

Sero-epidemiological and Virological Investigation of Dengue Infection in Oaxaca, Mexico, during 2000-2001

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Abstract

A sero-epidemiological-cum-virological investigation was carried out in Oaxaca, Mexico, during 2000-2001 to assess the incidence of dengue infection and the circulating viruses.

A total of 200 serum samples reportedly from dengue patients, based on clinical diagnosis, were collected from Oaxaca's Central Laboratory of Public Health (in the capital city of the state of Oaxaca). The samples were initially collected from ten regional health centres located across Oaxaca. The sample population for the study included both sexes and age groups with clinical signs compatible with dengue infection. All samples were tested for the presence of dengue virus, mainly by MAC-ELISA and RT-PCR.

Ninety-four out of 100 serum samples suspected of dengue were confirmed to be positive. Thirty-two were found positive by MAC-ELISA and 58 were positive by RT-PCR. In addition, the RT-PCR analysis showed that the prevalent serotype in the localities in the study area was DEN-2. However, one isolate of DEN-1 and another of DEN-4 were also detected. The number of infected females was higher than that of infected males and the most affected age group was of people aged under 35 years. The study also highlighted that the sensitivity and specificity of diagnostic tools were crucial for epidemiological studies.

Keywords: Serodiagnosis, MAC-ELISA, RT-PCR, DEN-2, Oaxaca, Mexico.

Introduction

In recent years, dengue fever (DF) / dengue haemorrhagic fever (DHF) has emerged as major health problem in Mexico. In 1960, the *Aedes aegypti* mosquito was eradicated but it reappeared in 1965^[1,2]. As pointed out

by Gubler^[3], factors such as demographic and social changes are responsible for the re emergence of dengue. Mexico is considered an endemic country for dengue and it is reported that major epidemics of DEN-1 occurred on the eastern coast of Mexico during 1979-1980. In 1984-1985,

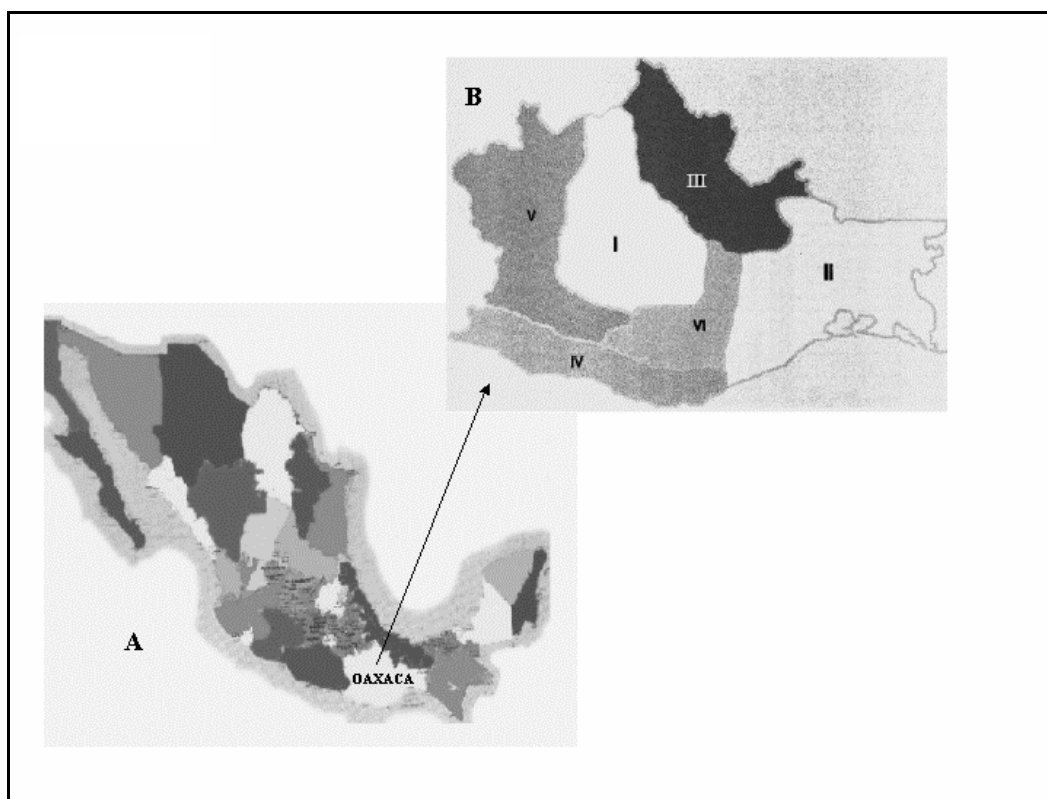
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dengue was diagnosed in 25 of the 32 states of Mexico. By then, DEN-1, DEN-2 and DEN-4 were present in the country, and in 1995, DEN-3 was circulating as well. Several cases of DHF were also confirmed^[4]. In subsequent years dengue achieved endemicity in the country.

