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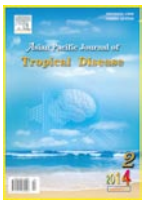

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Assessment of vertical dengue virus transmission in *Aedes aegypti* and serotype prevalence in Bantul, Indonesia

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ABSTRACT

Objective: To determine the reality of dengue virus (DENV) serotype circulation in Bantul and the potential impact of a vertical transmission in the maintenance of dengue.

Methods: Mosquitoes were captured using ovitraps in the vicinity of households of patients diagnosed with severe forms of dengue. DENV was detected in mosquitoes by immunochemistry and typed by reverse transcription-polymerase chain reaction.

Results: *Aedes aegypti* mosquitoes infected with DENV were found in 14 out of 17 districts in the Bantul Regency. Vertical transmission was demonstrated and serotype prevalence was coherent with the current clinical situation and the previous reports. DENV-3 was the most common serotype (12 districts), followed by DENV-2 (3 districts) and DENV-4 (1 district). No DENV-1 was found.

Conclusions: Unlike continental countries such as Cambodia or Thailand, where the replacement of serotypes is the rule, maintenance of DENV-3 is a major feature in Java. Vertical transmission is likely to play a major role along with the archipelago structure of Indonesia, which might help maintaining local mosquito populations. Regular survey of circulating DENV and prevalence will help predicting and controlling outbreaks.

1. Introduction

Dengue is an expanding mosquito-borne arboviral disease with a 30-fold rise in the number of human cases reported in the last 50 years. It is also extending to initially dengue-free countries[1]. The estimated number of cases occurring annually ranges from 50 to 100 million with about 2.5 billion people at risk, 75% of whom are located in the South East Asian and Pacific region[1]. Dengue disease can take different clinical forms. The most frequent form is the dengue fever, from which the patients recover quite rapidly. However, a small percentage of patients devolve the development to severe forms, dengue haemorhagic fever (DHF) and dengue shock syndrome (DSS).

Dengue virus (DENV), a member of the family Flaviviridae, genus *Flavivirus*[2], comprises four genetically and antigenically distinct serotypes (DENV-1, DENV-2, DENV-3 and DENV-4). The genome is a single stranded positive-

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strand RNA of ca. 11 kb coding for a single polyprotein further processed into structural proteins (capsid, premembrane/membrane, envelope; C, prM/M, E) and non-structural proteins (NS1, NS2A, NS2B, NS3, NS4A, NS4B and NS5) [3,4].

DENV is classified among arboviruses (or arthropod-borne viruses). DENV is vectored by mosquitoes from the genus *Aedes* and in particular by *Aedes aegypti* (*Ae. aegypti*). The dynamic of dengue outbreaks is rather complex and characterized by frequent replacement of virus lineages and serotypes[5-10]. The reasons for such a replacement are largely unknown, multiparametric, and involve purifying selection relating to host immune responses[11], lineage bottlenecks and environmental impact on mosquito populations[9,10,12].

Replacement lineages were suspected to be associated to the spread of small population of infected mosquitoes surviving in local pockets of life. Furthermore, the transmission of DENV can occur horizontally from mosquitoes to humans and humans to mosquitoes but can also occur vertically from mother to larvae through the transovarial route[13-16]. Vertical transmission can participate to the maintenance of the virus over time during low epidemic periods[13,17]. *Aedes* mosquitoes do not migrate over large areas and are usually limited to a radius of 150 m from their breeding site[18,19]. They, therefore, represent a good indicator of the diversity of the circulating DENV serotypes or lineages in a region and close to households with declared cases. Surveillance of infected mosquitoes with DENV could thus provide an early warning sign for the risk of transmission in an area and the specific predominant circulating serotype in the vector population. This approach is particularly effective when there is no systematic record and analysis of dengue and serotype identification in health centers and hospital.

2. Materials and methods

2.1. Mosquito eggs collection

Mosquito eggs were collected from 17 districts in the Bantul Regency. Ovitraps were distributed at the DHF highest incidence at Bantul Regency, Yogyakarta Province, Central Java, Indonesia (7°44′04″-8°00′27″ S and 110°12′34″-110°31′08″ E). A total of 55 to 70 ovitraps were distributed for each subdistrict in a radius of 100 m from households of patients suffering from DHF/DSS selected randomly. A total of 978 ovitraps were installed. *Ae. aegypti* mosquitoes hatched from collected eggs were reared until the imago stage at the Laboratory of Entomology, Center for Tropical Medicine, Gadjah Mada University.

