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VIROLOGICAL STUDY OF A DENGUE TYPE 1 EPIDEMIC AT RIO DE JANEIRO

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A dengue outbreak started in March, 1986 in Rio de Janeiro and spread very rapidly to other parts of the country. The great majority of cases presented classical dengue fever but there was one fatal case, confirmed by virus isolation.

Dengue type 1 strains were isolated from patients and vectors (Aedes aegypti) *in the area by cultivation in* A. albopictus *C6/36 cell line. The cytopathic effect (CPE) was studied by electron microscopy.*

An IgM capture test (MAC-ELISA) was applied with clear and reproducible results for diagnosis and evaluation of virus circulation; IgM antibodies appeared soon after start of clinical disease, and persisted for about 90 days in most patients. The test was type-specific in about 50% of the patients but high levels of heterologous response for type 3 were observed.

An overall isolation rate of 46,8% (813 virus strains out of 1734 specimens) was recorded. The IgM test increased the number of confirmed cases to 58,2% (1479 out of 2451 suspected cases).

The importance of laboratory diagnosis in all regions where the vectors are present is emphasized.

Key words: dengue virus-dengue epidemic – flavivirus – Rio de Janeiro

This paper describes virological aspects of a dengue epidemic in Rio de Janeiro first detected in March, 1986 and confirmed by laboratory tests in the following months. It soon spread to other parts of the country and it is estimated that more than two million clinical cases have occurred specially along the east coast of Brazil, during the year following the first virus isolations.

Only dengue virus type 1 was isolated so far, in all the areas investigated.

MATERIALS AND METHODS

Virus isolations – Serum specimens of acute clinical cases, were received from different sources: out-patient units, home-by-home surveys and hospitalized patients. Convalescent serum specimens were obtained from many patients. Post mortem tissues from possible fatal cases, were also received for virus isolation.

The sera were diluted 1/10 in tissue culture medium L-15 to eliminate toxicity, and inoculated (50 μl) in the *Aedes albopictus* C6/36 cell culture strain (about 10^6 cells/tube) maintained with L-15 medium, supplemented with 2% fetal bovine serum, 1% non-essencial amino-acids and 10% tryptose phosphate broth. The tissues were ground in tissue culture medium and inoculated as a 20% suspension, without further dilution.

A *Toxorhynchites amboinensis* cell line TRA 284 was employed for virus isolation from some of the specimens.

The inoculated cell cultures were kept at 28°C and observed daily for viral cytopathic effect (CPE). Tubes showing CPE were processed for identification by an indirect fluorescence test, using dengue serotype-specific monoclonal antibodies and anti-mouse IgG conjugate. Tubes showing no CPE after 10 days of inoculation were processed for direct fluorescent antibody

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test using a fluorescein isothyocinate (FITC) conjugate prepared from pooled human sera with high haemagglutination-inhibition (HI) antibody titres. Monoclonal antibodies were obtained from CDC/Atlanta (Gubler et al., 1984).

Virus isolation from vectors – Adult females of *Aedes aegypti* were captured in an epidemic area (Nova Iguaçu county), in May, 1986. About 120 mosquitoes were ground in L-15 tissue culture medium and, after centrifugation and antibiotic treatment, inoculated into cell cultures as described above.

Serology – Acute and convalescent sera, were tested by an IgM capture assay (MAC-ELISA) as described by Kuno et al. (1987).

Electron microscopy – Tissue cultures of *A. albopictus*, clone C6/36 infected with dengue virus were collected in sucessives days after infection, fixed with 1% glutaraldehyde in phosphate buffer and processed for transmission electron microscopic observations.

Virus titration – Patient sera, known to be virus positive by previous isolation, were diluted and 50 μl of each dilution inoculated into tubes of *A. albopictus* and of TRA 284 cell lines. The tubes were observed and handled as described for virus isolation. Titers were calculated as $TCID_{50}$ (Reed & Muench, 1938).

