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SEROLOGICAL DIAGNOSIS OF DENGUE BY AN ELISA INHIBITION METHOD (EIM)

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An ELISA Inhibition Method (EIM) was proposed for the serologic diagnosis of dengue, comparing its results with the Hemagglutination Inhibition (HI) and the IgM capture-ELISA (MAC-ELISA). Advantages and disadvantages of both methods are discussed according to sensitivity, specificity, performance and usefulness.

As a conclusion we recommend the complementary inclusion of the EIM and MAC-ELISA substituting the HI for laboratories engaged in the diagnosis and surveillance of dengue.

Key words: ELISA Inhibition Method – MAC-ELISA – dengue

Indirect micro ELISA systems have been widely used for detection of antibodies to different viral antigens (Voller &Bidwell, 1977).

In early studies using indirect micro ELISA systems for detection of antibodies to dengue (Dittmar et al., 1979; Fernández et al., 1983 a, b; Vázquez et al., 1986), yellow fever (Deubel et al., 1983) and other flaviviruses (Roehring, 1982; Okamoto et al., 1986) this technique was shown to be more sensitive and advantageous when compared to other classical, serologic diagnostic techniques. For quite a long time now, various investigators have stated the possibility of substituting some of the classical techniques for surveillance of certain arboviruses by other more rapid diagnostic methods as IgM detection (Burke, 1983; Gadkari & Shaikh, 1984; Bundo & Igarashi, 1985). To this end, a MAC-ELISA system was proposed for dengue surveillance on the basis of monosera study (Gubler et al., 1986). In the last few years, other investigators (Figueiredo & Shope, 1987) have developed an indirect enzymatic immune assay for detection of anti-dengue IgG and IgM antibodies, using dengue virus-infected mosquito cell cultures as antigen, as an alternative to the need for purification of the virus in similar systems. Also, an IgM capture ELISA has been recently proposed to characterize dengue infections where dengue and Japanese encephalitis co-circulate (Innis et al., 1989).

In a previous study conducted in our laboratory (Vázquez & Fernández, 1989) a description was mede of overall aspects relating to standardization and use of an EIM for serologic diagnosis of dengue, and some preliminary data were given on the diagnostic value of the method.

The purpose of this study is to assess the utility of the proposed method in comparison with other techniques currently used in the diagnosis and surveillance of dengue.

MATERIALS AND METHODS

Specimens – A study was done of 35 paired sera collected from clinically suspected cases of dengue during the dengue epidemic in Nicaragua in 1985. These sera were studied previously by HI (Clarke & Casals, 1958) and then kept at –20 °C until used.

Antigens – The source of antigens for ELISA consisted of 20% raw suspensions of infected suckling mouse brain in 0,02 M Tris solution, pH 8.6, which were centrifuged at 6000 rpm for 30 min. Subsequently, the supernatant was divided into aliquots and kept at –70 °C until used.

Viruses used include Dengue-1 (Hawaii Strain), Dengue-2 (New Guinea C strain),

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Dengue-3 (H-87), Dengue-4 (H-247), Yellow Fever (sucrose-acetone extracted antigen CDC) and Saint Louis Encephalitis (donated by the Virology Institute of Bratislava, Czechoslovakia).

EIM – In our experience, we used the method described in a previous study (Vázques & Fernández, 1989), which is as follows:

Polystyrene plates M 29 A (Dynatech) were adsorbed with anti-dengue human immunoglobulins at 1 μg/ml concentration in coating buffer pH 9.6 overnight at 4 °C. In the following day, the surface was blocked with 1% bovine serum albumin (BSA) in coating buffer for 1 h at 37 °C, and then washed 3 times in PBS + 0.05% Tween 20 (PBS-T20). At each well was added 100 μl of the diluted antigen (1/40) in PBS-T20. The plates were incubed for 1 h at 37 °C, after which, they were washed again and 100 μl of the respective serum dilutions were added to each well. PBS-T20 was left as control in an equal volume to sera.

