteriology, University of Singapore, Singapore.

[2] Assistant Lecturer, Department of Bacteriology, University of Singapore, Singapore.

[3] Formerly, Lecturer, Department of Paediatrics, University of Singapore, Singapore.

[4] Physician, Medical Unit I, General Hospital, Singapore.

who became ill at the same time and with a very similar clinical picture and haemorrhagic phenomena died, but from none of these was a virus isolated.

This paper describes the isolation of dengue-type viruses from patients in the acute phase of illness, and the methods employed in their identification. Cross complement-fixation tests were employed mainly, with cross-neutralization tests as confirmatory tests. A new test, called "the sensitized erythrocyte agglutination test", was also employed.

METHODS

Virus isolations

Attempts were made to isolate virus from the blood of patients during the first five days of illness. Infant mice 1-2 days old were inoculated intracerebrally with either blood or serum, positive results being obtained with either. The brains of infant mice falling sick or found dead were passaged further in infant mice and attempts made at various passage levels to adapt virus to older infant mice and subsequently adult mice. Blind passage was not attempted where the primary inoculation did not cause illness. It is likely that if blind passage had been performed, more isolations could have been achieved.

Virus identification

Virus identification was by complement-fixation tests (CFT) and neutralization tests (NT), mostly by the former. A few tests were performed by the method of sensitized erythrocyte agglutination.

Prototype viruses used were:

Dengue type 1, Hawaiian strain,
Dengue type 2, New Guinea strain,
Dengue type 3, H-37,
Dengue type 4, H-241,
Japanese encephalitis virus (JEV), Nakayama strain.

Immune sera were produced in adult mice by courses of at least five doses intraperitoneally of uninactivated infant mouse brain suspensions. Since the patient's serological reactions indicated that the disease was due to a dengue-type virus, attention was directed to the identification of type.

Considerable difficulty was experienced in the identification of some virus isolates because their immune sera were not specific enough and did not give a clear indication of the virus relationships. The difficulty could be due to broader antigenicity of these isolates as an inherent strain character which may be modified by continued passages.

Complement-fixation test

Cross complement-fixation tests were carried out by the drop method in plastic panels. Antigens were prepared by extraction of infected infant mouse brains with saline, five cycles of freezing and thawing and clarification by centrifugation at 10 000 r.p.m. for 30 minutes. In one test, antigens were prepared by acetone-ether extraction. Both these methods have been described by Casals (1947).

In the tests, the conventional checkerboard method of the simultaneous titration of antigen and serum was used. One drop (0.02 ml) each of six twofold dilutions of antigen and of serum were mixed with 0.02 ml of guinea-pig complement (2MHD) and held in a domestic refrigerator overnight. 0.04 ml of 0.1% sensitized cells was added and results were read after incubation at 37°C for one hour.

Each mixture giving at least nearly complete fixation of complement was recorded as a positive square on a 6×6 grid on squared paper.[1] Usually the serum dilutions used were from 1/4 to 1/128 and occasionally the end-points exceeding the latter were not observed. Antigen dilutions were from undiluted to 1/32 and were rarely reactive beyond this dilution. The number of positive squares resulting from a test indicated the antigenic relationship between the antigen and the immune serum in the test. The positive squares, ranging from 0 to 36, usually outlined a rectangle but irregular areas were not infrequent, probably being due to experimental error. To avoid further error due to variation from day to day, only results from tests done on one day were compared directly.

Neutralization test

Virus infective antigens were infant mouse brain suspensions. Virus-serum mixtures were held at 37°C for one hour and then inoculated into groups of six adult mice each where the virus had been adapted to adult mice; otherwise infant mice were used. In some tests, guinea-pig or rabbit immune sera were used. The 50% mortality end-points were calculated by the method of Reed & Muench (1938).

[1] The grid subdivisions correspond to the six successive twofold dilutions of serum and antigen tested; these subdivisions are not shown in Fig. 1-5 below, but only the number of positive squares.

Sensitized erythrocyte agglutination test

This method was developed from the work described by Hale & Pillai (1960).

Washed erythrocytes from 1-2-day-old chicks were suspended at 4% concentration in virus, adjusting diluent for pH 6.8. Lyophilized virus haemagglutinin was reconstituted in pH 9 borate saline buffer. Equal volumes of chick erythrocyte suspension and virus haemagglutinin were mixed and allowed to stand in the cold for two hours. The sensitized erythrocytes were washed three times in saline and might then be stored for several days. For use, the erythrocytes were resuspended at 1% concentration in virus, adjusting for pH 7.6.

Dilutions of mouse immune serum or patient's serum were made in pH 9 borate buffer saline plus 0.4% bovine albumin. 0.25-ml aliquots of serum dilutions were distributed in plastic haemagglutination trays and equal volumes of antigen added. Tests were read after two hours at room temperature and again after overnight storage in the cold. Positive and negative results were similar to those observed in the conventional pattern test for virus haemagglutination, although it should be emphasized that in the test described a positive result indicated presence of antibody. The technical details are to be published.[1]

RESULTS

Altogether 21 viruses were isolated from patients in the years from the middle of 1960 to early 1963. The particulars of adaptation are given in Table 1. The virus isolates were identified in groups by various tests as soon as they had been sufficiently adapted.