As per the records of the Mexican Health Office^[5] (Secretaría de Salud, SS), a higher number of dengue cases were recorded in the states of Nuevo León, Tamaulipas, Veracruz and Oaxaca during 1998-2001.

Oaxaca is located in the subtropical region of Mexico at about 1,600 metres above sea level (Figure 1A). There is high demographic pressure and migration to different urban zones is common, resulting in the establishment of scattered human settlements with deficient public services. All these factors contributed to the propagation of the *Aedes aegypti* mosquito, resulting in dengue outbreaks every year in most of Oaxaca's communities^[6].

Figure 1. Oaxaca, Mexico
(A) United States of México. Oaxaca is located in the west coast
(B) Oaxaca is divided by the health authorities in six jurisdictions (I-VI)



To assess the dengue situation, epidemiological studies were undertaken to make an estimate of the incidence of dengue virus infection and the circulating serotypes in some selected endemic areas of Oaxaca during 2000-2001.

Materials and methods

Population study

The state of Oaxaca is located on the west coast of Mexico. The Mexican Health Office has divided it into six jurisdictions: (I) Central Valleys, (II) Tehuantepec isthmus, (III) Tuxtepec, (IV) The Coast, (V) The Mixteca, and (VI) The Sierra. The presence of dengue virus has been registered in all six jurisdictions (Figure 1B). This study was carried out in ten municipalities distributed in five jurisdictions in the state of Oaxaca. The study population included both sexes and all age groups^[7]. Two population groups were included in this study: one group consisting of 200 serum specimens from patients manifesting signs and symptoms of dengue infection, and another group of 50 serum samples from healthy controls, all from the same jurisdictions.

Sample collection and diagnosis of dengue

Human sera were obtained from 200 patients presenting clinical manifestations of dengue and tested for anti-dengue IgM antibodies. Serum samples were collected by venipuncture, using Vacutainer tubes (Becton-Dickinson). The clinical samples corresponded with dengue cases reported during 2000-2001. Dengue-infected samples were obtained during the first five

days of the onset of fever and were processed for anti-dengue IgM detection using IgM capture ELISA (MAC-ELISA) as described by Vorndam et al.^[8] Samples from healthy donors were obtained at about the same time.

As a routine practice and with the idea of recording epidemic data, the suspected dengue samples already clinically diagnosed in community health centres were sent to the Central Laboratories in the city of Oaxaca (Laboratorio Estatal de Salud Pública del estado de Oaxaca, Secretaría de Salud). In this laboratory, the presence of dengue virus was confirmed by MAC-ELISA and RT-PCR.

Dengue virus isolates

Aedes albopictus C6/36 cells were grown in 48-well tissue culture plates as described by Igarashi^[9]. Briefly, 2×10^5 cells were plated in 1 ml of minimum essential medium (Gibco-BRL, Grand Island, N.Y.) supplemented with 7% fetal bovine serum (Sigma Chemical Co., St. Louis, Mo) and 1% glutamine, vitamins and nonessential amino acids. After 24 hours of culture, 100 μ l of every sera diluted 1:10 was added to the corresponding well. The mixture was then gently shaken and incubated for 60 minutes at room temperature. Cells were then washed with serum-free medium and cultured at 28 °C with complete medium for at least 10 days. Cells were harvested for RT-PCR diagnosis.

RNA extraction

Total RNA was extracted either from 100 μ l of serum or from cultured cells by using Trizol LS (GIBCO BRL, Gaithersburg, MD.) according to the manufacturers'

recommendations. Ethanol-precipitated RNA was recovered by centrifugation and air-dried. The RNA pellet was re-suspended in 50 µl of Diethyl-pyrocabonate (Sigma)-treated water (DEPC water) and used as a template for RT-PCR.

RT-PCR

Synthetic oligonucleotide primer pairs were designed based on published sequence data for each of the four serotypes of dengue^[10,11]. Four fragments of an expected size of 482 bp (DEN-1), 392 bp (DEN-4), 290 pb (DEN-3) and 119 bp (DEN-2) were obtained by using the SuperScrip™ One Step RT-PCR kit in conjunction with Platinum^R Taq polymerase (Invitrogen, Life Technologies). A mixture of 5 µl of RNA, 25 µM of sense and anti-sense PCR primers, and DEPC water to a total volume of 50 µl was incubated at 85 °C for 5 minutes and then chilled on ice. The tubes-reaction mixture containing 2X PCR buffer containing 0.4 mM of each dNTP, 2.4 mM MgSO₄ and Super Scrip™ RT/platinum^R Taq Mix, as recommended by the manufacturer (Invitrogen TM Life Technologies), was added to the RNA and primers-containing tube. The reverse transcription reaction was performed at 50 °C for 30 minutes. Thermocycling began with a hot start at 94 °C for 2 minutes followed by 40 cycles of annealing at 55 °C for 30 seconds, and extension at 72 °C for one minute and denaturing at 94 °C for 15 seconds.