RESULTS

Epidemiological background – An acute febrile disease resembling classical dengue virus infection was observed in March, 1986 in Nova Iguaçu county near Rio de Janeiro city (23°S/43°W). *Aedes aegypti* was present in the area as well as along the east coast of the country, after its reintroduction in 1975 in Salvador/Bahia (13°S/39°W).

The epidemic soon spread to surrounding areas, including the cities of Rio de Janeiro and Niterói and also to other states later in the same year (Alagoas, 10°S/36°W and Ceará 4°S/39°W).

In the Rio de Janeiro area, the epidemic declined in winter but a large number of cases were detected again in summer, during the first months of 1987 (Fig. 1). A decline in the number of cases was recorded after April, 1987 and only a few cases were observed by June, 1987.

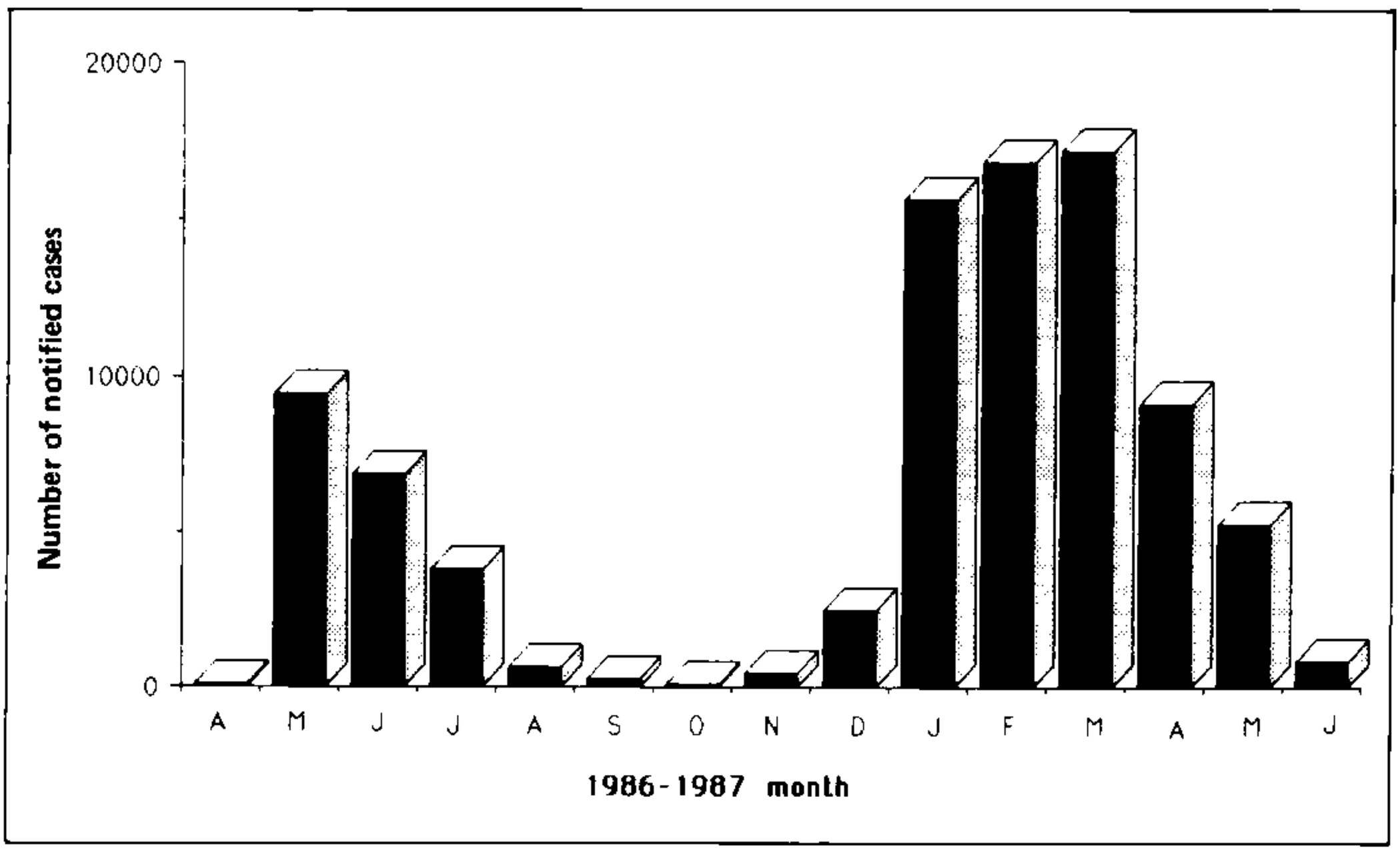

Fig. 1: notified cases of dengue in Rio de Janeiro State, April, 1986 – June, 1987. Source: SUCAM, Ministry of Health.

Signs and symptoms of classical dengue fever were observed but an increase of hemorrhagic manifestations was observed over the course of the epidemic.

Virological results – Only dengue virus type 1 was isolated from clinical specimens or mosquito pools. The isolation rate reached about 100% in the first specimens obtained declining later on. CPE in tissue cultures appeared as numerous syncytia, 3 to 4 days after the start of the lesion; cell vacuolation was observed and also seen by electron microscopy. The later (Fig. 2) revealed viral particles inside compartments of variable sizes and partially associated with internal membrane systems. These features were seen in early infection. Small vesicles with nearly two or three times the diameter of the endoplasmic reticulum, containing incomplete and a few complete virus particles, were detected; the cell nucleus was displaced to the periphery of the cell. The multivesiculated vacuoles increased in volume and number of internal elements; finally they fused into a unique large cell compartment but virus particles were rarely found inside them. At final stages of infection, the cells broke up and released the vesicles with virus particles, which remained associated to cell membranes; this may be one of the reasons why it was difficult to find isolated virus particles by negative staining of cell homogenates.