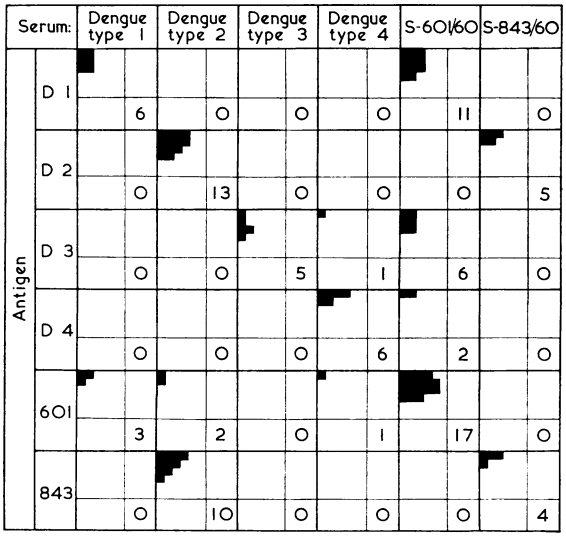
1960 virus isolates

Complement-fixation tests. Only two viruses were isolated in 1960. These were compared by cross complement-fixation tests and the results, recorded as described, are shown in Fig. 1. In this experiment alone, the antigens were prepared by acetone-ether extraction. Inspection of Fig. 1 shows that virus isolate S-601/60 was most closely related to dengue type 1 virus, whereas virus isolate S-843/60 was most closely related to dengue type 2 virus.

The conclusions drawn from the results are more clearly seen when the data are analysed by the method described by Fulton & Dumbell (1949). The

FIG. 1

CROSS COMPLEMENT-FIXATION TESTS OF VIRUS ISOLATES S-601/60 AND S-843/60



number of squares corresponding to the degree of complement-fixation given by a serum against an antigen is indicated beside the areas shown in Fig. 1. The result obtained against each antigen is divided by the result obtained against the homologous antigen. These ratios of heterologous/homologous fixation indicate the degree of antigenic relationship between the antigens. The ratios obtained are given in the upper part of Table 2. The two ratios that are obtained for each pair of viruses compared in this way are averaged and the results, which we call the " mean antigenic relationship " (MAR), are given in the lower part of Table 2.

Allowing for experimental error, the MAR value for identical viruses should be unity, whereas that for dissimilar viruses should be zero. Although numerical analysis by this method ignores such features as zone phenomena and non-reciprocal relationships it provides a scale of values for viruses that are being compared.

The mean antigenic relationship of virus isolate S-601/60 to the other viruses in the test was found to be greatest for dengue type 1 virus. There was a certain degree of cross-relationship. The MAR value of S-843/60 for dengue virus type 2 was practically unity and no cross-reactions were detected.

It should be noted that the lower part of Table 2 gives the mean antigenic relationship between

[1] Lim, K. A. & Pong, W. S. *Agglutination by antibody of erythrocytes sensitized by virus haemagglutinin* (to be published).

TABLE 1

DENGUE-TYPE VIRUSES ISOLATED IN SINGAPORE, 1960-63

Isolate No.	Patient's sex and age (years)	Date of onset	Duration of disease when virus isolated (days)	Incubation period in infant mice at 1st passage (days)	Dengue type	Interval between serum specimens (days)	CF antibody rise [a]	
							JEV	D1
1960								
S-601/60	M. 25	22. 4. 60	2	11	1	29	0/256	0/256
S-843/60	M. 24	20.10.60	1	9	2	41	0/0	0/256
1961								
S-132/61	F. 9	20. 3. 61	2	9	4	16	0/256	0/256
S-212/61	M. 23	4. 5. 61	2	9	4	25	0/256	0/256
S-378/61	M. 23	27. 7. 61	2	12	2	19	8/64	8/256
S-430/61	M. 17	27. 8. 61	1	10	2	15	0/128	0/128
S-554/61	M. 7	11.10.61	2	12	4	32	0/16	0/32
1962								
S-389/62	M. 11	14. 8. 62	2	9	1	15	0/0	0/16
S-429/62	F. 14	3. 9. 62	1	10	2	14	0/256	0/256
S-437/62	M. 6½	5. 9. 62	2	9	1	24	0/0	0/64
S-438/62	M. 6	5. 9. 62	2	10	2	28	0/0	0/256
S-452/62	M. 10	9. 9. 62	3	11	2	15	0/0	0/16
S-461/62	M. 18	13. 9. 62	2	9	2	8	0/0	16/256
S-509/62	M. 4	3.10.62	1	11	2	15	0/64	0/256
S-521/62	F. 20	7.10.62	2	10	2	33	0/16	8/256
S-575/62	M. 16	21.10.62	2	9	2	22	0/32	0/64
S-630/62	M. 14	10.11.62	2	10	2	15	0/0	0/256
S-699/62	M. 24	4.12.62	2	9	2	12	0/0	0/256
1963								
S-36/63	M. 55	7. 1. 63	3	10		14	0/0	0/256
S-210/63	M. 11	26. 3. 63	2	9		14	0/0	0/32
S-212/63	M. 33	25. 3. 63	3	14		16	0/0	0/128

[a] Expressed as reciprocals of titres of 1st specimen/2nd specimen.