The PCR conditions for serotype assessment were as follows: 40 cycles of denaturing at 94 °C for 30 seconds, annealing at 55 °C for 1 minute, and extension at 72 °C for 1 minute and, a final extension at 72 °C for 7 minutes. The

reaction mixtures were electrophoresed and visualised under UV light after ethidium bromide staining of the gels.

Results

Diagnosis of the samples by MAC-ELISA

Two hundred serum samples initially reported as suspected positive for dengue, based on clinical reports from the hospital where patients were hospitalised, were submitted for diagnosis based on anti-dengue IgM antibodies detection by MAC-ELISA. From these, only 34 samples were positive for IgM antibodies^ψ. As expected, the 50 negative-control samples resulted negative for anti-dengue IgM antibodies (Table 1).

Diagnosis by RT-PCR

Once the serum samples were tested for anti-dengue IgM antibodies, the results were confirmed by RT-PCR. In this case, only 25 samples from healthy donors were tested. By this method 58 samples proved to be positive for dengue, i.e. 24 more than by MAC-ELISA. Interestingly, all samples positive for MAC-ELISA were also positive by RT-PCR. Those samples showing positivity for DEN by RT-PCR were further tested for the four serotypes (DEN-1, -2, -3 and -4). It was found that the main circulating serotype in Oaxaca during 2000-2001 was DEN-2. Two other serotypes (DEN-1 and DEN-4) were also found (only one case each) (Table 1).

^ψ Out of 200 samples, originally sent, only 100 samples were found in good condition for evaluation by MAC-ELISA or RT-PCR. Other samples deteriorated under transportation/storage conditions.

Table 1. Positivity for dengue by MAC-ELISA and RT-PCR

From one hundred samples tested, from patients with clinical diagnosis of dengue, 36% proved positive by MAC-ELISA and 61% by RT-PCR. DEN-2 was the prevailing circulating serotype

Date of collection	Jurisdiction	Locality	Number of cases	MAC-ELISA	RT-PCR	Serotype	Serotype
Nov 2000	II	Salina Cruz	18	13+/5-	9+/9-	DEN-2	DEN-2
Nov 2000	III	Tuxtepec	9	2+/7-	5+/4-	DEN-2	DEN-2
Nov 2000	II	Tehuantepec	10	4+/6-	5+/5-	DEN-2	DEN-2
Jul 2000	III	Temascal	8	3+/5-	5+/3-	DEN-2	DEN-2
Nov 2000	II	Juchitan	3	2+/1-	2+/1-	DEN-2	DEN-2
Total cases				24+/24-	26+/22-		
May-Jun 2001	IV	Huatulco	21	6+/15-	12+/9-	DEN-2	DEN-2
Jun-Nov 2001	I	Oaxaca	7	2+/5-	7+/0-	DEN-2	DEN-2
Apr 2001	II	Juchitan	2	0+/2-	2+/0-	DEN-2	DEN-2
May 2001	II	Salina Cruz	1	0+/1-	1+/0-	DEN-2	DEN-2
Feb 2001	III	Tuxtepec	7	2+/5-	2+/5-	DEN-2	DEN-2
Feb 2001	V	Tonalá	2	0+/2-	2+/0-	DEN-2	DEN-2
Feb 2001	V	Huajuapán	6	0+/6-	6+/0-	DEN-2	DEN-2
Total cases				10+/36-	32+/14-		

Table 2. Distribution of dengue cases by age and sex

Age (in years)	Male	Female
1	0	1
2-4	0	2
5-9	7	10
10-14	5	6
15-20	2	3
21-30	0	5
>30	6	6
Total	20 (38%)	33 (62%)

Prevalence of infection by age and sex

An analysis by age and sex revealed a higher prevalence (61%) of infection in females than in males (39%) and that the most affected group of people was the under-35-year-olds (Table 2).

Discussion

The *Aedes aegypti* mosquito's adaptability to changing environmental conditions has contributed significantly to the increase in dengue epidemics in the world. Mexico is considered an endemic country where the

four serotypes of the dengue virus are in circulation. This study provides some insight into the dengue epidemic situation in Oaxaca state, Mexico, in an attempt to contribute to the prevention and control of outbreaks of DF/DHF.