Twofold determinations were made. Incubation was performed for 1 h at 37 °C, and after an equal series of washings, the anti-dengue human IgG-peroxidase conjugate, diluted at 1/1500 in PBS-T20 with 10% calf serum, was added. The final series of washings was performed after 1 h of incubation at 37 °C, and the substrate containing orthophenylenediamine (OPD) was then added. The reaction was left to run for 30 min, after which, it was stopped with 12.5% H_2SO_4. The reading was performed at 492 nm in a Multiskan (Flow).

The estimate of the inhibition percentage and the positivity criterion for the EIM (1:10 dilution) were considered according to the previously mentioned study (Vázquez & Fernández, 1989).

That serum showing an inhibition equal to or higher than 50% was considered positive. Serum pairs were considered positive when they fulfilled any of the following 3 variants: seroconversion (first serum negative and second serum positive), significantly increased quantity of antibodies between two positive sera expressed as an increase of 10% or more in the inhibition percentage of the second serum in relation to the first one, and those showing high fixed inhibition values equal to or higher than 85% in both sera of the pair.

The ELISA titer was established as the reverse of the last dilution whose inhibition is equal to or higher than 50%, and the positivity criterion for the pair in that case is a significant increase in the quantity of antibodies between the two sera of the pair, expressed as a fourfold increase or more in the titer of antibodies.

MAC-ELISA – Basically, we followed the method proposed by Gubler (Gubler et al., 1986), with some minor modifications. We used the same conjugate used for EIM (anti-dengue human IgG-peroxidase) diluted at 1/1500 in PBS containing 20% normal human serum (acetone-treated). The substrate used in our experiment contained OPD as chromogen, instead of the ABTS used in the reference study.

The positivity criterion used in IgM detection was established for optical density values equal to or higher than the mean of a group of negative control sera for anti-dengue IgM plus 3 standard deviations.

RESULTS

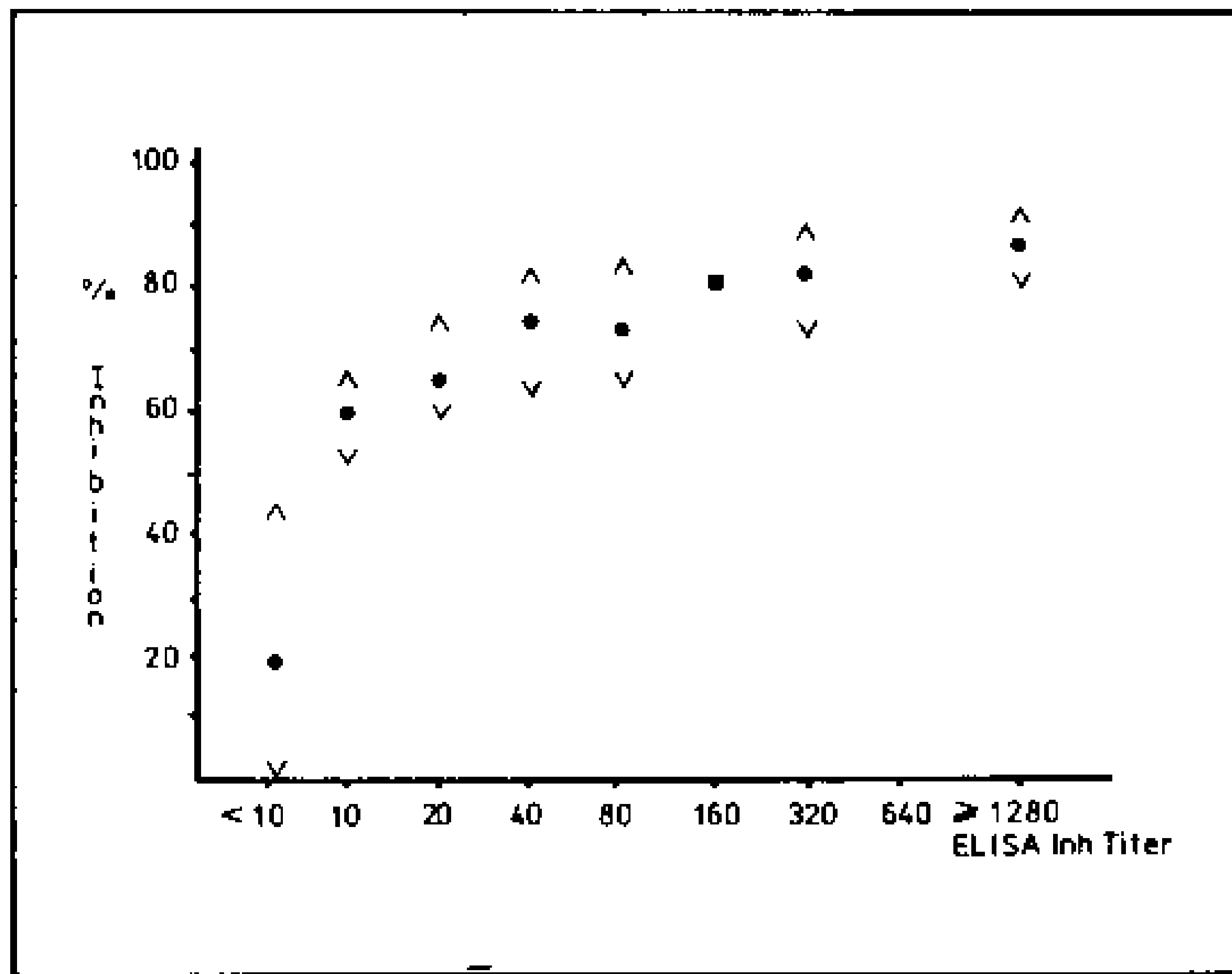
The comparative study between the diagnosis by ELISA inhibition percentage and HI in 35 paired sera resulted in a high coincidence (94%), with differences only in 2 pairs of serum in favor of a higher sensitivity in ELISA.

