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Molecular investigations of dengue virus during outbreaks in Orissa state, Eastern India from 2010 to 2011

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ABSTRACT

Dengue is one of the most important arboviral diseases in India. Orissa state in Eastern India reported the first dengue outbreak in 2010, followed by extensive outbreaks in 2011, affecting large number of people. Detailed entomological, serological and phylogenetic investigations were performed in mosquitoes and patients serum collected from dengue virus (DENV) affected areas of Orissa. The combination of DENV specific IgM capture-ELISA and reverse-transcription PCR (RT-PCR) detected high DENV positivity in serum samples. DENV was detected in mosquitoes reared from field caught pupae by RT-PCR, which confirmed the vertical transmission of DENV that may have an important role in the recurrence of dengue outbreaks. Phylogenetic analyses revealed the circulation of Indian lineage of DENV-2 (genotype-IV) and DENV-3 (genotype-III) in vectors and patients serum in Orissa from 2010 to 2011, DENV-2 being the prevailing serotype. Selection analyses within the C-prM region showed that the emergence of DENV-2 and DENV-3 in Orissa was constrained by purifying selection which suggested the role of ecological factors like mosquito density and behavior in the recurrent outbreaks. *Aedes albopictus* was found to be the most abundant vector in the areas surveyed, followed by *Aedes aegypti*. Indoor breeding spots (earthen pots) were most abundant, with high pupal productivity (38.50) and contributed maximum *Aedes* species in the affected areas. The DENV infection rate estimated by maximum likelihood estimate (MLE) was high for indoor breeding *Aedes* (4.87; 95% CI: 1.82, 10.78) in comparison to outdoor breeding *Aedes* (1.55; 95% CI: 0.09, 7.55). The high MLE in *Ae. albopictus* (4.72; 95% CI: 1.94, 9.80) in comparison to *Ae. aegypti* (1.55; 95% CI: 0.09, 7.54) indicated that *Ae. albopictus* was the main DENV vector responsible for the outbreaks. The results indicated the circulation of two virulent serotypes of DENV in Orissa, mainly by *Ae. albopictus* with the implication for implementation of intradomecile vector control measures to prevent the spread of dengue.

1. Introduction

Dengue, one of the major arboviral diseases in the world is caused by four antigenically and genetically distinct, single-stranded positive sense RNA viruses designated dengue virus serotypes 1–4 (DENV-1–4) (Gubler, 1998; Halstead, 2007).The DENV serotypes are further classified into multiple genotypes based on their genomic diversity (Weaver and Vasilakis, 2009). DENV infection is characterized by a spectrum of illness from mild, self limiting dengue fever to life threatening dengue haemorrhagic fever (DHF) and dengue shock syndrome (DSS). Recently the WHO/TDR, 2009 proposed a new classification of dengue, i.e. dengue (D), dengue with warning signs (DW) and severe dengue (SD) in order to re-evaluate the current classification for better management of high case fatalities (WHO/TDR, 2009). Several hypotheses, like antibody dependent enhancement (ADE) in heterotypic secondary dengue infections, involvement of a virulent viral genotype, and host factors have been suggested to explain the mechanism of pathogenesis of DHF and DSS (Mc Bride and Bielefeldt-Ohmann, 2000). Some genotypes of DENV have been associated with the occurrence of frequent DHF epidemics, while some others have been linked mostly to mild clinical manifestations. Thus, the circulation of certain genotypes might increase the risk for the development of DHF cases. Therefore, molecular surveillance is required in DENV affected areas to accurately monitor the introduction and circulation of new and more aggressive viral strains, especially during recurrent outbreaks (Rico-Hesse, 2007).

A member of family *flaviviridae,* DENV is transmitted to humans by the bite of an infected female mosquito. *Aedes aegypti* is the principal vector involved in transmission of DENV, with *Aedes albopictus* mainly serving as secondary vector. However, historically, *Ae. albopictus* has been responsible for dengue transmission in countries where *Ae. aegypti* was absent, e.g. Japan, China(Macao

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region) and United States (Hawaii state) (Shroyer, 1986; Gratz, 2004; Almeida et al., 2005; Effler et al., 2005). Spread of the mosquito vector and the virus has led to the resurgence of dengue epidemics and the emergence of dengue in new areas. Mosquito control is currently the only way to curb the spread of dengue, and vector control will remain integral in controlling the disease even after the eventual implementation of a safe and effective vaccine. Vector control is presently evaluated using surveillance techniques based on larval indices to determine risk and to guide mosquito control activities (PAHO, 1994). However, recently there has been a movement towards pupal indices, considering early instars as too immature to be representative of true mosquito productivity, because survival from early stage larvae to pupae is variable and a majority of larvae do not survive to adulthood, whereas most pupae survive to emerge as adults (Focks and Chadee, 1997). Hence identification and genotyping of DENV from field collected mosquitoes and pupae are required for successful epidemiological surveillance.

The DENV genome is approximately 11 kb in length and encodes eleven distinct proteins comprising 3 structural and 8 non-structural proteins in the order 5′-C-PrM/M-E-NS1-NS2A-NS2A-NS2B- NS3-NS4A-NS4B-NS5–3′. Although several regions within the DENV genome has been used for molecular studies, the capsid premembrane junction (C-prM) has been mainly used for rapid serotyping and genotyping of DENV in patients serum and mosquitoes because it harbours epidemiologically important sequence information and uses a single set of primer pair which could be used for amplification and subsequent sequencing for all four serotypes of DENV (Urdaneta et al., 2005). Thus rapid and economical molecular serotyping and genotyping of DENV can be accomplished using the C-prM region during epidemiological investigations.

Epidemics caused by the 4 DENV serotypes have been continuously reported from many parts of India (Dash et al., 2004; Dar et al., 2006). Although dengue outbreaks have prevailed throughout India, Orissa state, in the eastern part of India had never reported any dengue outbreak, in spite of the presence and circulation of important *Aedes* vectors like *Ae. aegypti* and *Ae. albopictus*. Recently, *Ae. albopictus* played a major role in the transmission of chikungunya in many parts of Orissa (Das et al., 2012a). In September 2010, the Public Health and Welfare Department of Orissa reported an outbreak of dengue for the first time in Malkangiri district. Since then, sporadic cases of DENV infection have been reported from different regions of the state, mainly Angul, Malkangiri, Dhenkanal, Sambalpur, Bolangir and Ganjam districts. From August 2011, the health authorities of Orissa reported repeated outbreaks of dengue viral infection in many districts affecting more than 10,000 persons with several deaths. The Angul district was the most affected contributing 80% of the total cases, followed by Malkangiri and other districts. In this study, we report the circulation of Indian lineage of DENV-2 (genotype IV) and DENV-3 (genotype III) and the involvement of indoor breeding *Aedes* species in the transmission of DENV in Orissa.