TABLE 2
ANTIGENIC ANALYSIS OF S-601/60 AND S-843/60

Heterologous/homologous ratio

Antigen	Serum					
	D 1	D 2	D 3	D 4	S-601/60	S-843/60
Dengue type 1	1	0	0	0	0.65	0
Dengue type 2	0	1	0	0	0	1.25
Dengue type 3	0	0	1	0.17	0.35	0
Dengue type 4	0	0	0	1	0.12	0
S-601/60	0.50	0.15	0	0.17	1	0
S-843/60	0	0.77	0	0	0	1

Mean antigenic relationship (MAR)

Virus	Virus					
	D 1	D 2	D 3	D 4	S-601/60	S-843/60
Dengue type 1						
Dengue type 2	0					
Dengue type 3	0	0				
Dengue type 4	0	0	0.08			
S-601/60	0.58	0.08	0.18	0.15		
S-843/60	0	1.01	0	0	0	

viruses and not, as in the upper part, between antigens and sera.

Neutralization tests. The conclusions arrived at by cross complement-fixation tests were confirmed by cross-neutralization tests, with the results shown in Table 3. Following the same procedure as in the treatment of complement-fixation test results, the MAR values have been calculated. Some of these values were approximate as the end-point in one test had not been observed and this affected a large number of other results. While inspection of the primary data of neutralization indices shows that the highest heterologous neutralizations were obtained by S-601/60 serum for dengue type 1 virus antigen, and by dengue type 1 serum for S-601/60 virus antigen, reference to the analysis shows that this relationship could also be expressed numerically in comparison to the relationships for the other virus types used in the test.

TABLE 3
NEUTRALIZATION TESTS AND ANTIGENIC ANALYSIS OF S-601/60 AND S-843/60

Neutralization index

Antigen	Serum [a]					
	D 1	D 2	D 3	D 4	S-601/60	S-843/60
Dengue type 1	3.4	0	1.3	1.1	1.7	0
Dengue type 2	1.2	2.8	1.9	1.5	1.1	2.1
Dengue type 3	0	0	1.8 [b]	0.9	0	0
Dengue type 4	0	0	0.9	4.5	0	1.1
S-601/60	2.6	0	1.8	1.5	1.5	0
S-843/60	0	2.5	1.2	1.8	0	3.9

Heterologous/homologous ratio

Antigen	Serum					
	D 1	D 2	D 3	D 4	S-601/60	S-843/60
Dengue type 1	1	0	0.72	0.24	1.13	0
Dengue type 2	0.35	1	1.05	0.33	0.73	0.54
Dengue type 3	0	0	1	0.20	0	0
Dengue type 4	0	0	0.50	1	0	0.28
S-601/60	0.76	0	1	0.33	1	0
S-843/60	0	0.89	0.67	0.40	0	1

Mean antigenic relationship (MAR)

Virus	Virus					
	D 1	D 2	D 3	D 4	S-601/60	S-843/60
Dengue type 1						
Dengue type 2	0.18					
Dengue type 3	0.36	0.53				
Dengue type 4	0.12	0.17	0.35			
S-601/60	0.95	0.37	0.50	0.17		
S-843/60	0	0.72	0.34	0.34	0	

[a] 0 = neutralization index < 0.5.
[b] Neutralization index less than stated.

MAR values in neutralization tests show wider variation than in complement-fixation tests since the former are subject to greater experimental error. However, the close though not identical relationship of S-601/60 virus isolate to dengue type 1 virus is confirmed, and similarly the close relationship of S-843/60 virus isolate to dengue type 2 virus. In this

TABLE 4
ANTIGENIC ANALYSIS OF S-378/61, S-430/61 AND S-554/61

Heterologous/homologous ratio

Antigen	Serum						
	D 1	D 2	D 3	D 4	S-378/61	S-430/61	S-554/61
Dengue type 1	1	0	0.29	0.07	0.75	0.25	0
Dengue type 2	0	1	0.19	0.07	3.00	0.83	0
Dengue type 3	0.29	0	1	0.38	1.00	0.25	0
Dengue type 4	0	0	0.19	1	0	0	4.0
S-378/61	0	0	0.24	0	1	0.50	0
S-430/61	0.14	0.83	0.33	0.15	2.0	1	0
S-554/61	0	0	0	0.46	0	0	1

Mean antigenic relationship (MAR)

Virus	Virus						
	D 1	D 2	D 3	D 4	S-378/61	S-430/61	S-554/61
Dengue type 1							
Dengue type 2	0						
Dengue type 3	0.29	0.10					
Dengue type 4	0.04	0.04	0.29				
S-378/61	0.38	1.50	0.62	0			
S-430/61	0.20	0.83	0.29	0.08	1.25		
S-554/61	0	0	0	2.23	0	0	

experiment, the dengue type 3 virus antigen used was of low titre and the relationship of this virus to the other viruses was not demonstrated clearly.