A comparison of the two mosquito cell lines available in the laboratory (*A. albopictus* clone C6/36 and *T. amboinensis* TRA-284) revealed that from 165 clinical specimens, virus was isolated from 52 cases in both cell lines, but 3 virus strains were isolated only in C6/36 line and one strain was detected only in TRA-284 cells, with a concordance rate of 97.5% (Table I).

TABLE I

Comparison of C6/36 and TRA 284 cell lines for virus isolation from 165 suspected of dengue in Rio de Janeiro

		TRA 284 Cell Strain	
		Positive	Negative
C6/36 Cell Strain	Positive	52	3
	Negative	1	109

In order to evaluate the virus content of the sera of dengue patients, 10 virus positive clinical specimens were titrated in C6/36 cell line. The titers found in these patients ranged from $10^{3.3}$ to $10^{8.3}$ $TCID_{50}$/ml, with a geometrical mean titer of $10^{6.15}$ $TCID_{50}$/ml. The IgM capture test, applied to 1066 sera from which clinical data were available, showed positivity after the 2nd day of infection and persistence of IgM antibodies for about 90 days (Table II).

TABLE II

Anti-dengue IgM in patients with signs and symptoms of dengue infection, Rio de Janeiro

Day after onset of disease	Nº patients analysed	Positive samples	%
2	113	14	12.3
3	104	27	26.0
4	94	21	22.3
5	84	48	57.1
6	66	52	78.8
7	69	61	88.4
8.10	103	95	92.2
11-15	107	104	97.2
16-20	152	149	98.0
21-35	47	42	89.3
26-30	40	39	97.5
31-60	75	71	94.6
61-90	12	9	75.0
Total	1066	732	–

The correlation between the IgM test and virus isolation, tested in specimens collected in the first week of clinical disease, is shown at Table III where the isolation rates and the IgM responses from the same patients are compared. A inverse correlation can be observed, with a decline in the isolation rate during the first week of disease.

