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Short Communication Open Access

Co-Habitation and Concurrent Infection of Dengue and Chikungunya Viruses in *Aedes Aegypti* Field Populations from India

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Abstract

Dengue and chikungunya have been identified as important re-emerging diseases in India. It has recently become a major health problem around the world, particularly in tropical and subtropical countries including India. Chikungunya fever is another re-emerging vector borne disease which is now being reported from areas previously unaffected with possibly changing epidemiology and severity of the disease. *Aedes aegypti* is the principal vector for the transmission of both of these arboviral infections.

Information on vector population in the field vis-à-vis co-habitation of dengue and chikungunya viruses is of great importance in order to understand the role of vectors in the transmission of co-infections, but such information is presently lacking in India. We carried out a pilot survey in the states of Delhi and Haryana to estimate the presence of co-infections in *Ae. aegypti* during pre-monsoon, monsoon and post-monsoon seasons. This study is the first to report co-habitation of DENV and CHIKV in *Ae.aegypti* field population.

Keywords: *Aedes aegypti*; Dengue, Chikungunya; Co-infection; Concurrent infectionsg

Short Communication

Aedes aegypti is an important vector mainly found in tropical and sub-tropical areas across the world [1] and is implicated in the spread of several arboviruses; most important of them being dengue virus (DENV) and chikungunya virus (CHIKV). Dengue caused by DENV and chikungunya caused by CHIKV are among the most important vector borne diseases affecting millions of people in India, with dengue contributing 34% (33 million infections) of the total global infections [2-4] and chikungunya contributing to spread of the infection to several other countries and serving as a transmission hub [5].

Dengue is an acute systemic viral disease that has established itself globally in both endemic and epidemic transmission cycles [6]. Dengue includes its two fatal syndromes, the dengue hemorrhagic fever (DHF) and dengue shock syndrome (DSS) [7]. With more than one-third of the world population living in areas at risk of this infection, dengue virus is a leading cause of illness and death in the tropics and subtropics (scientific working group report on dengue, WHO 2006 [8]. Global distribution map of dengue has estimated and predicted India to be the worst affected [9]. Chikungunya, on the other hand, is a self-limiting disease causing morbidity in the affected individuals and occurs in two phases- an acute febrile phase and an arthralgic chronic phase. In India, chikungunya re-emerged in 2006 [10] after a gap of 32 years and since then has been occurring either as single infection outbreaks or as co-infections with dengue in different parts of India [11-15]. In the recent decades, all four serotypes have been circulating together in Delhi making it into a hyper-endemic state [16] and presenting with concurrent infections. Several clinical reports provide information on these co-infections [15,17]. Chikungunya tends to cluster geographically and overlap with dengue because both share some common symptoms [17].

Material and Methods

Aedes immatures (larvae and pupae) were collected during three different seasons in Delhi, ie, pre monsoon, monsoon and post monsoon. Collections were made once a month from May 2012-October 2012 from four study sites; two from the urban localities of Delhi (South Delhi and West Delhi) and rest two from semi urban localities of Haryana (Bahadurgarh and Bhadana), India. Each study site were around 20-25 kms apart from each other with South Delhi and Bhadana being the farthest localities. Details of the study sites used for the survey are shown in Table 1. Study sites were identified mainly on the basis of incidence of dengue and chikungunya cases reported in the previous years (MCD, NVBDCP), water storage conditions and socio-economic factors of the population in those localities.

After collections, the immatures were reared in the lab and upon emergence, adults identified [18-21]. After species identification, *Aedes aegypti* mosquitoes were separated on the basis of their sex and collection sites. Mosquitoes were pooled (n≤10 each for male and female) breeding site-wise and stored in Trizol at -80°C until further use. Using primers listed in Table 2, RT PCR for DENV serotypes and CHIKV were performed. The amplified products were purified, sequenced and phylogenetic analysis performed.

Study site	Locality type	Longitude	Latitude
Delhi - South Delhi, West Delhi (2 districts)	Urban	28°36' N	77°12' E
Haryana - Jhajjar, Bahadurgarh (2 districts)	Semi urban	27°39' N	77°36' E

Table 1: Details of study sites.