2. Materials and methods

2.1. Study area

The state of Orissa is divided into four distinct physiogeographical regions; northern plateau, central tableland, coastal plains and eastern ghats. The study was conducted in Malkangiri district (Eastern Ghats) and Angul district (Central tableland) from 2010 to 2011 (Fig. 1). The areas affected by DENV infection of the representative districts were considered as case sites and areas, about 1.5 km away from the affected areas with no reported case of DENV infection, were taken as control sites. Detailed entomologic and molecular investigations were carried out in both case and control sites in Malkangiri and Angul districts.

2.2. Entomological studies

2.2.1. Entomological collection

Collection of adult mosquitoes was carried out in the case and control sites by using carbon dioxide baited traps and battery-operated aspirators. Collections were performed twice a day: 6.00 AM to 7.30 AM and 3.00 PM to 6.30 PM in the case and control sites. For collection of larvae/pupae, all containers with water were searched in indoor/domestic and outdoor areas and larvae/pupae collected using dip method or by Pasteur pipette. All water containing indoor and outdoor breeding containers were thoroughly searched for the presence of *Aedes* larvae/pupae. Adult mosquitoes, larvae and pupae collected from field were brought to the laboratory. Pupae were left to emerge as adults for identification. *Aedes* species were identified from the collected samples using standard keys of identification (Barraud, 1934) and pooled according to species, sex and the type of container habitat. The data on larval/pupal survey was analyzed and calculated in terms of different entomological indices, i.e. container index (CI) house index (HI) and breteau index (BI) as per the WHO procedure (WHO, 2003). Abundance of indoor and outdoor containers with *Aedes* pupae at the case and control sites was assessed in the study to know the most productive container in the areas surveyed. Productivity of a container type (the number of pupae in each container type divided by the total number of pupae in all containers) (Focks and Chadee, 1997; Barrera et al., 2006) was estimated for each container that harboured *Aedes* pupae. It will help to identify the most productive containers harbouring *Aedes* pupae during epidemiological surveillance.

2.2.2. Mosquito processing

Each mosquito pool ($\leqslant$10 mosquitoes), i.e. field collected adults and those emerged from pupae was subjected to laboratory processing for DENV identification. The infection rate of each DENV positive mosquito pool was estimated using a maximum likelihood estimate (MLE) statistical method for unequal pool sizes that calculated 95% Confidence Interval (CI) per 1000 mosquitoes (http://www.cdc.gov/ncidod/dvbid/westnile/software.htm) (Biggerstaff, 2009). The MLE estimate is more accurate and robust than minimum infection rates, which estimates the lower bound of the infection rate (Gu et al., 2008).

2.3. Molecular studies

2.3.1. Patients' serum collection

Blood samples (5 ml) were collected from suspected dengue patients (n = 185) after detailed clinical diagnosis by trained medical staff of community health centres, primary health centres and subcentres of Malkangiri and Angul districts. A tourniquet test was done on all the patients to confirm dengue infection clinically. The blood samples were stored at 15 °C in portable coolers and brought to Regional Medical Research Centre, Bhubaneswar within 8 h of collection. Serum was extracted from the blood by centrifugation at 5000 rpm for 10 min at 15 °C. It was stored at −80 °C until further processing.

2.3.2. Serology

All serum samples were tested for the presence of dengue virus specific IgM antibodies using Dengue-IgM capture ELISA (Innis et al., 1989). To rule out CHIKV infection/coinfection, all the serum samples were also tested for the presence of specific IgM antibodies using Chikungunya-IgM capture ELISA (Hundekar et al., 2002).

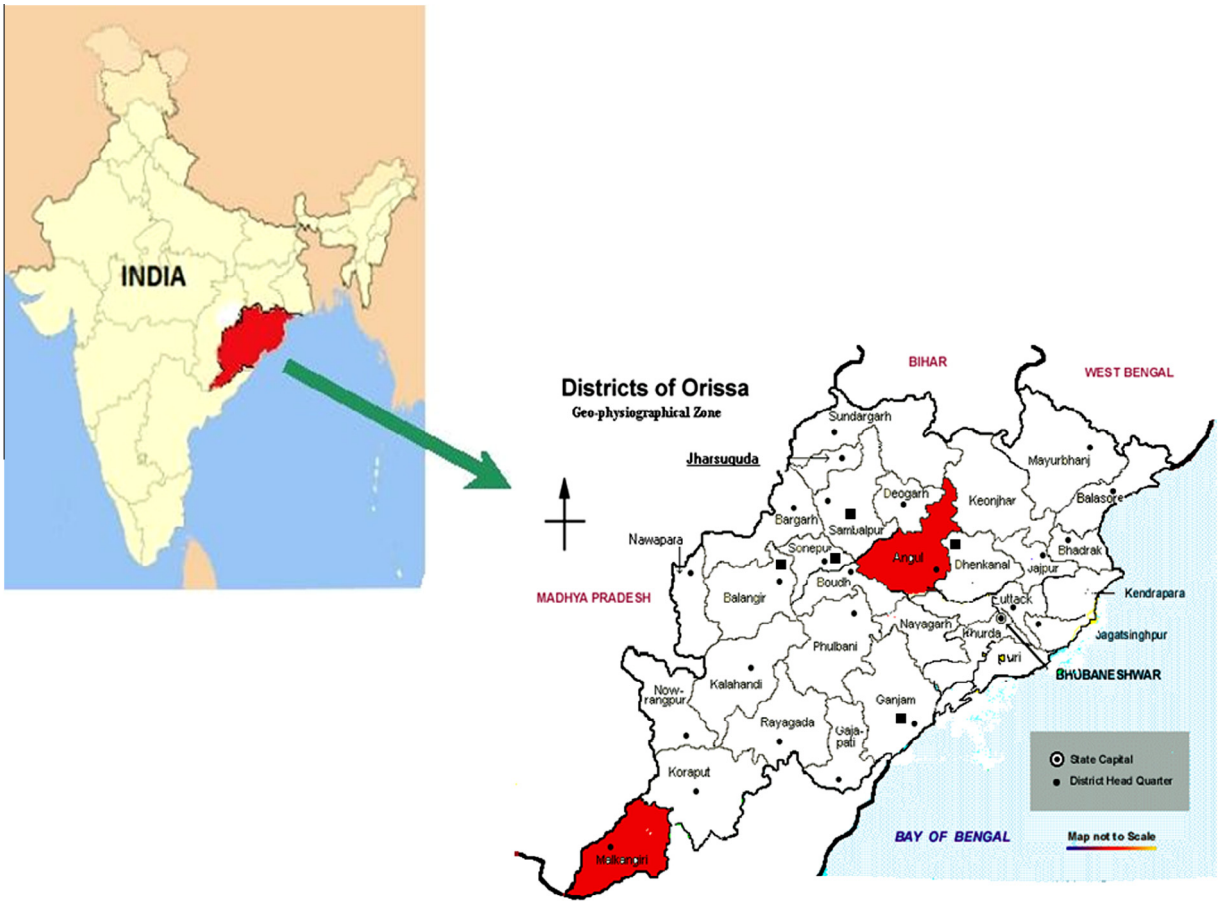

Fig. 1. Map of India and Orissa state showing the study areas (Malkangiri and Angul district) in red color. Solid squares represent the areas where sporadic cases occurred. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this book.)

