Communication/Comunicação

Cocirculation of two dengue virus serotypes in individual and pooled samples of *Aedes aegypti* and *Aedes albopictus* larvae

Cocirculação de dois sorotipos do vírus dengue em amostras individuais e *pools* de larvas de *Aedes aegypti* e *Aedes albopictus*

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ABSTRACT

Introduction: To detect dengue virus, eggs of *Aedes sp* were collected in the city of Belo Horizonte, Brazil, in 2007. **Methods:** Egg samples were subsequently hatched and the larvae were tested for the presence of dengue virus RNA by RT-PCR. **Results:** Among the *Aedes aegypti* larvae samples, 163 (37.4%) out of 435 were positive, including 32 (10.9%) of 293 individual larvae samples concomitantly positive for two serotypes. **Conclusions:** Virological surveillance detecting coinfected vectors in the field could represent an important strategy for understanding the numerous factors involved in the transmission and clinical presentation of dengue.

Keywords: Dengue. Virus. Serotypes. Vectors.

RESUMO

Introdução: Para a detecção do vírus da dengue, ovos de *Aedes sp* foram coletados em Belo Horizonte, Brasil, em 2007. **Métodos:** Amostras de ovos eclodiram e suas larvas foram testadas para a presença de RNA do vírus dengue por RT-PCR. **Resultados:** Das amostras de larvas de *Aedes aegypti*, 163 (37,4%) de 435 foram positivas, incluindo 32 (10,9%) das 293 amostras individuais que foram concomitantemente positivas para dois sorotipos. **Conclusões:** A vigilância virológica de vetores no campo poderia representar uma estratégia importante para a compreensão dos diversos fatores envolvidos na transmissão e apresentação clínica da dengue.

Palavras-chaves: Dengue. Vírus. Sorotipos. Vetores.

Dengue is an acute viral disease transmitted primarily by *Aedes aegypti* and the infectious agent is one of four serotypes of the dengue virus (DENV): DENV-1, DENV-2, DENV-3 or DENV-4. The incidence of dengue fever and dengue hemorrhagic fever (DHF) has increased in tropical regions of the Americas[1]. In Brazil, over the past 22 years, the geographic expansion of the vector has enhanced the cocirculation of serotypes DENV-1, DENV-2 and DENV-3 in 25 of the 27 Brazilian states[2].

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In the fall of 1996, DENV-1 was introduced to the City of Belo Horizonte, the capital of the State of Minas Gerais, resulting in a localized outbreak. In 1997, other outbreak occurred in the city during the summer and fall.

After a silent period during the winter and spring of 1997, a new and large epidemic cycle began in Belo Horizonte and continued throughout the summer and fall of 1998, during which the mean incidence rate was 381 per 10,000 inhabitants[3-5]. At that time, DENV-2 was isolated cocirculating with DENV-1.

A seroepidemiological survey was conducted by our group using a serum-neutralization assay. A seroprevalence of 23% was determined, with one in four of these cases demonstrating concurrent seropositivity for DENV-1 and DENV-2[5]. Other studies during the same period, using reverse transcription-polymerase chain reaction (RT-PCR) in pooled larvae collected in the nine administrative districts of the city, also detected samples positive for DENV-1 and DENV-2[6].

In 2002, DENV-3 was isolated from human serum for the first time in the city. Since then, cocirculation of DENV-1, DENV-2 and DENV-3 has occurred in the State of Minas Gerais and this cocirculation has been documented molecularly and by isolation, demonstrating the genotypic variation of DENV in the state[7].

During epidemiological weeks 13 and 14 of 2007 (March 25th to April 7th), eggs were collected from positive ovitraps (of 486 placed) in three administrative districts of Belo Horizonte (Central-South, East, and Venda Nova). The larvae of *A. aegypti* and *A. albopictus* that hatched from these eggs were reared to 4th instar, identified at a species level and examined for DENV.

In order to determine whether more than one serotype could be present in a single larva, the hatched larvae were distributed into individual and multiple larval pools. The *A. aegypti* pools had an average of eight larvae (range 2 to 10) and the two *Aedes albopictus* pools had 3 and 6 larvae each. The samples were then sent to the Molecular Virology and Bioproducts Laboratory of the Ezequiel Dias Foundation for RT-PCR analysis. The silica method was used to extract RNA from the larva samples[8]. Approximately 1μg to 5μg of extracted RNA was added to 50pmol of oligonucleotide antisense primers and 200U of reverse transcriptase (SuperScript II RT – Gibco/Life Technologies) to generate the cDNA. The oligonucleotide primers used for the detection of DENV have been previously described[9]. As a positive control for the assays, cDNA prepared from RNA extracted from the supernatant taken from the

cellular culture of C6/36 cells infected with standard specimens of DENV-1, DENV-2 and DENV-3 was used. For negative controls, sterile water was used. The amplification cycles were: one cycle at 95ºC (3min), 30 cycles at 95ºC (/15s each), one cycle at 55ºC (15s) and finally one cycle at 72ºC (30s).