1961 virus isolates

Complement-fixation tests. Five viruses isolated in 1961 were compared by complement-fixation tests with prototype viruses and the 1960 virus isolates.

In an early experiment, the results obtained were considered unsatisfactory because the degree of fixation obtained was generally low (see Fig. 2). Surprisingly, the antigenic analysis shown in Table 4 gives conclusions consistent with those obtained in subsequent experiments. Virus isolates S-378/61 and S-430/61 were most closely related to dengue type 2 virus, whereas virus isolate S-554/61 was most closely related to dengue type 4 virus. The high value of the MAR for the latter is a reflection of the error possible when dealing with small numbers.

The above conclusions were confirmed by tests between virus isolates S-378/61 and S-430/61 and dengue type 2 viruses, the results being shown in Fig. 3 and Table 5.

Similarly, the close relationship of virus isolate S-554/61 to dengue type 4 virus was shown in tests of this virus isolate against the prototype viruses. The results are shown in Fig. 4 and Table 6.

S-132/61 and S-212/61 were tested against dengue type 3 and type 4 prototype viruses and virus isolates S-601/60 and S-843/60, the latter standing in, as it were, for dengue type 1 and type 2 prototype viruses. The results obtained (Fig. 5; Table 7) were not very satisfactory as, for some reason, the test did not distinguish dengue type 3 from dengue type 4. However, the experiment did show that

TABLE 5

ANTIGENIC ANALYSIS OF S-378/61 AND S-430/61

Heterologous/homologous ratio

Antigen	Serum		
	D 2	S-378/61	S-430/61
Dengue type 2	1	1.42	0.95
S-378/61	0.75	1	0.84
S-430/61	1.10	1.96	1

Mean antigenic relationship (MAR)

Virus	Virus		
	D 2	S-378/61	S-430/61
Dengue type 2			
S-378/61	1.09		
S-430/61	1.03	1.40	

TABLE 6

ANTIGENIC ANALYSIS OF S-554/61

Heterologous/homologous ratio

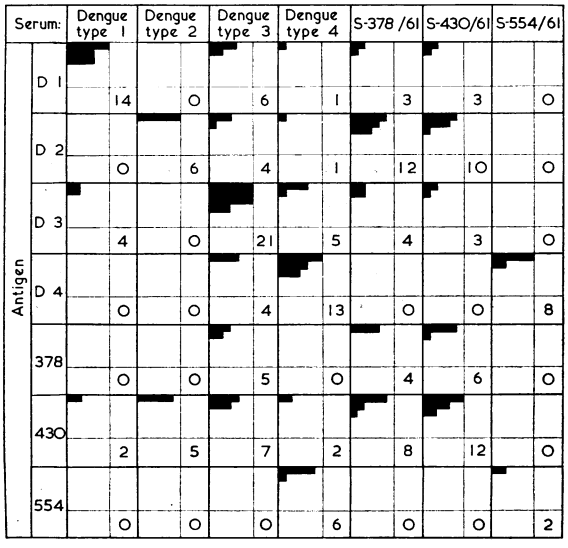
Antigen	Serum				
	D 1	D 2	D 3	D 4	S-554/61
Dengue type 1	1	0.27	0.57	0.22	0
Dengue type 2	0.50	1	0.48	0	0
Dengue type 3	0.21	0.12	1	0.39	0
Dengue type 4	0.07	0	0.43	1	0.77
S-554/61	0	0	0.13	0.83	1

Mean antigenic relationship (MAR)

Virus	Virus				
	D 1	D 2	D 3	D 4	S-554/61
Dengue type 1					
Dengue type 2	0.39				
Dengue type 3	0.39	0.30			
Dengue type 4	0.15	0	0.41		
S-554/61	0	0	0.07	0.80	

FIG. 2

CROSS COMPLEMENT-FIXATION TESTS OF VIRUS ISOLATES S-378/61, S-430/61 AND S-554/61



S-132/61 and S-212/61 were closely related (MAR= 1.03), were closer to dengue type 4 than to type 3, but were not closely related to either of the 1960 isolates (MAR from 0.19 to 0.44).

To verify the identification of virus isolates S-132/61, S-212/61 and S-554/61 as dengue type 4