2.2. Immunocytochemistry detection of DENV

DENV was detected in adult mosquitoes by immunocytochemistry as previously described using DSSC7 monoclonal antibody as primary antibody and anti-mice-peroxidase secondary antibody[20].

2.3. RNA extraction

Viral RNA was extracted from adult mosquitoes using the High Pure Viral Nucleic Acid Kit according to the manufacturer's instructions (Roche). Viral RNA was stored at -20 °C prior to reverse transcription.

2.4. Reverse transcription-polymerase chain reaction (RT-PCR) and nested PCR

Ten microliters of stabilized RNA extract were used as a template in a 25 μL volume retro-transcription reaction using primers D1 and D2 (Table 1) corresponding to the region of the virus genome spanning from the capsid gene (*C*) to the premembrane gene (*prM*)[21]. RT-PCR was conducted using the Superscript™ III One-Step RT-PCR system (Invitrogen). Reverse transcription and further PCR DNA amplification were conducted in a single tube. Synthesis reaction was conducted at 42 °C for 60 min and 94 °C for 2 min, followed by 40 amplification cycles of 94 °C for 30 seconds, 60 °C for 1 min and 68 °C for 2 min, with a final extension at 68 °C for 7 min. Serotyping was performed in one multiplex PCR reaction using 1 μL of cDNA and five different primers: D1 as forward primer and 4 serotype-specific (TS1, TS2, TS3 and DEN4) as reverse primers as described by Kosasih *et al*. (Table 1)[22]. PCR was done at 94 °C for 2 min, followed by 30 amplification cycles of 94 °C for 1 min, 55 °C for 1 min and 72 °C for 2 min, with a final extension at 72 °C for 10 min. Amplifications were visualized on 2% agarose gel with ethidium bromide. Serotypes were identified according to PCR fragment size: 482 bp, 119 bp, 290 bp and 389 bp for DENV-1, DENV-2, DENV-3 and DENV-4, respectively.

Table 1
Primers nucleotide sequences.

Primer	Sequence	Position	Size (bp)
D1	5′-TCA ATA TGC TGA AAC GCG CGA GAA ACC G-3′	134-161	511
D2	5′-TTG CAC CAA CAG TCA ATG TCT TCA GGT TC-3′	616-644	511
TS1	5′-CGT CTC AGT GAT CCG GGG G-3′	568-586	482
TS2	5′-CGC CAC AAG GGC CAT GAA CAG-3′	232-252	119
TS3	5′-TAA CAT CAT CAT GAG ACA GAG C-3′	400-421	290
DEN4	5′-TGT TGT CTT AAA CAA GAG AGG TC-3′	506-527	389

3. Results

3.1. Incidence of dengue in the Bantul Regency in 2011 and 2012

Epidemiological data on the incidence of dengue in humans in this regency of Indonesia are partial. Cases reported are only those corresponding to the severe forms, *i.e.* DHF and DSS. Regular dengue fever cases are not systematically tested and diagnosed and thus are not recorded. Furthermore, systematic serotyping is not conducted at the clinical level. This is very likely to lead to an underestimation of the actual impact of dengue in the 17 districts of the Bantul Regency. Data are shown in Table 2. Time distribution of cases and relative importance of DHF and DSS varied depending on the year considered. Relative importance of DSS was higher in 2011 with 19 cases reported when compared to the 4 cases reported in 2012. Similarly, DHF was more important in 2011 with an overall total of 226 cases when compared to a total of 144 cases reported for 2012. The main difference between both years was the seasonality of occurrence. In 2011, the peak was reached during the first three months, perhaps continuing a peak started at the end of 2010, after what the number of cases decreased to remain low during the second semester. Conversely, in 2012, the number of cases was low at the beginning of the year and increased gradually to reach a peak between May and July and decreased afterward.

Table 2
Occurrence of severe forms of dengue in patients from the Bantul Regency in 2011 and 2012.