A total of 13,436 *Aedes sp* eggs were detected in 247 (50.8%) of the 486 ovitraps, with an egg density Index (EDI)[10] of 54.4. Of the 3,604 (26.8%) eggs that hatched, 3,587 (99.5%) were *A. aegypti* and 17 (0.5%) were *A. albopictus.* Due to logistical constraints, a sample of 1,400 (39%) of the *A. aegypti* larvae were tested with RT-PCR. These larvae were selected using a stratified sampling process to assure proportional representation from all three administrative districts **(Table 1)**. All 17 *A. albopictus* larvae were tested.

The 1,417 larvae that were tested were divided into 445 samples, 435 *Aedes aegypti* (293 individual samples and 142 pools) and ten *A. albopictus* (eight individual samples and two pools). Among the *A. aegypti* samples, 163 (37.4%, 95%CI: 32.9%-42.2%) were positive for DENV. Among the *A. albopictus* individual and pool samples, five (50%, 95%CI: 20.1-79.9) were positive **(Table 2)**.

Among the *A. aegypti* larvae, DENV-2 was most frequently detected (n = 93, 21.4%). DENV-1 and DENV-2 were detected concomitantly in 29 samples (6.7%): 22 in individual larvae and seven in pooled specimens. DENV-2 and DENV-3 coinfection was detected in 14 (3.2%) samples, nine of which were individual samples. Among the *A. albopictus* larvae, DENV-2 was the most frequently isolated serotype (n = 2, 4%), with one sample coinfected with DENV-2 and DENV-3. DENV-1 and DENV-3 coinfections were not detected in either species of mosquito.

Infection rates (IR) for the various dengue viruses were determined using an Excel add-in[11]**.** The infection rates of the different dengue viruses, expressed as maximum likelihood estimates (MLE) are presented in **Table 3**. The overall IR for dengue virus in the *A. aegypti* larvae was 138.6/1,000 or 13.9% and DENV-2 was the predominant serotype with an overall IR of 11.1%. The IR of DENV-2 was 7.3% when was detected alone in pools, 2.1% when detected in combination with DENV-1 and 1.1% when detected in combination with DENV-3. The second most frequently detected was DENV-1 with an overall IR of 3.3%. The IR of DENV-1 was 1.2% when was detected alone in pools and 2.1% when detected in combination with DENV-2. The least frequently detected was DENV-3 with an overall IR of 1.8%. The IR of DENV-3 was 0.8% when detected alone in pools and 1% when detected in combination with DENV-2. No DENV1 and DENV-3 coinfections were detected.

TABLE 1 - Frequency distribution of placed ovitraps, ovitraps with eggs, and hatched eggs for two mosquito species, Belo Horizonte, 2007.

Ovitraps placed	486
Ovitraps with eggs	
n	247
%	50.8
Total eggs	13,436
egg density index (EDI)	54.4
Hatched eggs	3,604 (26.8%)
Aedes aegypti	3,587 (99.5%)
Aedes albopictus	17 (0.5%)
***Aedes aegypti* larvae tested with RT-PCR**	1,400 (39%)
individual samples	293
pooled samples (pool average = 8; n = 2 to 10)	142
total samples	435
***Aedes albopictus* larvae RT-PCR tested**	17 (100%)
individual samples	8
pooled samples (n = 3 & 6)	2
total samples	10

RT-PCR: reverse transcription-polymerase chain reaction.

TABLE 2 - RT-PCR test results among larvae of dengue vectors, according to dengue virus serotype, for two mosquito species, Belo Horizonte, 2007.