2.3.3. Viral RNA Isolation

Viral RNA was isolated from 140 μL serum employing QIAamp viral RNA mini kit (Qiagen, Hilden, Germany) and eluted in 50 μL elution buffer following manufacturer's protocol. For isolation of viral RNA from mosquito pools, each pool was crushed and homogenized thoroughly in 0.2 ml of 10% phosphate buffer saline (pH 7.8) containing 4% bovine albumin and antibacterial and antifungal antibiotics (100 U/mL penicillin, 100 μg/mL streptomycin and 0.25 μg/mL amphotericin B). The homogenate was clarified by centrifugation and used for viral RNA isolation. The extracted viral RNA was stored at −80 °C till further processing.

2.3.4. Dengue virus mosquito controls for RT-PCR

Ae. albopictus mosquito pools spiked with known concentration of DENV-1, DENV-2, DENV-3 and DENV-4 were used as positive controls for RT-PCR. The dengue viruses were kindly provided by the virology laboratory, Defence Research and Development Organization, India.

2.3.5. Reverse transcription-polymerase chain reaction (RT-PCR)

Detection and typing of DENV in mosquito pools and patients serum was carried out by RT-PCR kit (Qiagen, Hilden, Germany) targeting the C-prM region according to the protocol of Lanciotti et al., 1992, with slight modifications in two steps: The first step involved the synthesis of viral cDNA from viral RNA by using the dengue complex-specific primers [D1(5′-TCAATATGCTGAAACGCGCGAGAAACCG-3′) & D2(5′-TTGCACCAACAGTCAATGTCTTCAGGTTC-3′)] in a single tube, exhibiting 511 bp band. RT was carried out at 42 °C for 90 min and inactivation of RT enzyme at 85 °C for 10 min, followed by amplification:- initial denaturation at 94 °C for 5 min, followed by 35 cycles of denaturation at 94 °C for 30 s, annealing at 56 °C for 40 s, extension at 72 °C for 1 min and final extension at 72 °C for 5 min. The second step was perfomed by nested PCR using 5 μL of diluted material (1/100) from initial amplification reaction as template alongwith serotype-specific primers (TS1/TS2/TS3/TS4) as downstream primer and D1 as upstream primer in four different reactions exhibiting 482 bp, 119 bp, 290 bp and 392 bp respectively. The temperature cycling conditions consisted of 18 cycles of 94 °C for 30 s, annealing at 55 °C for 1 min, extension at 72 °C for 2 min and final extension at 72 °C for 10 min The amplicons were resolved on 1.5% agarose gel, column purified and used as template in sequencing reactions.

2.3.6. Sequencing and phylogenetic analysis

Of the samples diagnosed positive for DENV infection by nested PCR, four DENV-2 mosquito pools and two DENV-2 patients serum from Malkangiri district, one DENV-2 patients serum, one DENV-3 mosquito pool and two DENV-3 patients serum from Angul district were sequenced in a 16 capillary (90 cm) automated DNA sequencer (Applied Biosystems, Foster City, Ca, USA) using dengue group specific primers (D1/D2) and performance optimized polymer 7 following the manufacturer's instructions. The nucleotide sequences were retrieved, edited and analyzed by SeqScape (Applied Biosystems) and EditSeq of Lasergene 5. Multiple sequence alignment was performed by employing Muscle (Edgar, 2004). The best

fit model of nucleotide substitution was selected by using Akaike Information Criterion (AIC) as implemented in Modeltest version 3.7 (Posada, 2006). Phylogenetic analysis was done using the maximum-likelihood method using generalized time-reversible model of nucleotide substitution with gamma-distributed variation among sites and a proportion of invariable sites (GTR + G5 + I) available in Mega 5 software (Tamura et al., 2011). The robustness of each node was estimated using 1000 bootstrap replications under the Nearest-Neighbor Interchange procedure, with input genetic distance determined by the maximum-likelihood method. The phylogenetic tree generated was imported in newick format to Figtree 3.1 software and a more uniform midpoint rooted phylogenetic tree was developed.

2.3.7. Selection pressure analyses

Site-specific selection pressures in the C-prM region of DENV-2 and DENV-3 isolates were analyzed as the ratio of non-synonymous (dN) to synonymous (dS) substitutions per site, estimated by using likelihood procedures including single likelihood ancestor counting (SLAC), random effects likelihood (REL) and fixed effects likelihood (FEL) (incorporating the HKY85 model of nucleotide substitution and phylogenetic trees inferred by using the neighbor joining method). Sites were considered to be under positive selection if at least two of the methods indicated this with high statistical significance ($p < 0.1$/Bayes factor >50). The analysis was carried out using the online Datamonkey facility (http://www.datamonkey.org/) of the HyPhy package. For selection analysis of DENV-2 isolates, four datasets were used: all genotype V (American) sequences ($n = 12$), all genotype IV (Cosmopolitan) sequences ($n = 32$), only Indian sequences of genotype V ($n = 9$) and genotype IV ($n = 25$). For selection analysis in DENV-3 isolates, whole dataset of sequences belonging to genotype III was divided into four parts: all Indian sequences ($n = 19$), all American sequences ($n = 6$), all Sri Lankan sequences ($n = 2$) and all Singaporean sequences ($n = 4$). In all the datasets, closely related sequences (>99.5% nucleotide identity) were removed from the analysis.

2.3.8. Statistical analysis

The non parametric Mann Whitney *U* test was used to calculate the two-tailed *p* values by comparing the abundance of indoor and outdoor containers harboring *Aedes* pupae in the case and control sites. The relative abundance of all indoor and outdoor containers harboring *Aedes* pupae in the case sites was analyzed by the non parametric Kruskal Wallis one way analysis of variance (ANNOVA) test. The Fisher Exact test was employed to compare the occurrence of DENV infected *Aedes* pool and to calculate the odds ratio in indoor and outdoor containers. A p value < 0.05 was considered to be statistically significant for all the tests. All the statistical tests were performed by using the Graphpad Prism (version 5.01) software.