FIG. 3

CROSS COMPLEMENT-FIXATION TESTS OF VIRUS ISOLATES S-378/61 AND S-430/61

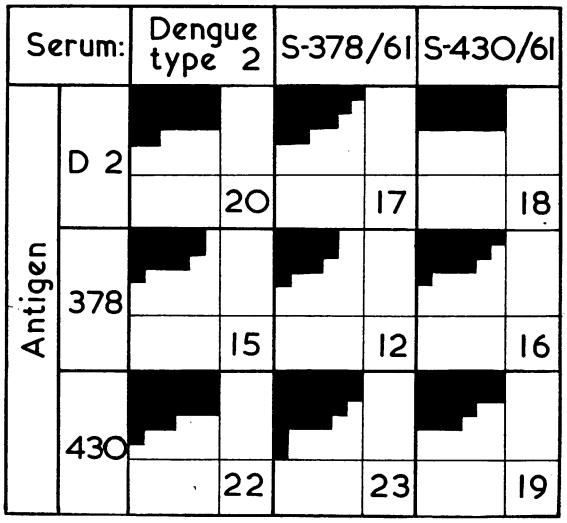


TABLE 7

ANTIGENIC ANALYSIS OF S-132/61 AND S-212/61

Heterologous/homologous ratio

Antigen	Serum					
	S-601/60	S-843/60	D 3	D 4	S-132/61	S-212/61
S-601/60	1	0.21	0.10	1	0.24	0.13
S-843/60	0.15	1	0	0.80	0.24	0
Dengue type 3	0.65	0.43	1	1.10	0.55	0.73
Dengue type 4	0.25	0.50	1.40	1	0.83	1.20
S-132/61	0.35	0.64	1.50	1.80	1	1.20
S-212/61	0.25	0.36	1.20	1.60	0.86	1

Mean antigenic relationship (MAR)

Virus	Virus					
	S-601/60	S-843/60	D 3	D 4	S-132/61	S-212/61
S-601/60						
S-843/60	0.18					
Dengue type 3	0.37	0.22				
Dengue type 4	0.63	0.65	1.25			
S-132/61	0.30	0.44	1.04	1.32		
S-212/61	0.19	0.18	0.96	1.40	1.03	

FIG. 4

CROSS COMPLEMENT-FIXATION TESTS OF VIRUS ISOLATE S-554/61

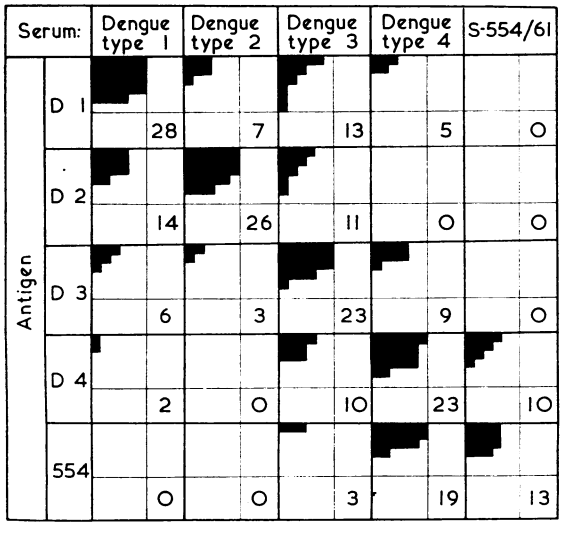
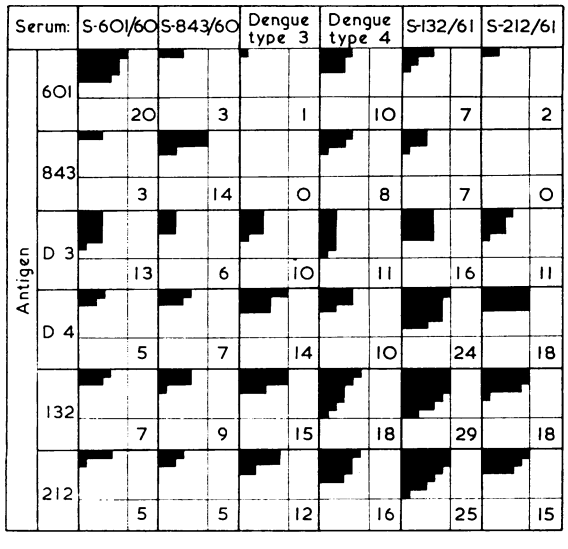


FIG. 5

CROSS COMPLEMENT-FIXATION TESTS OF VIRUS ISOLATES S-601/60, S-843/60, S-132/61 AND S-212/61



viruses, they were tested against dengue type 3 and type 4 prototype viruses. The areas of fixation and the MAR values are given in Table 8.

Neutralization tests. The results obtained by complement-fixation tests were confirmed by cross-neutralization tests (see Table 9). For economy, a complete 9×9 cross-neutralization test was not attempted and the results indicate only the relationships of each virus isolate to the prototype viruses. In this experiment only mouse immune sera were used. The calculation of the heterologous/homologous ratios is not shown, but the MAR values of relevance are given in Table 10.

In this experiment, dengue type 3 prototype viruses S-132/61, S-378/61, S-430/61 and S-554/61 had not yet been adapted to adult mice. The performance of neutralization tests in infant mice results in even greater experimental errors and so the mean antigenic relationship values obtained must be interpreted with caution. However, the identification of the virus isolates by complement-fixation tests may be regarded as confirmed by the neutralization test results.

1962 virus isolates

Eleven viruses were isolated in 1962. Identification was by complement-fixation tests and sensitized erythrocyte agglutination tests.

