Dot enzyme immunoassay: an alternative diagnostic aid for dengue fever and dengue haemorrhagic fever*

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A dot enzyme immunoassay (DEIA) for the detection of antibodies to dengue virus was tested for use as a tool in the presumptive diagnosis of dengue fever and dengue haemorrhagic fever. Paired sera from the following groups of patients were tested using the DEIA and the haemagglutination inhibition (HI) test: those with primary dengue fever; those experiencing a second dengue infection; and febrile patients who did not have dengue. The data obtained show that the DEIA can be effectively used at a serum dilution of 1 : 1000 to confirm presumptive recent dengue in patients with a second dengue infection. However, demonstration of seroconversion proved necessary for patients with primary dengue. At a serum dilution of 1 : 1000 the DEIA has a specificity of 97.3%. The role of this simple and rapid test in improving the effectivity of programmes for the control of dengue virus infection is discussed.

Introduction

Dengue fever and dengue haemorrhagic fever, once important mainly in south-east Asia, are becoming serious causes of morbidity and mortality in many tropical and subtropical areas. Both conditions are caused by infection with any one of the four dengue virus serotypes, and the life-threatening dengue haemorrhagic fever is associated with a secondary immune response to a dengue virus serotype, as discussed by Halstead (1).

Dengue virus is primarily transmitted by the mosquito vector, *Aedes aegypti*, and the advent of modern forms of transport and the increase in international travel have led to the circulation of more than one dengue virus serotype in most endemic areas.

Control of these diseases is thus critically dependent on effective vector control efforts, a task that is notoriously difficult to achieve. In countries where such vector control is practised, the emphasis has been on a reduction at source. However, surveillance of vector activity must be directed or focused to be able also to control virus activity. Public health staff have to depend for disease notification on medical personnel who are already very busy managing patients. As a result, cases of dengue may not be notified sufficiently early for vector control to prevent further transmission.

One of the reasons for such late notification is the lack of readily available rapid diagnostic methods for the confirmation of dengue virus infections. Clinicians therefore do not obtain prompt feedback on their diagnoses and the retrospective results of tests do not have any value in on-the-spot monitoring of disease and virus activity.

The present study was carried out to investigate whether the dot enzyme immunoassay (DEIA) for the determination of antibodies to dengue virus antigens (2, 3) could be used effectively as a rapid diagnostic method for disease control and patient management purposes.

Methods

Viral antigens

The dengue virus antigens used were a mixture prepared from the supernatants of C6/36 cells that were infected with dengue virus type 1 (Hawaii), type 2 (strain 16681), type 3 (H87), and type 4 (H241). Monolayers of C6/36 cells were grown at 28 °C in Leibovitz 15 medium (L15) containing a 10% tryptose phosphate broth, 10% heat-inactivated fetal bovine serum, 50 U/ml penicillin, and 50 μg/ml streptomycin sulfate. When the cells were confluent, the growth medium was removed and the monolayers were inoculated with dengue virus at a multiplicity of infection of 0.1 plaque-forming units per cell. The supernatants were harvested when a cytopathic effect was observed and centrifuged at 200g for 10 minutes at 4 °C.

Preparation of antigen-coated membranes

Drops (2 μl) of dengue virus antigens or uninfected control antigens were dotted onto nitrocellulose

membranes[a] (0.45 μm pore size) and allowed to dry at room temperature. Unbound sites were then blocked by soaking in skimmed-milk buffer (SMB) containing 5% skimmed milk in phosphate-buffered saline (PBS) (pH 7.6), and the membranes were rinsed in distilled water, air-dried, and stored at 4 °C until use.

Test procedures

The antigen-coated membranes were incubated for 60 minutes at room temperature with serum dilutions in SMB. The membranes were then washed three times with PBS and incubated for 60 minutes at room temperature with protein A conjugated with horseradish peroxidase[b] diluted 1 : 4000 in SMB. After a further three washes with PBS, the bound antibodies were visualized by being developed for 30 minutes at room temperature in a chromogenic substrate containing 4-chloro-1-naphthol/hydrogen peroxide. The reaction was stopped by addition of water, and the membranes were allowed to dry. The results were read visually.