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Results and Discussion

A total of 7007 immatures were collected during the six months. Details of the collection are described elsewhere. Testing for co-habitation in pools of mosquitoes in the urban sites revealed presence of DENV and CHIKV viral RNA (VNA) in ten pools out of the 38 pools tested. In case of per-urban sites, DENV and CHIKV positivity was seen in three of the 24 pools tested. Detail of pool positivity is provided in Table 3. These results clearly confirmed co-habitation of dengue and chikungunya viruses in the field mosquitoes (Figure 1). Sequencing of the amplified products and BLAST analysis of the sequence revealed DENV 2 serotype in all the samples. Phylogenetic analysis of CHIKV samples showed that the strains belonged to ECSA genotype.

Studying reports of DENV occurrence in Delhi in the recent years revealed that there has been a serotype switch between 2011 and 2013. As our collection was done in the year 2012, we sought to test if there was concurrent infection of different DENV serotypes in the field mosquitoes. For this purpose, we selected samples from two time points, one prior to dengue cases occurrence in 2012, and the second one in the later part of the year. Serotyping of the mosquito samples yielded interesting results. Concurrent infection in the pooled mosquitoes was evident by specific bands through RT PCR for DENV 1, 2, 3. Furthermore, it was seen that samples collected in the month of July showed strong positivity for DENV 1 as evidenced by a thick band while DENV 2 and 3 showed poor amplification. In those samples collected in the month of October, however, DENV 2 showed a much distinct band while DENV 1 showed poor amplification (Figure 2). The amplicons were cloned, sequenced and BLAST analysis performed. Phylogenetic analysis was performed to confirm the genotypes and serotypes (Figure 3a-3c). In order to understand the pattern of DENV serotypes in the mosquitoes, we inspected the clinical cases that were prevalent in Delhi between 2011- 2012. Observed that DENV-1 was the circulating serotype in Delhi during 2010-2011. However, in 2012, reports on DENV prevalence in Delhi showed that DENV-2 along with DENV-3 replaced DENV-1. Post 2012, there has been a major shift in the incidence of DENV-2 (86%) in Delhi as reported. In our study, we performed the PCR for all the serotypes over the months. In the month of July, the samples were strongly positive for DENV 1 while DENV 2 and DENV 3 showed very poor amplification. However, in the samples collected in the later months of the year, DENV 2 showed a distinct amplification pattern and DENV 1 showed poor amplification. The results clearly corroborates with circulating DENV serotypes in the years. The samples in July were positive for DENV 1 from the previous year. However, due to factors presently unknown, DENV 2 and DENV 3 dominate over DENV 1 in the later months of the study. Those factors that facilitate this switch need to be studied in detail in order to understand this phenomenon. Our study has clearly established the presence of both DENV and CHIKV in the mosquito population. One previous study reported the presence of both CHIKV and DENV from *Ae. albopictus* collected from a co-infection patient's residence.

This is the first report of concurrent infection of DENV serotypes along with co-infection with CHIKV from same pool of *Ae. aegypti* collected

Origin	Gene	Sequence (5' -> 3')	Amplicon size	Source
CHIKV E1 gene	Forward	TACCCATTTATGTGGGGC	298 bps	[14]
	Reverse	GCCTTTGTACACCACGATT		
CHIKV E1 gene	Forward	GCTCCGCGTCCTTTACC	555 bps	[54]
	Reverse	ATGGCGACGCCCCCAAAGTC		
DENV Universal	Forward	TGGCTGGTGCACAGACAATGGTT	510bps	[55]
	Reverse	GCTGTGTCACCCAGAATGGCCAT		
DENV1	Forward	GGGGCTTCAACATCCCAAGAG	405 bps	
	Reverse	GCTTAGTTTCAAAGCTTTTTCAC		
DENV2	Forward	ATCCAGATGTCATCAGGAAAC	346 bps	
	Reverse	CCGGCTCTACTCCTATGATG		
DENV3	Forward	CAATGTGCTTGAATACCTTTGT	196 bps	
	Reverse	GGACAGGCTCCTCCTTCTTG		
DENV4	Forward	GGACAACAGTGGTGAAAGTCA	143 bps	
	Reverse	GGTTACACTGTTGGTATTCTCA		

Table 2: List of primers used in this study.