3. Results

3.1. Entomology

A total of 2156 *Aedes* mosquitoes [258 (77 males & 181 females) field caught and 1898 pupae reared] were collected from case and control sites and processed in 121 pools. The collection comprised 60.9% *Ae. albopictus,* 29.8% *Ae. aegypti* and 9.3% *Ae. vittatus* (Table 1). Indoor containers (earthen pots, buckets and plastic drums) were the most abundant (68.2%) and contributed maximum *Aedes* species in case sites and *Aedes* species was found in most outdoor containers (tires, small wastes, large wastes, tree holes, stony pits & cement tanks) in control sites (72.9%) in both the districts. The average productivity of indoor containers was high (21.77) in comparison to outdoor containers (5.77) in the case sites, with earthen pots registering the highest value (38.50) (Table 2). In control sites, the average productivity was high for the outdoor containers (11.80), (especially cement tanks) in comparison to indoor containers (9.71) (Table 3). DENV RNA was detected in 6 of the 75 *Ae. albopictus* pools and 1 of the 35 *Ae. aegypti* pools by virus isolation and RT-PCR. One field caught adult pool and five mosquito pools (reared from pupa) of *Ae. albopictus* and one mosquito pool (reared from pupa) of *Ae. aegypti* were found to be positive for DENV RNA. DENV-2 was detected in 4 *Ae. albopictus* pools collected from Malkangiri, 1 *Ae. albopictus* pool and 1 *Ae. aegypti* pool obtained from Angul outbreak. DENV-2 and DENV-3 was detected in one *Ae. albopictus* pool collected from Angul in 2011. Out of 6 DENV positive mosquito pools (reared from pupa), 5 (85.7%) pools were detected in indoor containers, i.e. earthen pots (4) and buckets (1) in the case sites, and 1 (14.3%) pool was detected in outdoor containers, i.e., in discarded tires in the control sites. The DENV infection rate estimated by maximum likelihood estimate (MLE) statistical method was high for indoor breeding *Aedes* species (4.87; 95% CI: 1.82, 10.78) as compared with outdoor breeding *Aedes* species (1.55; 95% CI: 0.09, 7.55). The high MLE of *Ae. albopictus* (4.72; 95% CI: 1.94, 9.80) in comparison to *Ae. aegypti* (1.55; 95% CI: 0.09, 7.54) indicated that *Ae. albopictus* was the principal vector

Table 1
Mosquitoes collected, total number of pools, dengue virus positive pools and MLE after RT-PCR analysis in case and control sites of Malkangiri and Angul district, Orissa in 2010–2011.

Distribution of mosquitoes	Case sites				Control sites			
	No. of specimens (pools)	DENV positive pools	Infection rate MLE (95% CI)	DENV serotypes	No. of specimens (pools)	DENV positive pools	Infection rate MLE (95% CI)	DENV serotypes
Mosquitoes collected from field								
Ae. albopictus female	112 (11)	1	8.92 (0.52, 43.39)	2	28 (3)	0	0	–
Ae. albopictus male	27 (3)	0	0	–	18 (2)	0	0	–
Ae. aegypti female	21 (2)	0	0	–	12 (1)	0	0	–
Ae. aegypti male	18 (2)	0	0	–	8 (1)	0	0	–
Ae. vittatus female	8 (1)	0	0	–	0	0	0	–
Ae. vittatus male	6 (1)	0	0	–	0	0	0	–
Total	192 (20)	1	0		66 (7)	0	0	
Pupae reared mosquitoes								
Ae. albopictus	687 (34)	4[a]	6.09 (1.99, 14.66)	2, 2, 2, 2&3	440 (22)	1[b]	2.27 (0.13, 11.06)	2
Ae. aegypti	381 (19)	1[a]	2.63 (0.15, 12.83)	2	203 (10)	0	0	–
Ae. vittatus	106 (5)	0	0	–	81 (4)	0	0	–
Total	1174 (58)	5	0	–	724 (36)	1	0	–

[a] DENV positive pools in indoor breeding spots.
[b] DENV positive pools in outdoor breeding spots.

Table 2
Abundance of indoor and outdoor containers with *Aedes* pupae alongwith their productivity and the type of DENV detected in case sites of Orissa.

Distribution	Receptacle type	Pupae			No. of containers	Productivity of container	Type of DENV detected
		n	Avg[a] (SD)	Max[b]			
Indoors	Earthen pots	452	22 (41)	121	438	38.50	DENV-2, 3
	Buckets	162	21 (53)	72	156	13.79	DENV-2
	Plastic drums	153	18 (32)	67	139	13.03	
Outdoors	Discarded tires	138	23 (31)	52	118	11.75	–
	Tree holes	52	8 (10)	27	45	4.42	–
	Cement tanks	77	23 (27)	47	51	6.55	–
	Discarded small wates (<3 l)	56	13 (25)	43	36	4.77	–
	Discarded large wastes (>10 l)	61	16 (27)	37	32	5.19	–
	Stony pits	23	7 (9)	18	17	1.95	–
	Total	1174			1032		

Productivity of container = No. of pupae in the container 100×/total No. of pupae.
[a] Average No. of pupae.
[b] Maximum No. of pupae in an individual container.

Table 3
Abundance of indoor and outdoor containers with *Aedes* pupae alongwith their productivity and the type of DENV detected in control sites of Orissa.

Distribution	Receptacle type	Pupae			No. of containers	Productivity of container	Type of DENV detected
		n	Avg[a] (SD)	Max[b]			
Indoors	Earthen pots	83	12 (21)	22	67	11.46	–
	Buckets	53	16 (19)	17	42	7.32	–
	Plastic drums	75	27 (31)	51	34	10.35	–
Outdoors	Discarded tires	153	26 (41)	40	137	21.13	DENV-2
	Tree holes	22	12 (14)	11	15	3.03	–
	Cement tanks*	175	28 (31)	53	164	24.17	–
	Discarded small wates (<3 litres)	61	21 (34)	36	51	8.42	–
	Discarded large wastes (>10 litres)	84	19 (26)	41	54	11.60	–
	Stony pits	18	7 (9)	12	11	2.48	–
	Total	724			575		

Productivity of container = No. of pupae in the container 100×/total No. of pupae.
[a] Average No. of pupae.
[b] Maximum No. of pupae in an individual container.

Table 4
Age distribution of DENV positive and negative cases.

Age distribution of patients (years)	Total No. of suspected cases ($N = 185$)		Total (%)
	No. of DENV +ve cases (%)	No. of DENV −ve cases (%)	
4–14	12 (9.0)	5 (9.6)	17 (92)
15–24	29 (21.8)	30 (57.7)	59 (31.9)
25–50	87 (65.5)	14 (26.9)	101 (54.6)
51–75	5 (3.7)	3 (5.8)	8 (4.3)
Total	133	52	185

responsible for the outbreaks (Table 1). Furthermore, maximum *Ae. albopictus* immature stages (larvae & pupae) were obtained from indoor containers, having high CI (>60) and BI (>100) in the case sites in comparison to low CI (<20) and BI (<30) in the control sites.