The haemagglutination inhibition (HI) test was performed as described by Clarke & Casals (4).

Panel of reference sera

These were sera pooled from more than 20 individuals per pool as described below.

—Pooled convalescent sera (PCS), consisting of convalescent sera pooled from patients with dengue infection diagnosed by the HI test (HI titre > 1 : 10 240 for all four dengue serotypes).
—Pooled positive sera (PPS), consisting of sera taken from normal adults with antibodies to dengue virus (HI titre = 1 : 80 for all four dengue serotypes).
—Pooled negative sera (PNS), consisting of sera taken from normal adults with an HI for all four dengue serotypes <1 : 10.
—A series of pools of acute sera (P1–P10) from patients with a clinical diagnosis of dengue or dengue haemorrhagic fever, with a range of anti-dengue HI titres.

Serum specimens

Paired serum specimens were collected from Malaysian patients with a clinical diagnosis of dengue or dengue haemorrhagic fever, or with a diagnosis of "viral fever". A total of 1144 samples of normal sera were obtained using specimens from blood donors that had been sent for screening for hepatitis B

surface antigen (HBsAg) or human immuno-deficiency virus (HIV) during 1987 and 1988. A total of 77 plasma specimens were also collected from children attending outpatient clinics with complaints of fever, conjunctivitis, or other minor problems.

Results

Panel of reference sera

The major antigens in the viral preparations that were used to coat the membranes for use in the DEIA consisted of the structural proteins E and prM, and the dimeric form of the nonstructural protein NS1 (NS1d), as determined by immunoblotting analysis of the prepared antigens.

It has been demonstrated that the DEIA based on antigen prepared from sucrose-gradient-purified dengue virus type 3 antigens from infected suckling mouse brain is more sensitive than the HI test for determining antibodies to dengue virus (2, 3). Since, however, the DEIA in the present study was prepared using cell-culture-derived antigens, we investigated its sensitivity using the panel of pooled reference sera, which was also characterized using the HI test (Table 1). The findings demonstrate that even when the dengue antigen used consists of a mixture of antigens derived from four dengue virus serotypes, the DEIA is not only highly sensitive, but just as specific as the HI test.

Data that we have reported previously (3) suggest that a 1 : 1000 serum dilution is a reasonable

Table 1: **Comparison of the dot enzyme immunoassay (DEIA) endpoint titres with the haemagglutination inhibition (HI) titres for dengue virus type 1 (DEN1) and type 3 (DEN3) antigens for a series of pooled sera**

Serum pool	Reciprocal HI titre		Reciprocal DEIA titre	DEIA score[a]
	DEN1	DEN3		
P1	2560	2560	10 000	'+ +
P2	160	160	1000	+/−
P3	10	10	<250	−
P4	>5120	>5120	100 000	+ +
P5	160	160	1000	+/−
P6	640	640	4000	+
P7	2560	2560	10 000	+ +
P8	80	80	500	−
P9	<10	<10	<250	−
P10	320	160	1000	+/−
PCS	>5120	>5120	50 000	+ +
PPS[b]	80	80	2000	+/−
PNS[b]	<10	<10	<250	−

[a] At a 1 : 1000 dilution cut-off.
[b] These are pooled normal sera; all other sera were from patients (PPS = pooled positive sera; PNS = pooled negative sera).

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cut-off point to distinguish patients suffering a current or recent dengue infection from those with previous infections. Table 1 shows the qualitative score for each pool of serum at the 1 : 1000 cut-off dilution. Four categories were used to denote titres that were negative (−), weakly positive (+/−), positive (+) or strongly positive (+ +) relative to a standard positive (+) (a reference serum with a dengue HI titre of 1 : 1280).