Virus positivity/pool						
	Urban			Peri-Urban		
Months	No. of pools tested #	No: of pools positive		No. of pools tested #	No: of pools positive	
		CHIKV	DENV		CHIKV	DENV
May	6	0	ND	3	0	ND
June	5	1	1	5	1	0
July	12	10	3*	4	4	1
August	5	5	ND	4	4	ND
Sept	5	5	1	4	4	1
October	5	5*	5*	4	4	1

ND - not done, # Number denotes the number of pools tested for CHIKV only. DENV RT PCR was performed randomly from positive CHIKV pools.
* Denotes RT PCR data from *Ae. aegypti* pools from same breeding sites.
Table 3: Details of pools positive for CHIKV and DENV through RT PCR.

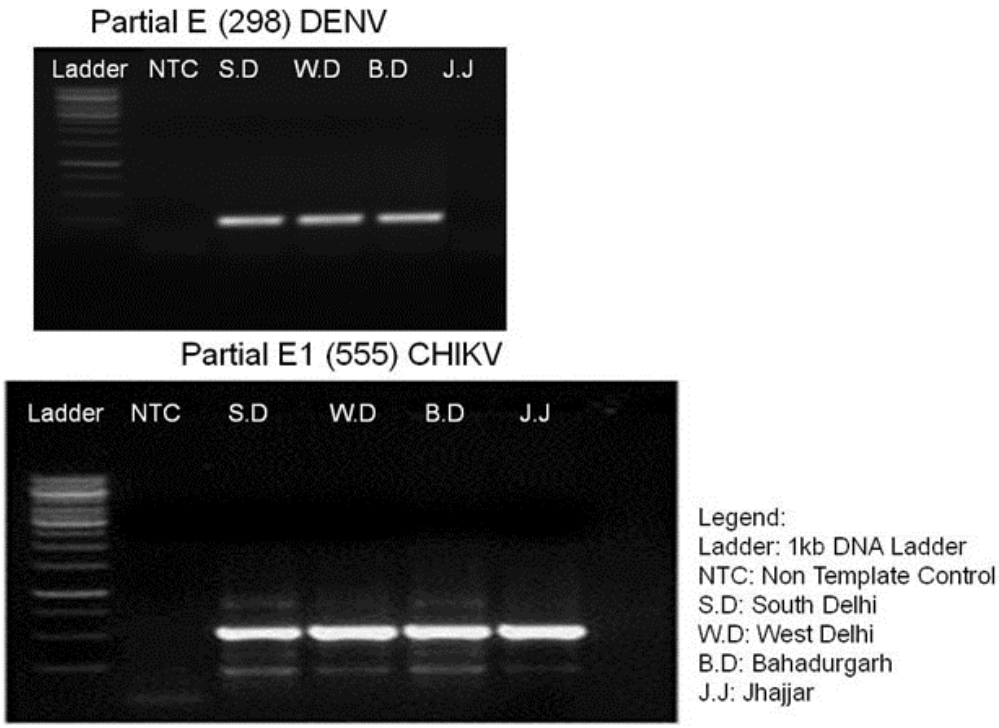

Figure 1: RT-PCR of DENV– CHIKV co-infection in *Aedes aegypti* pooled samples: Random mosquito pools belonging to each of the four study zones were taken and subjected to Reverse Transcriptase PCR for the amplification of (a) partial E1 gene (555bps) of CHIKV and (b) partial E gene (346bps) of DENV.