TABLE III

Dengue virus type 1 isolation and anti-dengue IgM in relation to onset of disease, Rio de Janeiro

Days after onset of disease	Dengue cases	% IgM +	% Isolation
2	78	17.9	82
3	70	38.5	61
4	66	31.8	68
5	79	60.7	39
6	60	86.6	13
7	67	91.0	9
8-10	96	99.0	1
Total	516	–	–

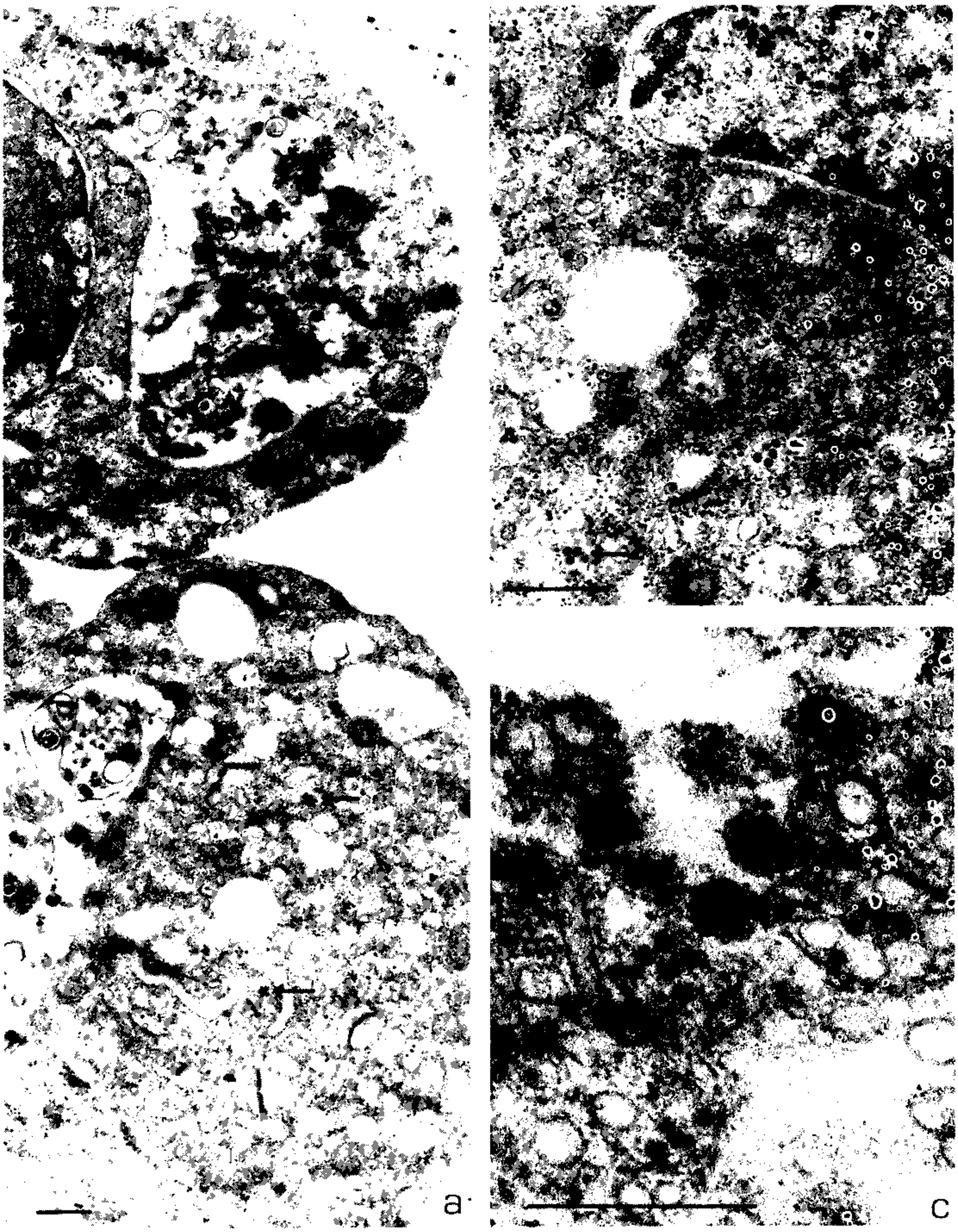

Fig. 2: electron microscopy. a – Dengue infected *Aedes albopictus* cell at initial stage (arrows show viral particles). Cell with normal appearance at upper corner (15.000x). b – Virus replication area with particles (arrows) inside rough endoplasmic reticulum (30.000x). c – Final infection stage: ruptured cell and aggregates of virus particles (65.000x). Bar = 0.5 μ.

In order to study the specificity of the IgM response in our patients, we tested sera previously known to be positive by MAC-ELISA for dengue 1, with all four dengue virus types. The results are presented at Table IV, where monospecific responses could be obtained in 50% of the patients, with a high heterologous response for type 3 in the other patients.

TABLE IV

Specific IgM response for dengue virus in clinical cases, Rio de Janeiro

Patients	IgM Response			
	Den 1	Den 2	Den 3	Den 4
68	68	8	34	3

The MAC-ELISA was also applied at the start of the outbreak, to blood specimens from the general population, including 709 specimens obtained in Nova Iguaçu county and in 416 specimens from Niterói city, collected by reandomized sampling methods; positivity rates of 16% and 4% respectively were observed showing different epidemiological patterns, at the moment of blood specimen collection.

Vaccinated with 17D yellow fever strain show no reactivity with dengue antigen in the MAC-ELISA, but dengue patients sera, cross react broadly with yellow fever antigen (Nogueira et al., unpublished data).

DISCUSSION

Dengue virus type 1 outbreak started in Rio de Janeiro State (Nova Iguaçu county) and the virus was isolated in April, 1986. The disease was characterized by fever, headache, retrobulbar pain, nackache, muscle and joint pain, marked weakness and prostration (Schatzmayr et al., 1986).