When performing titration by EIM of 70 monosera corresponding to 35 paired sera, distributed according to their titers (Table I), the mean was estimulated for inhibition percentage and showed and overall trend towards increased inhibition percentage of a serum in direct proportion to its titer, as shown in the Figure.

TABLE I

Sera distribution by titer as determined by EIM

Titer by ELISA inhibition	Quantity of sera	Mean of % inhibition (dil. 1:10)	Lower limit value	Upper limit value
<10	30	19.22	0	45
10	7	59.00	53	67
20	5	66.00	59	77
40	4	74.50	62	82
80	2	73.00	63	83
160	1	80.00	–	–
320	2	81.50	73	90
640	0	–	–	–
⩾1280	19	86.40	80	93



Values of sera in EIM percentage (1:10 dilution) in relation to EIM titer.
● mean values.

∧ ∨ limit values (maximum and minimum).

■ unique value.

The analysis on relationship between results from EIM according to inhibition percentage criteria for dilution 1/10 and titer in 35 paired sera (Table II) shows a diagnostic coincidence in 32 of them (91%); there are 3 non-coincident pairs of serum which were positive according to inhibition percentage criterion, and negative by the titer positivity criterion.

TABLE II

Relationship between results from EIM according to inhibition percentage (dil. 1:10) and titer criteria in 35 pairs of sera

		EIM (% dil 1:10)		
		+	–	Total
EIM (titer)	+	21	0	21
	–	3	11	14
	Total	24	11	35

Data on differing sera in the EMI according to percentage and titer variants are shown in Table III, where percentage seroconversion is observed in 3 cases. The convalescent sera of the 3 pairs show discreetly positive values, however, it was possible to detect IgM antibodies in them which, together with the evidence of seroconversion by HI in 2 of the pairs (324 and 775) strongly support the diagnostic seropositivity criterion, eventhough it has not been possible to observe significant increase of antibody titers by EIM.