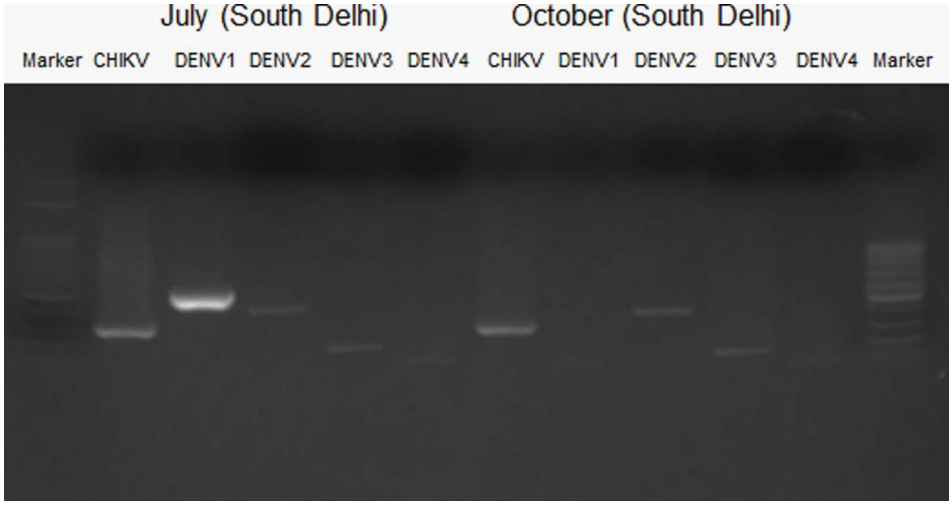

Figure 2: RT-PCR for genotyping of DENV serotypes and CHIKV co-infection in *Aedes aegypti*: Mosquito pools positive for DENV E gene 346bps further genotyped for serotypes along with CHIKV.

from field sites. Ideally, detecting both viruses from a single mosquito establishes co-infections within the mosquito that was beyond the scope of the methodology of this study as samples were pooled at the time of sample collection itself. But importantly it is to be noted that several of the pools that were positive were acquired from the same breeding sites. Since our collections were of only immature stages, it is reasonable to assume that these pools could have been generated from a single mother, thereby providing support to our hypothesis that both CHIKV and DENV can co-habit in *Ae. aegypti*.

The most interesting aspect of these experiments was the presence of several serotypes within the same pool of samples and the gradual switching over of serotypes within the mosquito. In-depth studies over

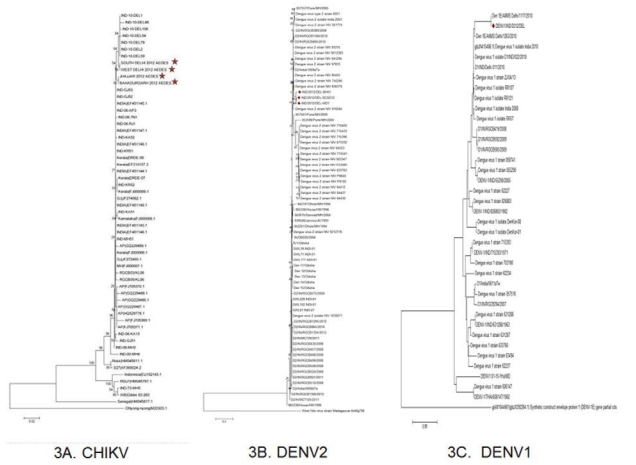

Figure 3: Phylogenetic analysis of Chikungunya and Dengue field collected samples (a) Phylogenetic analysis of the sequenced CHIKV samples isolated from field collected mosquitoes from four study sites viz. Urban sites: South Delhi and West Delhi, Peri-urban sites: Bahadurgarh and Jhajjar (b) Phylogenetic analysis of the sequenced DENV2 samples isolated from field collected mosquitoes from three of four study sites viz. Urban sites: South Delhi and West Delhi, Peri-urban sites: Bahadurgarh and Jhajjar and (c) Phylogenetic analysis of the sequenced DENV1 samples isolated from field collected mosquitoes from one of four study sites viz. South Delhi.

a larger population of mosquitoes both in control conditions as well as from field population can reveal the dynamics of these viruses within the vector and the transmission potential of the vector of these viruses which could be achieved by studying the disease incidence from these localities during disease outbreaks. There is no doubt however; this pilot study has paved way for several interesting questions as to the role of vector in chikungunya and dengue transmission.

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