TABLE 8

COMPLEMENT-FIXATION TESTS AND ANTIGENIC ANALYSIS OF S-132/61, S-212/61 AND S-554/61

CF result (No. of squares)

Antigen	Serum				
	D 3	D 4	S-132/61	S-212/61	S-554/61
Dengue type 3	33	17	19	19	6
Dengue type 4	4	17	15	17	11
S-132/61	8	31	20	22	16
S-212/61	10	28	22	24	15
S-554/61	3	28	21	20	13

Mean antigenic relationship (MAR)

Virus	Virus				
	D 3	D 4	S-132/61	S-212/61	S-554/61
Dengue type 3					
Dengue type 4	0.56				
S-132/61	0.60	1.29			
S-212/61	0.55	1.18	1.01		
S-554/61	0.28	1.25	1.14	0.99	

TABLE 9

NEUTRALIZATION TESTS OF 1961 VIRUS ISOLATES

Antigen	Serum [a]								
	D1	D2	D3	D4	S-132/61	S-212/61	S-378/61	S-430/61	S-554/61
Dengue type 1	4.0	NT	NT	NT	2.6	2.2⁻	2.0⁻	2.8	2.1
Dengue type 2	NT	3.5	NT	NT	3.2	2.2	2.8	3.5	1.9
Dengue type 3	NT	NT	4.4⁺	NT	3.0	2.1⁻	2.0⁻	2.4	2.2⁻
Dengue type 4	NT	NT	NT	4.9⁺	4.9⁺	4.2	2.7	2.7	3.9
S-132/61	1.7	1.1	0.9	2.2	4.0	NT	NT	NT	NT
S-212/61	1.5	1.5	NT(I)	2.5	NT	2.5	NT	NT	NT
S-378/61	2.5	3.0	2.6	2.3	NT	NT	3.0	NT	NT
S-430/61	2.4	4.0	3.0	1.3⁻	NT	NT	NT	4.1	NT
S-554/61	0.5⁻	0.9⁻	0.9⁻	2.6	NT	NT	NT	NT	2.4

[a] NT = not tested; NT(I) = not tested because of insufficient materials. The signs + or − indicate end-points were not obtained in these experiments and the neutralization indices are greater or less than those stated. The figures given, however, were used in calculating the MAR.

TABLE 10

MEAN ANTIGENIC RELATIONSHIPS OF 1961 VIRUS ISOLATES TO DENGUE PROTOTYPE VIRUSES, AS DETERMINED BY NEUTRALIZATION TESTS

Virus isolate	Prototype virus			
	D 1	D 2	D 3	D 4
S-132/61	0.54	0.56	0.48	0.84
S-212/61	0.63	0.66	(0.84) [a]	1.10
S-378/61	0.65	0.90	0.63	0.69
S-430/61	0.64	1.00	0.64	0.47
S-554/61	0.51	0.53	0.56	1.08

[a] Heterologous/homologous ratio for S-212/61 serum against dengue type 3 virus; *not* a mean value.

Complement-fixation tests. Table 11 gives the fixation areas obtained in cross complement-fixation tests between the virus isolates and the prototype viruses. The virus isolates were not compared against each other. Table 12 gives MAR values determined from the results obtained. Unfortunately, some virus isolates—e.g., S-437/62, S-452/62 and S-521/62—had not been adapted sufficiently to mice at the time of testing. CF antigens prepared from these strains were of low potency and the small amount of homologous fixation obtained resulted in exaggerated heterologous/homologous ratios. None the less, the antigenic analysis (Table 12) shows that two virus isolates were dengue type 1 viruses and eight virus isolates were dengue type 2 viruses. In the case of virus isolate S-521/62, the test could not distinguish it from either dengue type 1 or type 2 virus. This virus isolate was, however, identified by another method.

Sensitized erythrocyte agglutination test. Table 13 shows the results of tests by this technique with the 11 virus isolates. As a control for specificity JEV antigen was included, but antigens were prepared for only five of the virus isolates. For convenience, the results are expressed as the number of serum dilutions (y), giving positive agglutination. Since the initial serum dilution was 1/20, the titres obtained by the mouse immune sera against each antigen are easily calculated as 10×2^y. The agglutinating

TABLE 11

COMPLEMENT-FIXATION TESTS [a] BETWEEN 1962 VIRUS ISOLATES AND PROTOTYPE VIRUSES

Antigen	Serum														
	D1	D2	D3	D4	S-389/62	S-429/62	S-437/62	S-438/62	S-452/62	S-461/62	S-509/62	S-521/62	S-575/62	S-630/62	S-699/62
Dengue type 1	39	28	27	27	40	22	33	24	5	25	27	34	22	27	30
Dengue type 2	26	40	24	20	29	32	22	36	19	36	33	32	29	35	36
Dengue type 3	37	28	46	29	19	24	28	25	13	25	23	18	29	25	25
Dengue type 4	23	25	27	32	26	26	19	17	11	22	18	15	16	21	24
S-389/62	34	21	18	14	35										
S-429/62	26	34	17	13		33									
S-437/62	11	9	8	4			8								
S-438/62	17	32	20	11				19							
S-452/62	11	12	8	4					6						
S-461/62	24	35	14	13						29					
S-509/62	25	40	25	12							23				
S-521/62	19	25	11	7								12			
S-575/62	40	41	34	27									30		
S-630/62	21	35	15	13										20	
S-699/62	21	31	14	11											22

[a] Results expressed as number of squares. Eight serum dilutions were tested against six antigen dilutions. The maximum area observable was 48 squares.