Sera from afebrile adult donors

To determine whether screening an afebrile population for antibodies at a 1 : 1000 titre cut-off would generate a large number of false-positive "presumptive recent dengue" cases, we used the dengue-antigen-coated membranes in the DEIA to screen for the presence of antibodies to dengue virus antigens in 1144 adult (> 20 years of age) blood donors. Altogether, 84.0% were positive or weakly positive at a serum dilution of 1 : 200, suggesting that the vast majority of adults tested had been exposed to dengue or a related flavivirus. However, when these antibody-positive donor sera were retested at the cut-off dilution of 1 : 1000, 109 of 1144 (9.5%) were weakly reactive (+/−) and only 14 (1.2%) were positive (+), i.e., equivalent to an HI titre of ⩾1 : 1280.

Sera from children with nondengue illnesses

Of 77 children aged 2–12 years who were attending outpatient clinics with nondengue illnesses, only 9 (11.7%) were positive or weakly positive for antibodies to dengue virus at a 1 : 200 dilution cut-off and all were negative when tested at a 1 : 1000 dilution.

Sera from patients with primary dengue

Paired sera from 49 cases of primary dengue, as determined using the HI test in conformity with WHO criteria (5), were tested using the DEIA at a 1 : 1000 cut-off dilution. Only 30 pairs (61.2%) seroconverted at this dilution, as indicated by a discernible difference in colour intensity between the acute and convalescent serum specimens. Thus a change from (−) to (+/−) was considered to be as much a seroconversion as one from (+/−) to (+) or from (+) to (+ +). No case exhibited (+) or (+ +) for the first or acute specimen. Hence if seroconversion is used as a marker of dengue infection, only 61.2% of known primary dengue cases were positive according to the results of the DEIA at a 1 : 1000 dilution; however, when these cases were retested at a 1 : 500 dilution the rate of seroconversion increased to 88.4% of the known primary dengue cases.

Sera from patients with secondary dengue

Paired sera from 51 cases of secondary dengue were tested by DEIA using a cut-off of 1 : 1000 dilution. A total of 39 acute-phase sera were positive (+) or strongly positive (+ +), i.e., 76.5% of patients with presumptive recent dengue were identified using acute-phase sera alone. The remaining 12 cases of secondary dengue had acute-phase sera that were weakly positive (+/−), but seroconverted on the second specimen (from (+/−) to (+) or (+ +)). Thus all the known cases of secondary dengue that were tested were correctly identified using the DEIA.

Sera from febrile patients negative for dengue in the HI test

Paired sera from 76 febrile cases that were dengue-negative according to WHO criteria were tested using the DEIA at a 1 : 1000 dilution cut-off. Two cases (2.6%) were positive (+) for both specimens, i.e., they were false positives. A further 73 (96.1%) cases had negative (−) or weakly positive (+/−) reactions for both specimens, denoting no seroconversion, and were therefore considered negative for current dengue infection. One case showed seroconversion by DEIA (from (−) to (+/−)), which was consistent on retesting at a 1 : 500 dilution cut-off. If this case is considered to be dengue-positive, the specificity of the DEIA test at a 1 : 1000 dilution cut-off was 97.3% (73/75).

Discussion

While the DEIA test as described in this article is clearly useful as a simple and rapid diagnostic tool for detecting cases of secondary dengue fever, it is somewhat less useful for cases of the primary disease. However, if paired sera are used, as is the current practice with the HI test, the DEIA can confidently be used to determine seroconversions in cases of primary dengue. Although by using serum dilutions of 1 : 500 we were able to detect seroconversions in 88.4% of the cases of primary dengue, in situations where primary dengue is dominant, lower serum dilutions, e.g., 1 : 250, could be used to increase the sensitivity of the test.

Generally in south-east Asia, patients who present with dengue usually have a secondary immune response to dengue virus. For example, in 1980 the Thai Ministry of Public Health reported 386 cases of primary dengue out of 5575 confirmed cases that were tested (6), i.e., 93.1% of these confirmed cases were experiencing a second dengue infection. This situation occurs also in Indonesia, and as cases of dengue fever become more frequent this pattern will become common in other endemic

countries. In such countries, DEIA could be extremely useful as a rapid diagnostic tool for dengue fever and dengue haemorrhagic fever, since acute-phase specimens alone can provide a reliable indication of recent infection.