TABLE III

Differing sera in results from EIM

Serum		HI	EIM (%)	EIM titer	ELISA IgM
324	1	<20	4	<10	–
	2	80	58	10	+
526	1	<20	25	<10	–
	2	20	53	10	+
775	1	<20	0	<10	–
	2	40	58	10	+

The overall results of IgM study allowed to observe IgM response in at least one of the sera of the pair in 20 of them, all positive according to the percentage criterion in EIM. No IgM response was observed in the 11 negative pairs, nor in 4 positive ones, which results in the different tests are shown (Table IV). This resulted in an 88.5% coincidence and an 83% IgM detection sensitivity in relation to EIM. No false-positive IgM response was observed.

TABLE IV

Differing sera in results from EIM and MAC-ELISA

Serum		HI	EIM (%)	EIM titer	ELISA IgM
306	1	640	90	⩾1280	–
	2	640	89	⩾1280	–
524	1	<20	16	<10	–
	2	2560	82	⩾1280	–
535	1	<20	42	<10	–
	2	640	63	80	–
537	1	<20	28	<10	–
	2	2560	82	⩾1280	–

As part of this study, tests were performed for specificity of the system in detection of antibodies to other flaviviruses (Den-1, Den-3, Den-4, Yellow Fever and St. Louis Encephalitis). The response was confirmed to be widely reactive among dengue viruses and other flaviviruses. This situation is similar to what occurs with HI and MAC-ELISA, which cannot be used for identification of the infective virus serotype.

DISCUSSION

In a preliminary study (Vázquez & Fernández, 1989) standardizing the EIM for anti-dengue antibody detection, the positivity criteria for the test were established by comparing it with HI in a group of 82 human sera; a high degree of coincidence (>83%) was obtained between both techniques, with a higher sensitivity for ELISA. The analysis also showed a trend towards increased inhibition percentage as serum titers increased by HI. The asypmtotic trend of the curve towards a maximum limit value in the inhibition percentage as titers increased (Fig.), as well as a relative overlapping in the distribution of inhibition percentage for adjacent titers make it difficult to interpret the relationship between the inhibition percentage and the titer above a 59% of inhibition, as confirmed also in later studies (unpublished data).

Additionally, it may be observed that the 10% increase positivity criterion for the inhibition percentage of one serum of the pair in relation to the other is insufficient because frequently for one titer distribution there may be sera with differences higher than 10%. Results point out the utility of performing an initial screening of pair of sera by EIM (50%) in 1/10 dilution, and if both sera are positive, they indicate the need of performing their titration in order to see if there are significant differences (⩾ 4 times) between antibody titers of both sera, as a diagnostic criterion.

MAC-ELISA allows to study a large number of specimens. It is at least as sensitive, and its specificity is similar to that of HI, especially if paired sera are tested. Its advantages include the utilization of monosera for rapid presumptive diagnosis of cases admitted to hospitals, which should be confirmed, however, in a second serum specimen because of problems in the diagnostic elucidation for the current infection, depending on persistence of anti-dengue IgM antibodies for a long time in some individuals. This method has also been used successfully for dengue surveillance in endemic and non-endemic areas. In the present study, MAC-ELISA showed a 16% of false-negative cases because of non-detection of IgM antibodies in 4 of 24 positive pairs of sera by EIM (dil. 1/10). As it is known, there is considerable variation among patients in the amount of IgM produced and in the rapidity with which IgM develops.

The sensitivity, economy and easy implementation of the proposed method, unquestionably higher than those of HI together with the utilization of raw viral suspensions and untreated human sera, make the EIM a method of choice for serologic diagnosis of dengue.

The possibility of using some common reactives in EIM and MAC-ELISA, as demonstrated in this study as well as their similarity in methodologies and use of the same equipment, together with some worthy possibilities of MAC-ELISA, allow to recommend the complementary inclusion of both systems substituting the classical HI for laboratories engaged in the diagnosis and surveillance of dengue.

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