3.2. Demography of patients

Of the 185 patients enrolled in the study, 90 were females and 95 males with ratio 1:0.94. Their age ranged from 4 to 80 years. Most cases of DENV infection belonged to the age group 25–50 years (Table 4). A predilection by sex was not noted among patients with and without dengue fever.

3.3. Serology and RT-PCR

Out of 185 patients collected, 133 were diagnosed with dengue viral infection either based on detection of IgM antibody by ELISA or RT-PCR. All serum samples were tested negative for the presence of chikungunya virus specific IgM antibodies by CHIK-IgM ELISA. Out of 133 patients, 80 (60.1%) confirmed the presence of C-prM gene of DENV by RT-PCR and 43 (32.3%) patients confirmed the presence of dengue IgM antibodies by ELISA. 10 (7.6%) patients proved positive both by RT-PCR and IgM ELISA in the same serum sample (Table 5). All the serum samples, which were positive by RT-PCR were collected between 2 and 6 days post onset of fever, thereby indicating acute stage of infection. Based on the amplification by nested PCR, DENV-2 (119 bp) was detected in 76 (85%) patients from Malkangiri and Angul districts and coinfection of DENV-2 and DENV-3 (119 bp & 290 bp) was detected in 14 (15%) patients from Angul district (Figs. 2 and 3).

3.4. Phylogenetic and sequence analyses

Phylogenetic analyses of C-prM gene sequences of DENV-2 viral isolates obtained from mosquitoes and patients serum from Orissa along with other viral sequences derived from GenBank showed that Indian DENV-2 isolates (pre1990) were grouped under genotype-V (American genotype) and the isolates (post1990) grouped under genotype IV (Cosmopolitan genotype). The only exception was India-60 isolate of 2001 which grouped under American genotype, which was further supported by the amino acid substitutions it harbored that belonged to American genotype. Further analysis revealed that genotype IV was subdivided into 2 clades: Indian lineage (Orissa DENV-2 isolates were grouped within this lineage) and Non Indian lineage (Fig. 4). Within the Indian lineage, the North Indian isolates and Orissa isolates were clustered together

Table 5
Serological and RT-PCR results of suspected DENV patients collected from different areas of Angul and Malkangiri district, 2010–2011.

Collection site (District)	No. of patients	No. of IgM positive	No. of RT-PCR positive	No. of IgM + RT-PCR positive	No. of DENV-2 positve sera by RT-PCR	No. of DENV-2 + DENV-3 sera by RT-PCR
Kalamachuin (Angul)	44	14	24	3	21	6
Bantala (Angul)	31	7	14	1	13	2
Talcher (Angul)	25	5	10	2	11	1
Beda (Angul)	12	7	3	0	3	0
Padmagiri (Malkangiri)	33	4	14	2	13	3
Main town (Malkangiri)	27	4	8	1	8	1
Block colony (Malkangiri)	13	2	7	1	7	1
Total	185	43	80	10	76	14

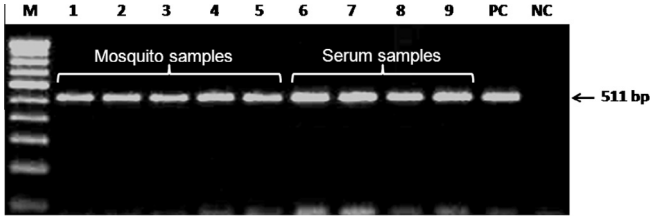

Fig. 2. RT-PCR gel: Ethidium bromide stained agarose gel showing the DENV specific C-prM gene region (511 bp) in mosquitoes and patients' serum (lanes 1–9) from Malkangiri and Angul outbreaks. M is the 100 bp DNA ladder. PC denotes DENV positive control and NC denotes negative control.

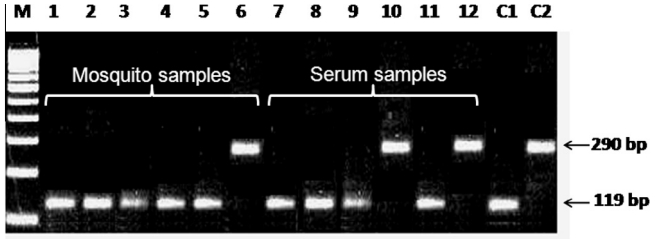

Fig. 3. Nested PCR gel: Ethidium bromide stained agarose gel showing the serotype DENV-2 (119 bp) in mosquitoes (lanes 1–4) and patients' serum (lanes 7–8) obtained from Malkangiri and Angul outbreaks. Lanes 5 & 6 represent serotypes DENV-2 (119 bp) and DENV-3 (290 bp) in the same mosquito sample from Angul district. Lanes 9 & 10 and lanes 11 & 12 represent two patients sera showing DENV-2 (119 bp) and DENV-3 (290 bp) from Angul outbreak. M is the 100 bp DNA ladder. C1 and C2 represent DENV-2 and DENV-3 positive controls.

and were distinct from South Indian isolates (Kerela 2008 isolates). From the phylogenetic tree, it can be inferred that there has been a shift in the occurrence of DENV-2 genotypes in the course of time, i.e., genotype-V (American genotype), mainly responsible for Indian dengue outbreaks prior to 1990 has been replaced by genotype-IV (cosmopolitan genotype) which caused dengue outbreaks post 1990 and has often been associated with the occurrence of recent DHF epidemics in India.

Phylogenetic analyses of C-prM gene sequences of DENV-3 viral isolates obtained from mosquitoes and patients serum from Orissa alongwith other viral sequences derived from GenBank showed that Indian DENV-3 isolates were grouped under genotype-III. More detailed analysis showed that genotype III was subdivided into 4 clades: clade I represented Indian lineage (Orissa DENV-3 isolates grouped within this clade), clade II represented American lineage, clade III represented Srilankan lineage and clade IV represented Singaporean lineage (Fig. 5). The Indian isolate (Gwalior-60) was an exception and grouped within the American lineage suggesting certain variations within the isolate that allowed it to group into non Indian lineage. However, there has been no change in the occurrence of DENV-3 genotypes with time (genotype-III, being the main prevailing genotype in India since its inception) unlike DENV-2 genotypes.