Even in countries where primary dengue is the norm, use of the HI test requires paired specimens and the demonstration of seroconversion. This is much more easily achieved using DEIA, and the serum dilution used can be selected according to the epidemiological status of each country. Alternatively, serial dilutions of serum can be used to determine seroconversion in paired sera in those instances when demonstration of seroconversion using a single dilution is equivocal. In our experience, however, seroconversions are readily identified using DEIA, with changes in colour intensity being unambiguous if paired sera are tested simultaneously. In those few instances where such changes in colour intensity are ambiguous, our experience indicates that seroconversion has not occurred.

Because the DEIA is more sensitive than the HI test, seroconversions can be detected with it on specimens collected only a few days apart. Thus, it is useful and administratively more efficient to obtain a first specimen from patients on admission to hospital and a second specimen on their discharge. It is advantageous to do this because many patients do not return to provide a second specimen, thus making it difficult to make a confirmatory laboratory diagnosis in most cases of primary dengue. This applies even if an IgM test is used.

One of the difficulties associated with the DEIA that we have described is uncertainty about the length of time that high levels of antibody to dengue virus persist after a patient is infected. At present we are carrying out a 3-year follow-up study of dengue patients to determine what proportion exhibit positive DEIA results at a 1 : 1000 dilution at various times after infection. Preliminary data suggest that cases of primary dengue exhibit positive results at 1 : 1000 dilution for as long as serum IgM persists. Also, more than 50% of cases with secondary dengue exhibit positive results for at least 6 months after infection (M.J. Cardosa et al., unpublished results, 1990).

Data we have obtained from studies of blood donors give an indication of the proportion of false-presumptive positives that are likely to be detected in blind screenings of normal adults using the DEIA at 1 : 1000. For example, in Penang, Malaysia, this proportion is about 1.2%, but since the population of febrile patients presenting with clinical features of dengue or dengue haemorrhagic fever differs from the general population, the proportion of false positives should be lower. Comparative studies of the results of DEIA and IgM assays should also provide information about this proportion.

It is also of interest to determine whether patients with antibodies to other flaviviruses show cross-reactions in the DEIA. If the assay is used to determine the presence of antibodies, it will clearly cross-react with antibodies against other flaviviruses, as do the HI and IgM tests. Using the 1 : 1000 dilution cut-off, we have, however, used the DEIA prepared with antigens against dengue virus or Japanese encephalitis virus to compare sera from patients with these conditions; our results indicate that although patients with secondary dengue may react positively with Japanese encephalitis virus antigens, patients with primary dengue do not. On the other hand, serum from patients with Japanese encephalitis does not cross-react in the dengue DEIA. Further investigations are required with sera from areas that are endemic for other flaviviruses; however, our impression is that, provided the cut-off dilution is carefully selected, cross-reactions should not pose a problem with the DEIA.

The DEIA that we have described is a very suitable replacement for the HI test in areas where there is no established supply of fresh gander erthyrocytes and suckling-mouse brain haemagglutinins. The simplicity of the DEIA and the stability of the antigen-coated membranes makes it suitable for use in relatively unsophisticated settings as a first-line screening test or for predicting impending outbreaks of dengue infection.

Ideally, a simple IgM test would be the method of choice for the rapid diagnosis of dengue. However, the IgM-capture enzyme-linked immunosorbent assay (ELISA) (7) is tedious and expensive to perform and has many parameters that require careful control. Although the IgM-capture ELISA can be used in the field, it can be performed optimally only in well-equipped reference and research laboratories.

Thus the test chosen for the detection of antibodies to dengue virus should be appropriate to the situation. A combination of tests should be considered, with the simple DEIA being selected for first-line screening in peripheral areas, while representative specimens are sent to reference laboratories for confirmation by IgM-capture ELISA. In a busy hospital laboratory use of the DEIA should be considered for first-line screening on a daily basis, with the IgM-capture ELISA being used for batchwise confirmation.

There is no longer any reason why the laboratory diagnosis of dengue should be the sole responsibility of central or reference laboratories. Effective control of the spread of the disease depends on the rapid identification of affected areas and im-

mediate and focused action. Cooperation between the public health sector, peripheral health services, and central laboratories can and must be the basis of a policy for the eradication of dengue.