TABLE 12

ANTIGENIC ANALYSIS OF 1962 VIRUS ISOLATES BY COMPLEMENT-FIXATION TEST

Virus isolate	Prototype virus				Identification
	D 1	D 2	D 3	D 4	
S-389/62	1.01	0.68	0.47	0.59	1
S-429/62	0.67	0.91	0.55	0.60	
S-437/62	2.22	1.50	1.84	1.26	1
S-438/62	0.83	1.35	0.85	0.62	2
S-452/62	0.56	1.73	1.17	0.98	2
S-461/62	0.74	1.06	0.58	0.59	2
S-509/62	0.91	1.22	0.77	0.58	2
S-521/62	1.67	1.66	0.87	0.74	?
S-575/62	0.84	1.0	0.87	0.69	2
S-630/62	0.95	1.32	0.79	0.73	2
S-699/62	0.95	1.21	0.73	0.72	2

titres of an immune serum are greatest for similar antigens. MAR values were not calculated as antigens were not prepared for all virus isolates. These results, however, confirm the identification of the virus isolates by complement-fixation tests; S-521/62 was shown to be a dengue type 2 virus.

SEROLOGICAL REACTIONS

Group B arbovirus cross-reactions

Many patients, including those from whom viruses were isolated, were tested for antibody with dengue type 1 antigens. Most patients had antibody rises for dengue type 1 antigens whether tested by the complement-fixation test, the neutralization test or the haemagglutination-inhibition test. Many also had concurrent rises of antibody for Japanese encephalitis antigen as well. In the absence of virus isolation, the confirmation of diagnosis by laboratory tests is handicapped by this group B cross-reaction. Even if the rise of antibody for the infecting

TABLE 13

SENSITIZED AGGLUTINATION TESTS[a] ON 1962 VIRUS ISOLATES

Mouse immune serum	Antigen										Identification
	JEV	D1	D2	D3	D4	S-438/62	S-452/62	S-461/62	S-521/62	S-699/62	
JEV	7	1	1	1	2	1	1	1	1	1	
Dengue type 1	2	7	2	2	3	1	2	2	3	3	
Dengue type 2	2	2	7	2	3	6	6	7	7	7	
Dengue type 3	2	3	3	8	4	1	1	1	2	2	
Dengue type 4	2	2	1	2	8	1	2	2	2	2	
S-438/62	2	3	6	2	2	5	5	6	6	5	
S-452/62	2	1	5	1	2	4	4	4	5	5	
S-461/62	2	2	6	2	2	6	6	6	7	7	2
S-521/62	2	2	6	1	1	5	5	6	7	6	2
S-699/62	2	3	7	3	2	6	6	7	7	7	2
S-389/62	2	5	2	1	2	1	2	2	2	2	1
S-429/62	2	3	6	3	2	5	5	5	5	5	2
S-437/62	2	7	3	1	2	3	3	3	3	2	1
S-509/62	2	2	5	1	1	5	5	5	5	5	2
S-575/62	2	2	5	1	1	4	4	4	5	5	2
S-630/62	2	2	6	1	1	6	6	6	6	6	2

[a] Each result is the number of serum dilutions giving positive agglutination = y. Initial serum dilution = 1/20. Serum titre = 10×2^y.

virus is assumed to be higher—and this is not always so—in practice the differences in the antibody titres observed are sometimes too small to allow any confident evaluation of results.

Similar difficulties also arise in the serological diagnosis of Japanese encephalitis infections, in which we have never succeeded in isolating virus from patients during life. Prior to 1960, although it was common to find both dengue as well as JEV antibodies in adult sera, the usual finding in children with JEV infection was antibody for this virus antigen only. Since 1960, the widespread circulation of dengue viruses in Singapore has broadened the antibody spectrum of children as well as of adults and caused difficulties in the interpretation of results.

Sensitized erythrocyte agglutination (SEA) diagnostic test

Following infection, SEA antibodies rise to very high titres, often exceeding 10 240. Although cross-reactions are present, they are usually of much lower titres. Invariably, the difference in titres of a patient's serum for dengue virus antigen and JEV antigen are of the order of 64-fold as compared to 4- to 16-fold for CF antibody differences. Table 14 shows the results obtained in tests on a number of sera for both dengue type 1 antigens and JEV antigens. The superiority of the SEA test is apparent, and with further experience it is hoped that it can be adopted as a routine diagnostic test. In application, it is the simplest of all tests to perform once the antigen has been made.