C-prM gene sequence analyses showed a high sequence identity (98.2–98.9%) among DENV-2 and DENV-3 isolates of Orissa (Supplementary Table S1) alongwith North Indian isolates, mainly Gwalior 2001 and Delhi 2006 isolates. Sequence divergence of 11.7% was noted between the early (pre1990) and recent (post1990) DENV-2 Indian isolates. Compared to DENV-2 isolates of the American genotype (including the Indian isolates before 1990), all Indian DENV-2 isolates (post1990) belonging to Cosmopolitan genotype, including Orissa isolates had the non-synonymous M104 V, V112A and T145A amino acid changes. However the Non Indian isolates belonging to Cosmopolitan genotype had certain unique substitutions, i.e., L108 M, D143 N and valine at position 112 which were not found in Indian isolates and thus responsible for clustering them into separate clade. The India-60 (P23850) isolate of 2001 grouped under the American genotype as it had the amino acid substitutions present in the isolates belonging to American genotype. Comparison of C-prM gene sequences in all Indian DENV-3 genotype-III isolates (including the Orissa isolates) revealed high sequence identity (98.4%), alongwith M108I and T112A amino acid changes. The only exception was Gwl-60 isolate, which revealed 86% sequence identity and grouped within the American lineage in the phylogenetic tree.

3.5. Selection pressure analyses

Selection analyses within the C-prM gene region of DENV-2 isolates did not detect strong positive selection in the Cosmopolitan (Genotype IV) or American (Genotype V) genotypes. In all the datasets, most codon positions were strongly conserved, with a relatively low ratio of non-synonymous to synonymous substitutions per site (dN/dS), suggesting high purifying selection within the DENV-2. However, some evidence of positive selection was noted at sites 104 (SLAC: dN/dS = 2.58, $p = 0.25$; FEL: dN/dS = 1.92, $p = 0.10$), 112 (SLAC: dN/dS = 3.09, $p = 0.14$; FEL: dN/dS = 2.84, $p = 0.17$) in the Indian dataset as well as whole dataset belonging to cosmopolitan genotype. Evidence of positive selection was also observed in site 143 (SLAC: dN/dS = 4.13, $p = 0.22$; REL: dN/dS = 0.46, Bayes factor = 195.56) in the American genotype (both Indian and whole datasets) of DENV-2 isolates. Selection analysis of DENV-3 (genotype III datasets) did not detect any positively selected site within the C-prM gene region, which suggests that DENV-3 isolates were under strong purifying selection.

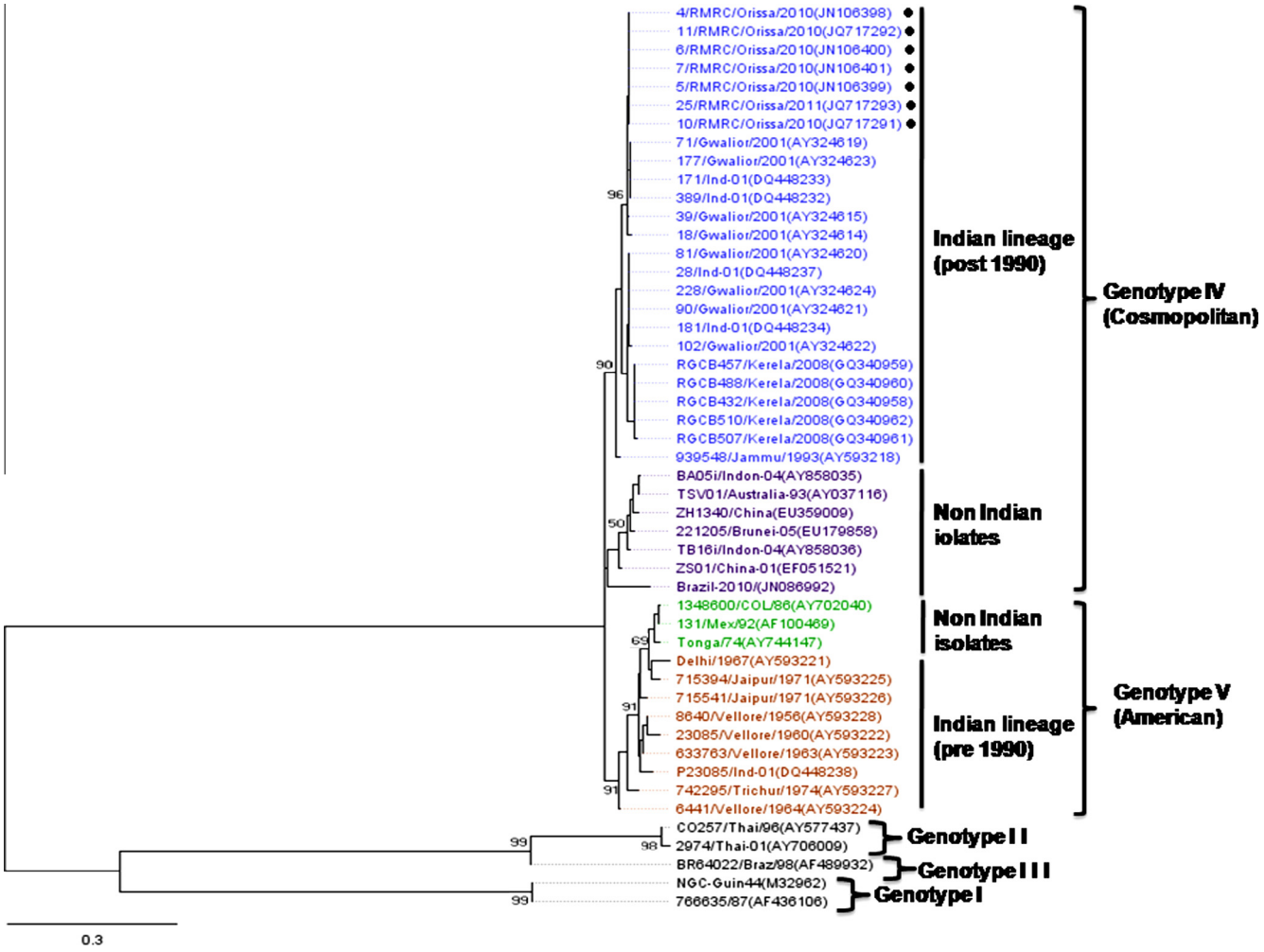

Fig. 4. Mid point rooted phylogenetic tree of DENV-2 viruses generated by maximum likelihood method based on C-prM gene (415 nt). Each strain is identified by its name and the year it was isolated, followed by GenBank accession numbers in parentheses. Solid circles denote viral RNAs isolated and sequenced from mosquitoes and serum obtained from Orissa. The tree depicted 2 broad genotypes containing Indian isolates: Genotype V (American) comprising Indian isolates before 1990 (red) and Non Indian isolates (green) and Genotype IV (Cosmopolitan) subdivided into Indian lineage after 1990 (blue) and Non Indian lineage (dark blue). Bootstrap values are indicated at the major branch points. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this book.)