From the 200 serum samples collected from suspected dengue patients initially considered for the study, only 100 could be used. The remaining 100 samples were presumably subjected to non-appropriate storage conditions. From the 100 suspected samples tested, nearly 100% proved to be positive for dengue (36% by MAC-ELISA and 61% by RT-PCR). However, the data reported here could be an underestimation considering the several factors that could influence the laboratory determination outcome, such as sample handling and the diagnosis systems performed at local hospitals. In some localities of Oaxaca, the diagnosis for dengue was being simultaneously carried out with the diagnosis for rubella and toxoplasma in a monoclonal antibodies-based multiplex assay. In rural communities, however, only the presence of anti-dengue IgM antibodies was tested.

In this regard, it is possible that some patients presenting an early secondary infection in the absence of strong clinical manifestations had undetectable levels of anti-dengue IgM antibodies, since IgG is the prevalent Ig isotype at this stage of the infection. For these cases, it would be necessary to consider some other diagnosis techniques such as virus isolation or RT-PCR. Unfortunately, these are difficult to carry out in rural hospitals due to high costs and lack of suitably trained personnel.

Additional effort is needed to ensure appropriate sample collection, handling

and storage in order to send them to the reference laboratory for adequate diagnosis. It is worth noting that in several Mexican states, health authorities are working on vector control as well as on facilities for sample collections to be sent to the Instituto de Referencia Epidemiológica (InDRE) in Mexico City for a proper diagnosis. It is still, however, a long way for good quality medical care to reach most Mexicans.

This report shows that by MAC-ELISA, 36% of the tested samples were found positive for dengue, whereas by RT-PCR up to 64% of the samples proved to be positive. Although the sensitivity and specificity reported for MAC-ELISA is reported to be good enough for a diagnosis system, it is likely that as a result of inadequate handling and storage conditions, some samples reported as negative could in fact be positive for dengue when tested by RT-PCR. No false positive results were found. This raises the question as to how many laboratory assays must be carried out on a suspected dengue sample before reporting it as negative.

The use of RT-PCR makes it possible to identify the dengue serotype involved; in this regard this study shows that in Oaxaca, Mexico, the prevalent serotype of dengue virus was DEN-2, although isolated cases of DEN-1 and DEN-4 infections were also found. Some other local reports had also mentioned the presence of DEN-3 and several cases of DHF.

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References

- [1] Novo S. Breve historia y antología de la fiebre amarilla. *Salud Pública de México*, 1995, 37: S99-S102.
- [2] Gómez H. Monografía sobre la epidemiología del dengue en México. Secretaría de Salud, 1992, Vol. 43.
- [3] Gubler JD. Epidemic dengue/dengue haemorrhagic fever as a public health, social and economic problem in the 21st century. *Trends in Microbiology*, 2002, 10: 100-103.
- [4] Briseño-García B, Gómez-Dantes H, Argott-Ramírez E, Montesano R, Vázquez-Martínez AL, Ibanez-Bernal S, Madrigal-Ayala G, Ruiz-Matus C, Flisser A and Tapia-Conyer R. Potential risk for dengue haemorrhagic fever: the isolation of serotype dengue-3 in Mexico. *Emerging Infectious Disease*, 1996, 2(2): 133-135.
- [5] Anonymous. Gaceta informativa de la Secretaría de Salud, SSA-Oaxaca, 1999.
- [6] Cisneros AS, Martínez US, Cruz GM, Tovar RG, Moreno MG, Díaz-Badillo A and Muñoz ML. Dengue fever in the state of Oaxaca, Mexico. The American Society of Tropical Medicine and Hygiene, 50th Meeting, 2001, Atlanta, Georgia, USA.
- [7] Martínez-Soriano U. Tipificación Molecular del virus del dengue en el estado de Oaxaca. B.Sc. Thesis, 2002, 19-58.
- [8] Vorndam V and Kuno G. Laboratory diagnosis of dengue virus infections. In: Gubler DJ and Kuno G (Eds.), *Dengue and dengue hemorrhagic fever*. CAB International, New York, NY, 1997, 313-333.
- [9] Igarashi A. Mosquito cell cultures and the study of arthropod-borne togaviruses. *Advances in Virus Research*, 1985, 30: 21-39.
- [10] Lanciotti RS, Calisher CH, Gubler DJ, Chang GJ and Vorndam V. Rapid detection and typing of dengue viruses from clinical samples by using reverse transcriptase-polymerase chain reaction. *Journal of Clinical Microbiology*, 1992, 30: 545-551.
- [11] Harris E. A low cost approach to PCR. Ed. Oxford University Press, USA, 1998, 96-105.