TABLE 14

REPRESENTATIVE SEROLOGICAL REACTIONS IN SUSPECTED ARBOVIRUS INFECTIONS, 1962

Serum No.	Patient's sex and age (years)	Clinical diagnosis [a]	Interval between serum specimens (days)	Serological reactions [b]						Confirmation of diagnosis [f]
				CF [c]		SEA [d]		HI [e]		
				JEV	D1	JEV	D1	JEV	D1	
437	M. 6	E	2 [g]	0	0	0	0	0	0	
			16	0	64	0	640	20	640	No
438	M. 6	HF	2 [g]	0	0	0	320	0	80	
			26	0	256	160	10 240	640	5 120	Yes
469	M. 2	E	6	0	0	0	0	Not tested		
			24	0	0	40	40			No
457	M. 50	E	6	8	8	640	0	10	0	
			33	64	64	1 280	20	80	10	Yes
460	F. 13	E	2	0	0	0	20	10	10	
			30	0	0	0	40	10	10	No
477	M. 19	E	4	8	8	0	0	0	0	
			22	32	32	0	0	0	0	No
479	M. 18	HF	9	0	0	0	40	0	20	
			26	0	0	0	40	0	20	No
493	M. 4	HF	4	256	256	0	10 240	2 560	5 120	
			20	256	256	40	10 240	5 120	5 120	Yes
494	M. 9	HF	4	0	0	0	160	20	160	
			20	64	256	80	10 240	2 560	5 120	Yes
498	M. 5	HF	7	256	256	80	10 240	5 120	5 120	
			21	256	256	40	10 240	2 560	5 120	Yes

[a] E = encephalitis; HF = haemorrhagic fever.
[b] Titres expressed as reciprocals. 0 = less than lowest dilution.
[c] Serum dilutions from 1/8 to 1/256.
[d] Serum dilutions from 1/20 to 1/10 240.
[e] Serum dilutions from 1/10 to 1/5 120.
[f] In all instances the laboratory confirmation or otherwise of the clinical diagnosis was consistent with the outcome of the illness.
[g] Virus was isolated from these serum specimens.

DISCUSSION

The isolation of dengue-type viruses in Singapore raises questions of epidemiological interest. Prior to 1960, although dengue fever had been recognized, there had never been an outbreak of so many cases of a dengue-like disease with haemorrhagic manifestations. It is clear that Singapore haemorrhagic fever, as the latter has been called, has recently appeared in Singapore.

Although the Singapore disease is significantly different clinically from the haemorrhagic fevers that have occurred in the Philippines and in Thailand, the etiological agents are closely related. Dr Hammon (personal communication) found that our virus isolates S-601/60 and S-843/60 were more closely related to viruses isolated in Thailand than to dengue type 1 and dengue type 2 prototype viruses. The Thailand isolates have not been used in our laboratory.

A reasonable hypothesis relating together the haemorrhagic fevers that have occurred in South-East Asian countries is that the disease originated in one locality and spread to neighbouring countries. The different clinical picture observed in the different areas must then be attributed to unknown factors. It should be noted, however, that whereas Chikungunya virus accounts for a large proportion of haemorrhagic fever in Thailand, indistinguishable from that due to dengue-type viruses, it has not been detected in Singapore.

The alternative hypothesis, that the outbreaks in various places were due to indigenous viruses, is not so plausible. One would have to postulate either that a number of " haemorrhagic " variants had evolved within a few years of each other in different places or that the disease had been occurring all the while without attention being drawn to it. The sudden appearance in Singapore of a large number of cases of a disease hitherto unrecognized would favour the theory that the disease was imported. Recent observations of a possible change in the clinical picture is consistent with the adaptation of a population to a new disease.

If indeed, haemorrhagic fever has spread from country to country the means by which this happened is of considerable interest. A study of the epidemiology of haemorrhagic fever in South-East Asian countries would cast light on the mechanism of the transmission of arboviruses.

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RÉSUMÉ

En 1960 apparut à Singapour une maladie ressemblant à la dengue et caractérisée par d'importantes manifestations hémorragiques; en moins d'un an, plus de 200 cas furent hospitalisés. Du fait de sa ressemblance, à bien des égards, avec les fièvres hémorragiques de Thaïlande et des Philippines, cette maladie fut appelée fièvre hémorragique de Singapour.

Vingt et un virus ont été isolés chez les malades hospitalisés de 1960 à 1962 au Singapore General Hospital. L'on a utilisé, pour l'identification, les tests conventionnels de fixation du complément et de neutralisation. En outre, un nouveau test, appelé agglutination d'érythrocytes sensibilisés, a été employé. Dans cette méthode, l'antigène est représenté par une suspension d'érythrocytes de poulets âgés de 1 à 2 jours, érythrocytes sensibilisés par l'hémagglutine virale en milieu acide (pH : 6,8). Il n'y a pas d'agglutination de l'antigène lorsque la suspension est à pH 7,6. C'est à ce pH que s'effectue le mélange avec l'anticorps.

La distribution des types de virus de la dengue selon l'année d'identification est la suivante: en 1960 ont été identifiés un virus de type 1 et un de type 2; en 1961 deux de type 1 et trois de type 4; en 1962 deux de type 1 et neuf de type 2. Trois virus isolés en 1963 n'ont pas encore été identifiés. L'on n'a pas retrouvé le virus Chikungunya, cause importante de fièvre hémorragique en Thaïlande.

Il est probable que les fièvres hémorragiques des Philippines, de Thaïlande et de Singapour sont dues aux mêmes virus ayant migré de pays en pays. La différence entre les tableaux cliniques observés dans les différents pays peut être imputable à des facteurs inconnus. L'étude épidémiologique de la fièvre hémorragique de l'Asie du Sud-Est contribuerait à élucider le mécanisme de la transmission des arbovirus.

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