3.6. Statistical results

The Mann Whitney *U* test analysis showed that indoor containers with *Aedes* pupae were most abundant in comparison to outdoor containers in the case sites (sum of ranks = 40, 15, $U = 1.00$, $p = 0.011$), whereas the abundance was high for outdoor containers as compared with indoor containers in control sites (sum of ranks = 15, 51, $U = 0$, $p = 0.004$) (Fig. 6). The Kruskal Wallis ANNOVA analyses showed that earthen pots (indoors) were the most abundant containers harboring *Aedes* pupae in the case sites (Kruskal Wallis statistic, $K = 33.6$, $p < 0.001$). Fisher exact test revealed that there was significant differences in the occurrence of DENV infected *Aedes* pools at indoor containers in comparison to outdoor containers and showed that the odds ratio of the occurrence of DENV infected *Aedes* pool was significantly high in indoor containers as compared with outdoor containers (OR = 6.00, $p = 0.042$, 95% CI = 1.12, 31.89).

4. Discussion

The DENV serotype and its genotype circulating in an area play an important role in the outcome of the disease. Some genotypes induce greater viremia and are transmitted more readily by vectors, thereby having a higher potential to cause large epidemic. Co-circulation of multiple serotypes along with their virulent genotypes of DENV is considered as the most common factor for the re-emergence of severe and fatal dengue in endemic areas. Coupled with high vector density, this could lead to concurrent infection in the same patient with multiple serotypes of the virus that might alter the clinical expression of the disease (Rico-Hesse, 2007). Hence, virologic surveillance based on the isolation and identification of DENV in the human population and vectors will provide an important means for early detection of any change in the prevalence of dengue virus serotype(s)/genotype(s) circulating in the affected areas.

In this study, cases with suspected DENV infection belonged to the age group 4–80 years with majority of symptomatic cases occurring in the age group 25–50 years. During the first 2 days of fever, viremia was detected by RT PCR in about 90% of patients, whereas IgM antibody was detected in approximately 10% of patients. During 4–12 days of fever, the IgM antibody developed in all patients, while DENV was still detectable by RT-PCR in approximately 15% of patients. The present study revealed high DENV positivity by using the combinations of RT-PCR and IgM ELISA, which could detect viremic individuals from the onset of disease up to 14 days of fever and thus proved to be very effective tools for early screening dengue patients during outbreaks.

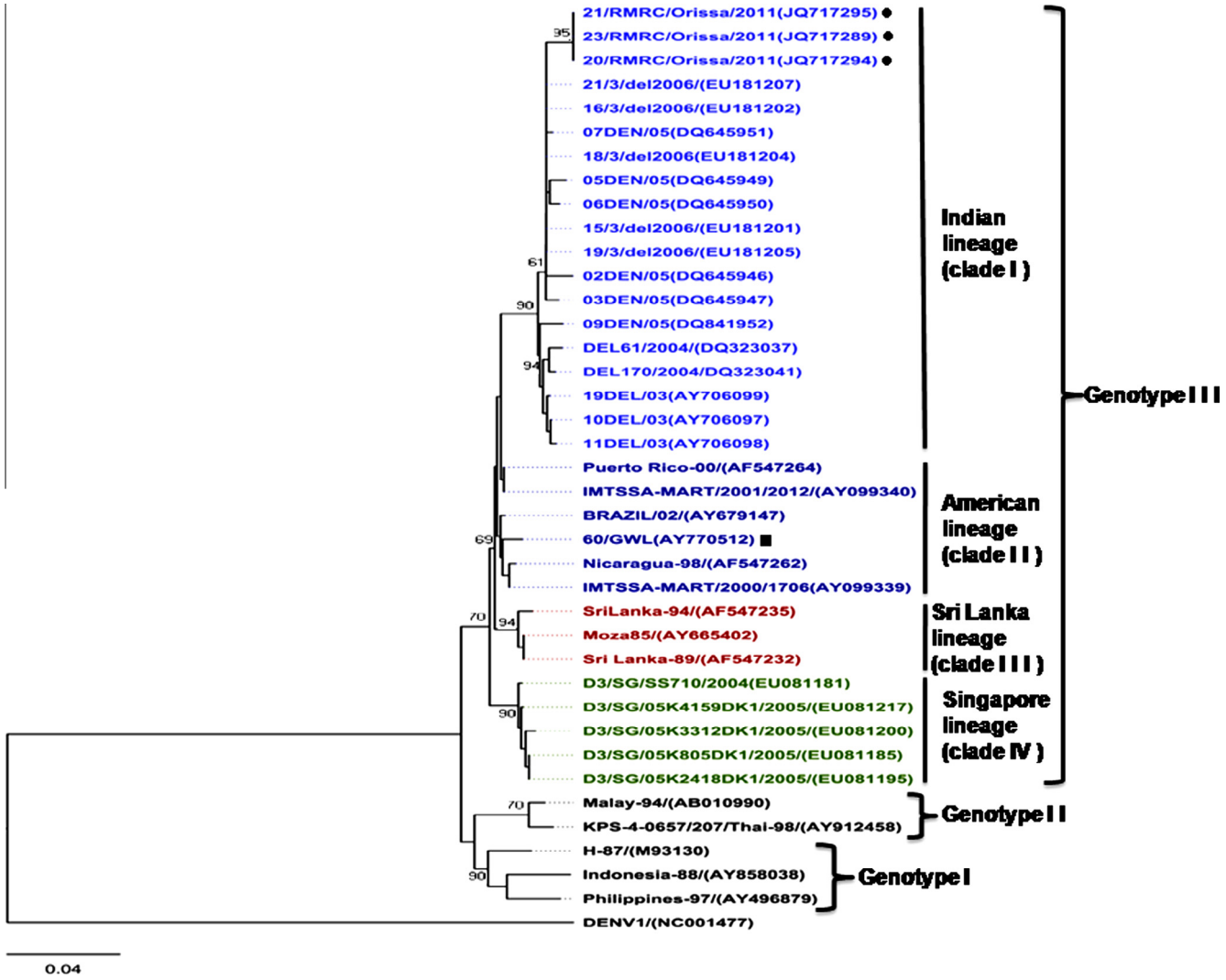

Fig. 5. Mid point rooted phylogenetic tree of DENV-3 viruses generated by maximum likelihood method based on C-prM gene (435 nt). Each strain is identified by its name and the year it was isolated, followed by GenBank accession numbers in parentheses. Solid circle denotes viral RNAs isolated and sequenced from serum and mosquitoes obtained from Orissa. The Indian isolates grouped into genotype III, which was subdivided into 4 clades: clade I represented Indian lineage (blue), clade II represented American lineage (dark blue), clade III represented Srilankan lineage (red) and clade IV represented Singaporean lineage (green). Solid square represented Gwalior-60 isolate of India which was exceptional and grouped within American lineage. Bootstrap values are indicated at the major branch points. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this book.)