Acknowledgements

Résumé

Le titrage immuno-enzymatique sur taches comme outil diagnostique pour la dengue et la dengue hémorragique

Cet article décrit les résultats de l'évaluation d'un titrage immunoenzymatique sur taches (DEIA) comme outil diagnostique pour la détection des anticorps dirigés contre le virus de la dengue. Les antigènes ont été préparés à partir de cellules C6/36 infectées et disposés en taches sur des membranes de nitrocellulose. Des cellules non infectées ont été traitées de la même façon et utilisées comme témoins négatifs. Les anticorps liés aux membranes traitées par l'antigène ont été visualisés au moyen d'une protéine A conjuguée à la peroxydase de raifort, avec coloration par un système chromogène 4-chloro-1-naphtol/peroxyde d'hydrogène.

Au total, 1144 donneurs adultes ont été testés à la recherche des anticorps dirigés contre les antigènes du virus de la dengue avec une dilution finale du sérum de 1 : 200 et 1 : 1000. A la dilution 1 : 200, 84,0% de cas donneurs présentaient des anticorps décelables vis-à-vis du virus de la dengue, mais seuls 1,2% étaient encore positifs à la dilution 1 : 1000. Sur 77 enfants testés, 11,7% étaient positifs à la dilution 1 : 200, mais aucun ne l'était plus à la dilution 1 : 1000.

Des échantillons de sérums de malades ayant un diagnostic clinique de dengue ou de dengue hémorragique, hospitalisés dans divers établissements de Penang, en Malaisie, en 1987 et 1988, ont été testés par inhibition de l'hémagglutination (HI) et classés comme suit: malades atteints de dengue primaire; malades atteints d'une réinfection par le virus de la dengue; malades négatifs pour la dengue. Tous ont été testés par DEIA à la dilution limite de 1 : 1000. Au total, 61,2% des malades atteints de dengue primaire présentaient une séroconversion à cette dilution. Lorsque les sérums étaient retestés à la dilution 1 : 500, le taux de séroconversion s'élevait à 88,4%. Sur les échantillons de phase aiguë obtenus chez les malades atteints d'une réinfection, 76,5% étaient nettement positifs et les cas restants présentaient une séroconversion, à la dilution 1 : 1000. Chez les malades considérés comme négatifs pour la dengue d'après les résultats de l'inhibition de l'hémagglutination, 96,1% étaient également négatifs par DEIA; un seul cas présentait une séroconversion dans cette dernière épreuve. La spécificité du DEIA à la dilution 1 : 1000 était de 97,3%.

References

1. **Halstead, S.B.** Dengue: haematologic aspects. *Seminars in haematology*, **19**: 116–119 (1982).
2. **Cardosa, M.J. et al.** Development of a dot enzyme immunoassay for dengue 3: a sensitive method for the detection of antidengue antibodies. *Journal of virological methods*, **22**: 81–88 (1988).
3. **Cardosa, M.J. et al.** A dot enzyme immunoassay for dengue 3 virus: comparison with the haemagglutination inhibition test. *Southeast Asian journal of tropical medicine and public health*, **19**: 591–594 (1988).
4. **Clarke, D.H. & Casals, J.** Techniques for haemagglutination and haemagglutination inhibition with arthropod-borne viruses. *American journal of tropical medicine and hygiene*, **7**: 561–573 (1958).
5. *Dengue haemorrhagic fever: diagnosis, treatment and control*. Geneva, World Health Organization, 1986.
6. **Kanai, C. & Sompop, A.** Dengue haemorrhagic fever surveillance in Thailand. In: Pang, T. & Pathmanathan, R., ed. *Proceedings of the International Conference on Dengue/Dengue Haemorrhagic Fever, 1–3 September 1983, Kuala Lumpur*. University of Malaya, Kuala Lumpur, 1983, pp. 159–165.
7. **Innis, B.L. et al.** An enzyme-linked immunosorbent assay to characterize dengue infections where dengue and Japanese encephalitis co-circulate. *American journal of tropical medicine and hygiene*, **40**: 418–427 (1989).