These cases were the first confirmed dengue infections in Rio de Janeiro State since the last outbreak, described years ago on clinical grounds (Antonio Pedro, 1923).

The last previously described dengue outbreak in Brazil, occurred in the Amazon area (Roraima territory), city of Boa Vista in 1981. Dengue virus type 1 and 4 were isolated and 7000 cases were estimated to have occurred (Osanai et al., 1983).

About 90% to 95% of the dengue type 1 strains isolated during the epidemic in Rio de Janeiro, show a clear and consistent CPE in *A. albopictus* C6/36 cell line; in some specimens CPE could be observed at 24-48 hours after inoculation.

Strain variation among dengue virus may influence reactivity with the monoclonal antibodies used, in particular with types 1 and 2 (Guber, 1987); although some weaker reactions could be rarely observed, no doubtful results were recorded by the use of the described immunofluorescence techniques.

Kuno et al. (1985) studying the sensitivity of mosquito cell lines for dengue virus isolation, found that the isolation rate was lower in C6/36 than in TRA-284; however our findings (Table I) show that *A. albopictus* cell line was at least as sensitive as the TRA-284, for the current dengue 1 strain. Since in our laboratory the former cell line has presented less maintenance problems and the CPE has been easier to recognize, it was decided to use only the C6/36 cell line for isolation. A high concordance of results on virus isolation in both cell strains, was observed but three virus strains were isolated only in C6/36 cell strain and one virus strain were obtained only in the TRA-284 cell line.

A total of 813 virus strains were isolated from 1734 specimens inoculated until June 1987. Our isolation rate of 46.8% has to be analyzed considering the epidemic situation and difficulties on specimens collection due to absence of previous experience with dengue infections in the area.

It should pointed out that from July to August, 1986 influenza A virus H1N1, was detected in Rio de Janeiro, circulating in the dengue epidemic area (WHO Wkly. Epidem. Rec.). This could have led to difficulties in the clinical diagnosis of acute febrile diseases.