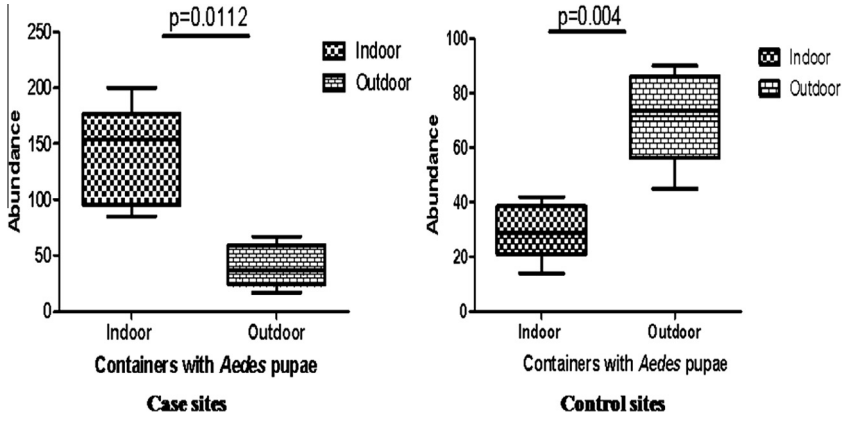

Fig. 6. Comparison of the abundance of indoor and outdoor containers harboring *Aedes* pupae at the case and control sites using non parametric Mann Whitney *U* test as shown in boxplot. The shaded boxes represent the abundance of various indoor and outdoor containers collected from case and control sites. Bars (whiskers) at the extreme ends of the boxes denote the maximum (top whisker) and minimum (bottom whisker) number of containers collected from different sites. The horizontal line in the box denote the median value.

The C-prM gene region was selected for phylogenetic analyses in the study because the nucleotide sequence of the C-prM region is reported to yield important evolutionary information and also provides an economic alternative, since a single set of primer pair could be used for amplification and sequencing of any of the four serotypes of dengue virus (Dash et al., 2004). Moreover, the Lanciotti's protocol, which was developed for rapid detection and typing of dengue viruses from clinical samples has also been used for detection and typing of dengue viruses from mosquitoes with high sensitivity and specificity (Urdaneta et al., 2005; Jittmittraphap et al., 2006). Therefore this method was used for DENV serotyping and genotyping from vectors and patients serum in the present study.

Molecular phylogenetic analyses using C-prM gene of DENV revealed the circulation of Indian lineage of DENV-2 (genotype-IV) and DENV-3 (genotype-III) in vectors and patients' serum during recurrent outbreaks in Orissa. DENV-2 was found to be the more prevailing serotype (85%) as compared with DENV-3 serotype (15%) in the cases. Both the genotypes are highly virulent and have been associated with repeated dengue outbreaks in India (Kumar et al., 2010; Sharma et al., 2011). Since co-circulation of virulent genotypes have been associated with the occurrence of recurrent DHF epidemics, hence co-circulation of DENV-2 (genotype-IV) and DENV-3 (genotype-III) may lead to more severe epidemics in Orissa unless proper anti-dengue measures are implemented. Selection analyses within the C-prM region of the DENV-2 and DENV-3 isolates revealed that most codons within the C-prM region were conserved, thereby indicating that DENV-2 and DENV-3 evolution in Orissa is constrained by purifying selection. This suggests the possible contributions of other ecological factors such as mosquito density and behavior and susceptible human population to the recurrence of dengue outbreaks in Orissa.

Entomological analyses revealed that *Ae. albopictus* was the most abundant species in the areas surveyed, followed by *Ae. aegypti*. Indoor breeding spots, especially earthen pots were most abundant and contributed maximum *Aedes* species in the affected areas. The high productivity of earthen pots in the affected areas was an important outcome of the study. The local tradition of storing water for long periods in earthen pots for several purposes within houses correlated with high pupal productivity in such containers. DENV RNA was detected in adult mosquitoes obtained after laboratory rearing of pupae, which confirmed the transovarial/vertical transmission of DENV in the affected areas. Vertical transmission of DENV is a major factor responsible for virus persistence and survival in nature for long periods, especially during adverse climatic conditions and inter-epidemic periods when the vector density is low and has very important role in the re-emergence of DENV during recurring outbreaks (Martins et al., 2012).

Statistical analyses showed that *Aedes species* preferably bred indoors in case sites so that its proximity to humans increased, thereby increasing vector-host interactions which in turn enhanced the risk of DENV transmission. The high DENV MLE infection rate of *Aedes* species obtained from indoor breeding spots in comparison to outdoor breeding spots indicated that indoor breeding *Aedes* species was responsible for transmission of DENV in Orissa. Further analyses showed that the MLE infection rate was high for *Ae. albopictus* in comparison to *Ae. aegypti*, which indicated it to be the principal vector responsible for the dengue outbreaks in Orissa. In our previous study, we found that *Ae. albopictus* breeds preferably in outdoor breeding habitats during non-epidemic periods (Das et al., 2012b). However, in the present study, *Ae. albopictus* was mainly found breeding indoors in the dengue affected areas, which suggests that *Ae. albopictus* exhibits a wide range of breeding behavior during epidemic and non epidemic periods. As a consequence, the characteristics of DENV circulation and their outbreak dynamics are likely to be modified (Tewari et al., 2004).

Our analyses of C-prM gene sequences from *Aedes* mosquitoes and patients serum infected with DENV confirmed the circulation of Indian lineage of DENV-2 (genotype-IV) and DENV-3 (genotype-III) in vectors and patients' serum of Orissa, DENV-2 being the predominant serotype during the outbreaks. Another important finding of the study was the detection of DENV in indoor breeding *Aedes* species, particularly *Ae. albopictus* in the case sites of the districts surveyed. Such type of adaptation by *Aedes* species to indoor/domestic environment will trigger increased human-vector contacts that will presumably stimulate feeding behavior and thus will produce more competent vectors (Dieng et al., 2010). This behavioral change will lead to increased vectorial capacity and enhance its ability to transmit different arboviral strains and virulent genotypes, thereby leading to more severe epidemics. Therefore greater emphasis should be placed on intradomecile vector control strategies, especially when the force of transmission is high during repeated outbreaks involving serotypes (Morrison et al., 2008). Overall, our results suggest that circulation of virulent genotypes of DENV and ecological factors pertaining to vectors were central in shaping the dynamics of the dengue outbreaks in Orissa. Hence the study suggests the concurrent surveillance of viral isolates, mosquito vectors (including the proportion of mosquitoes infected with DENV in indoor/outdoor containers) and periodic surveys of seroprevalence rates of the population to get a complete depiction of the dengue outbreaks in order to plan for successful implementation of strategic control measures.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.meegid.2013.03.016.

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