Samples	DENV (all serotypes)	DENV-1	DENV-2	DENV-3	DENV-1 and DENV-2	DENV-2 and DENV-3	NEG	Total	Positive %	95% CI	
Aedes Aegypti											
individual	110	10	60	8	22	10	183	293	37.5	32.0	43.4
pool	53	6	33	3	7	4	89	142	37.3	29.5	45.9
total n (%)	163 (37.4)	16 (3.7)	93 (21.4)	11 (2.5)	29 (6.7)	14 (3.2)	272 (62.6)	435 (100.0)	37.4	32.9	42.2
Aedes Albopictus											
individual	4	---	3	----	----	1	4	8	50.0	17.4	82.6
pool	1	---	1	----	----	---	1	2	50.0	2.7	97.3
total n (%)	5 (50.0)	---	4 (40.0)	---	---	1 (10.0)	5 (50.0)	10 (100.0)	50.0	20.1	79.9

---: negative samples, DENV: dengue virus.

TABLE 3 - Infection rate per 1,000 (expressed as maximum likelihood estimation with 95%CI) of the various dengue virus serotypes in *Aedes aegypti*, Belo Horizonte, 2007.

	Infection Rate	Lower Limit*	Upper Limit*	Scale	Point Est Method	CI Method	Num Pools	Num Pos Pools	Num Individuals
DENV-1 only	11.61	6.94	18.31	1,000	Bias Corrected MLE	Corrected Score	435	16	1,400
DENV-2 only	72.90	60.40	87.30	1,000	Bias Corrected MLE	Corrected Score	435	93	1,400
DENV-3 only	7.88	4.19	13.57	1,000	Bias Corrected MLE	Corrected Score	435	11	1,400
DENV-1 & DENV -2	20.96	14.51	29.34	1,000	Bias Corrected MLE	Corrected Score	435	29	1,400
DENV-2 & DENV -3	10.09	5.80	16.39	1,000	Bias Corrected MLE	Corrected Score	435	14	1,400
All DENV -1	33.18	24.88	43.39	1,000	Bias Corrected MLE	Corrected Score	435	45	1,400
All DENV -2	111.00	95.92	127.96	1,000	Bias Corrected MLE	Corrected Score	435	136	1,400
All DENV -3	18.12	12.14	26.07	1,000	Bias Corrected MLE	Corrected Score	435	25	1,400
All DENV (1, 2 & 3)	138.60	121.84	157.29	1,000	Bias Corrected MLE	Corrected Score	435	163	1,400

MLE: maximum likelihood estimates, DENV: dengue virus, *95%.

In 1996 and 1997, only DENV-1 was detected in human sera submitted to the Belo Horizonte City Health Department Epidemiological Surveillance Service. Beginning in 1998, DENV-2 was also isolated[3]. Since 2002, DENV-3 has been predominantly isolated. It is interesting to note that in the present study, DENV-2 was the most frequently detected serotype.

Sabin demonstrated the occurrence of transovarial transmission of DENV-2 by *A. aegypti* in nature by the isolation of dengue virus from naturally infected mosquito larvae showing a minimum infection rate of 1:2,067 for DENV-2[12]. Natural transovarial transmission of DENV-4 by *A. aegypti* was demonstrated from adult mosquitoes reared in the laboratory from eggs collected in Trinidad, with a minimum infection rate of 1:1,855[13]. The results of laboratory experiments described above with *A. aegypti* showed a very inefficient minimum infection rate. Cecilio et al determined an infection rate for DENV-2 of 1:35.45, indicating that transovarial transmission of DENV by *A. aegypti* could provide a mechanism for the maintenance of DENV when continuous mosquito breeding is interrupted[6]. Transovarial transmission was confirmed by the detection of DENV-2 in male mosquitoes hatched from eggs which were collected in nature and reared in controlled conditions.

The detection of coinfection by two serotypes in a single larva of the vector means that the new generations of mosquitoes could be coinfected by transovarial transmission. To our knowledge, this is the first report in the Brazilian literature of double infection by different serotypes of the dengue virus detected in individual larvae.

Human cases of concurrent infections by two serotypes have been reported in Brazil: in the City of Cuiabá, State of Mato Grosso (DENV-1 and DENV-2)[14] and more recently in the City of Fortaleza, State of Ceará (DENV-2 and DENV-3)[15].

The true epidemiological significance of the transmission of two or more serotypes by vectors that acquired the virus by transovarial transmission has yet to be established. The present results raise several questions related to the infectivity of these coinfected vectors, their significance in transmission during *silent* periods (interepidemic) and the relation between simultaneous infection by two serotypes and the clinical severity of cases. Virological and entomological surveillance detecting coinfected vectors in the field could represent an important strategy for increasing current understanding of the various factors involved in the transmission and clinical presentation of dengue and improving the monitoring of the dynamic of its occurrence.

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CONFLICT OF INTEREST

The authors declare that there are no conflicts of interest.

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