The observed increase of hemorrhagic manifestations as the outbreak progressed has been previously described (Morens et al., 1986).

Dengue virus was isolated from a post-mortem liver specimen obtained from a patient

with an acute clinical disease; infortunately, no blood specimens were available for serological testing. The virus was reisolated from the liver fragments kept at – 80°C. No virus has been obtained from post-mortem specimens collected from other possible dengue fatal cases.

IgM antibodies were detected in the early phase of disease, reaching a high level after the second week and declining after the 60th day of infection. Titers as high as 1/5000 were found in some patients, between the second and third weeks from the onset of disease. A very low circulation of flavivirus in the area, before the outbreak (Pinheiro et al., 1975) may be responsible for the high IgM response in some our patients.

It is well known that response of IgM for dengue virus is not specific enough to identify the virus serotype responsible for the infection (Gubler, 1987). However among the 68 patients studied (Table IV) a monospecific response for dengue 1 was detected in 50% of the patients. About half of the patients also showed heterologous response for type 3 and much lower titers for types 2 and 4.

A decreasing isolation rates in the course of the first week of disease was observed in 516 patients. This may be related to the increasing amount of antibody in the sera. The influence of dengue haemagglutination-inhibition (HI) antibodies in middle to high titer range (1/160) making the isolation in tissue cultures less feasible, has been described (Gubler et al., 1981).

We have been able to identify 31 patients with virus and IgM antibodies present in the same specimen. An evaluation of these sera show that 19 had titers lower than 1/80 by MAC-ELISA; similar results using HI test have been observed in the dengue outbreak in Cuba (Guzman et al., 1984).

Within a total of 2541 patients studied until now dengue infection could be confirmed by virus isolation and/or serology (MAC-ELISA) in 1479 (58.2%). The value of laboratory studies in the characterization of dengue suspected cases is emphasized. This becomes specially important in view of the risk of other dengue serotypes being introduced in Brazil.

Virus surveillance has to be expanded to all areas under risk of dengue infection in the country and efforts are being made to establish a network of laboratories able to carry out flavivirus diagnosis.

In our experience, the MAC-ELISA test has been extremely useful both for the diagnosis of individual cases and for epidemiological surveys as it detects both apparent and inapparent recent infections.

RESUMO

Aspectos virológicos de uma epidemia de dengue no Rio de Janeiro – Uma epidemia de dengue iniciou-se em março de 1986, no Rio de Janeiro, alcançando rapidamente outras partes do país. Febre dengue clássica foi observada, porém o vírus do dengue foi isolado de um caso fatal.

As amostras de dengue 1 isoladas de pacientes e de fêmeas adultas de *Aedes aegypti* capturadas na área apresentaram efeito citopático na linhagem de células *A. albopictus* clone C6/36. A lesão celular foi estudada também em microscopia eletrônica, sendo descritos os dados observados.

Um teste de captura para IgM (MAC-ELISA) foi utilizado durante a epidemia tanto para o diagnóstico como para avaliar a circulação de vírus, com resultados claros e reprodutíveis; os anticorpos IgM apareceram precocemente após o início da doença clínica, permanecendo por cerca de 90 dias na maioria dos pacientes. A reação mostrou-se tipo específico em cerca de 50% dos pacientes, porém, um alto nível de respostas heterológas para dengue tipo 3 foi observado nos demais pacientes.

Observou-se uma taxa total de isolamento de 46,8% (813 amostras de vírus de 1734 pacientes). Com a reação de IgM houve um aumento de casos confirmados: 1479, em 2541 casos suspeitos (58,2%).

A possível influência dos anticorpos dos pacientes sobre a taxa de isolamento de vírus é discutida, porém a questão não está esclarecida completamente.

Os autores enfatizam a importância do diagnóstico laboratorial dos casos suspeitos de dengue em todas as regiões do país, onde os vetores são encontrados.

Palavras-chave: vírus dengue-epidemia de dengue – flavivírus – Rio de Janeiro

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