Month	2011		2012	
	DSS	DHF	DSS	DFH
January	11	89	0	9
February	6	35	0	14
March	2	28	1	17
April	0	16	0	15
May	0	13	2	26
June	0	11	0	25
July	0	6	0	19
August	0	3	0	8
September	0	6	0	7
October	0	5	0	0
November	0	5	0	0
December	0	9	1	4
Total	19	226	4	144

3.2. Assessment of dengue infection in mosquitoes using immunochemistry

In the absence of consolidated epidemiological data in humans, the study of distribution of DENV in mosquitoes can provide useful additional information to characterize the subtype of virus circulating in ecosystems and to predict the risk of outbreaks. The number of positive ovitraps varied depending upon the district considered from 5 to 31 (Table 3). Dengue detection in mosquitoes was conducted using an immunological approach[21]. Out of the 17 districts making the Bantul regency, 12 were positive for dengue-infected *Ae. aegypti* individuals and five were negative, *i.e.* Sanden, Sedayu, Bambanglipuro, Piyungan and Dlingo. No correlation could be found between the number of *Ae. aegypti* positive mosquitoes and the relative number of *Ae. aegypti* individuals collected. Nevertheless, the detection of dengue in adult mosquitoes hatched from ovitrap-collected eggs was an indication of the vertical maintenance in local *Aedes* populations.

Table 3
Ovitraps distribution and hatched adult mosquitoes screening.

District	Total ovitrap	Positive ovitrap	Ovitrap index (%)	Mosquitoes analyzed by RT-PCR	Dengue-positive mosquitoes	Infection index (%)
Pajangan	58	27	46.55	8	1	12.50
Sewon	56	12	21.43	8	2	25.00
Bantul	56	19	33.93	5	2	40.00
Kasihan	55	18	32.78	31	2	6.45
Sedayu	70	5	7.14	7	0	0.00
Pandak	57	15	26.32	12	2	16.70
Srandakan	56	31	55.36	10	4	40.00
Sanden	55	14	25.45	7	0	0.00
Bambanglipuro	55	14	25.45	7	0	0.00
Kretek	63	22	34.92	8	3	37.50
Pundong	57	24	42.11	9	3	33.30
Jetis	58	14	24.14	9	2	22.20
Imogiri	57	20	35.09	11	7	63.60
Pleret	60	25	41.67	11	3	27.30
Banguntapan	55	21	38.18	10	3	30.00
Piyungan	51	8	15.90	8	0	0.00
Dlingo	59	13	22.03	5	0	0.00

3.3. RT-PCR detection and typing of DENV in mosquitoes

The identification of the DENV types and their relative distribution in the Bantul Regency was conducted using RT-PCR (Figure 1). When using PCR detection, mosquitoes infected with dengue were found in 14 districts out of 17 (82.3%) in the Bantul Regency, and the three additional districts were Sanden, Sedayu, and Piyungan; whereas when using immunocytochemistry, infected mosquitoes were detected in only 11 districts (64.7%). Mosquitoes from 12 districts (85.7%) were found to be infected with DENV-3, out of which 11 were hosting only DENV-3 and one, Pandak, showed a cocirculation of DENV-2 and DENV-3 (Figure 1). Two additional districts were found to harbor mosquitoes

infected with other dengue serotypes. Mosquitoes collected from Kasihan were infected only with DENV-2 and in the district of Bantul a cocirculation of DENV-2 and DENV-4 was recorded (Figure 1). All together DENV-3 was found in 12 districts (76.4%), DENV-2 was recorded in 3 districts (17.6%), DENV-4 was present in only one district (5.9%) whereas 3 districts (17.6%) provided no infected mosquitoes. No DENV-1 infected mosquitoes were found.

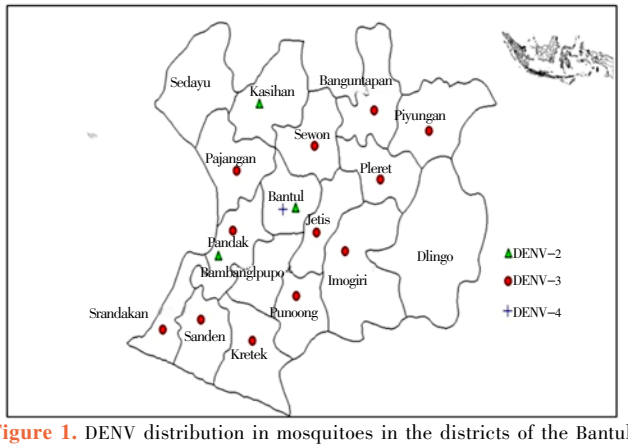

Figure 1. DENV distribution in mosquitoes in the districts of the Bantul Regency.

4. Discussion

The data reported in this work confirm the presence of vertical transmission and maintenance of DENV in *Ae. aegypti* populations close to households of HFD diagnosed patients and also indicate that DENV-3 is the most prevalent serotype in mosquitoes captured in the Bantul Regency. These environmental data corroborate previous clinical observations indicating a dominance of DENV-3 in patients with a severe dengue syndrome[23,24]. In addition, DENV-2 and DENV-4, although present as circulating serotypes, represent marginal occurrences in the captured mosquitoes as in infected patients. Although, this indicates the cocirculation of different serotypes of DENV in the same *Ae. aegypti* populations or different populations sharing the same area in at least two provinces, it nevertheless suggests a stability of the serotypes relative incidence over time. DENV-3 was shown to be the predominant serotype followed by DENV-4 and DENV-1[23,24]. The predominance of DENV-3 in Central Java in hospitalized patients has been reported for a long time[25,26]. The four serotypes were also found in the Jakarta Area from 1972 to 1992 with DENV-2 and DENV-3 as dominant serotypes, followed by DENV-1 and DENV-4[25]. In 2004, a RT-PCR survey conducted during a dengue outbreak showed that DENV-3 was the most dominant (57.0%), followed by DENV-4 (20.7%), DENV-2 (13.7%) and DENV-1 (5.6%). Double DENV-3/DENV-1 infections were recorded in patients in 3.7% of cases[26]. Similar data were obtained by a survey conducted in Semarang where DENV serotypes in *Aedes* species were dominated by DENV-3, followed by DENV-2 and DENV-1, with few DENV-4, whereas no relationship could be established between the distribution of DENV serotypes and isolation of *Aedes* mosquitoes with DHF endemicity levels[27]. However, the occurrence of severe forms of dengue has been the subject of contradictory conclusions and hypotheses depending upon time and places. They were associated to DENV-2 in South America but to DENV-3 peaks in Asia, with the exception of Thailand[10,28,29], to secondary infection with differing serotypes[30-33], to virulent viral forms[33,34], and to individual immunological reactions[33]. Interestingly, a shift from DENV-2 to DENV-1 associated with a 3-time increase in DHF cases in patients from the seaport city of Surabaya (Eastern Java) in 2008 was accompanied by the presence in wild-caught mosquitoes of DENV-1 genotypes[16,35]. However this shift took place within only one month from October to November, 2008, suggesting that a climatic event and/or the import of exogenous mosquito populations through the seaport might have occurred. Unlike other regions of continental South East Asia, such as Cambodia or Thailand, where the replacement of serotypes was shown to occur frequently[8-10], the maintenance of DENV-3 at a higher prevalence seems to be a major feature at least in Java Island. Vertical transmission and maintenance of DENV in mosquito populations is very likely to participate to this feature along with the archipelago structure of Indonesia, which might also help maintaining local mosquito populations.

Indonesia is the country who has experienced the highest increase of dengue incidence in South East Asia since 2004[35]. Understanding the dynamic of dengue outbreaks in the country and developing risk management scenarios are therefore a key issue. However, being an archipelago with a patch mosquito population structure and specific movement of populations associating high mobility in seaports and commercial nodes and relative stasis inland, Indonesia seems to be a mosaic of individual situations making the development of a global model for prediction and management difficult. This work indeed also underlines the difficulty of analyzing the dynamic of the disease evolution in the absence of key clinical and epidemiological information and thus of any possibility of conducting a true integrated analysis. Like previous

analyses, it is merely a snapshot, which cannot reflect the overall situation and the spatio-temporal evolution of dengue. The lack of recurrent data recording on the dengue serotypes isolated from patients makes it difficult to correlate clinical occurrence and cocirculation in mosquito populations. Furthermore, since only severe forms requiring hospitalization were recorded in the district health center there is a clear underestimation of the dengue burden in the Bantul Region.

This work is part of a concerted transdisciplinary and integrated approach aiming at developing dynamic models and simulation tools to be implemented at the community scale, which is the most suitable one to implement efficient prediction and control actions. Partial analyses and incomplete surveillance and reports will undoubtedly lead to misleading conclusions and inappropriate and unsuccessful actions. There is also a true need for the implementation of accurate and permanent surveillance and diagnostic taking into account the clinical occurrence of the various serotypes and lineages in human cases and their association with various forms of the disease. The circulation of serotypes and lineages in mosquito populations, the dynamics of mosquito populations themselves as well as the impact of human activity, *i.e.* the movement of people and goods and urbanization, and the climatic and environmental factors must altogether be considered in detail. In face of the complexity of dengue dynamics, only such a comprehensive modeling will be able to bring elements of understanding and options for efficient control hints for control. However, this also requires a call for international and coordinated financial and scientific efforts, the perhaps only way to efficiently tackle this spreading major arboviral disease.

Conflict of interest statement

We declare that we have no conflict of interest.

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