Nation-wide vector surveillance did not indicate transmission of the “American lineage pandemic ZIKA virus” in India

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Abstract

Background In wake of the global health emergency declared by the World Health Organization (WHO) during 2016, on the outbreak of ZIKA pandemic, Indian Council of Medical Research (ICMR) carried out countrywide vector surveillance for ZIKA and DENGUE viruses (ZIKV & DENV) in India, as a preparedness measure. **Methods** The study incorporated high-risk zones distributed to 49 Districts in 14 states/ Union Territories (UT) of India during 2016-2019. Seven ICMR Institutions undertook the study, following a uniform Standard Operating Protocol. Aedes specimens sampled on weekly intervals were processed by multiplex Reverse transcriptase PCR for ZIKV/DENV and Real time RT-PCR of ZIKV, among few samples distributed to all the Districts. **Results** Altogether, 79492 specimens of Aedes mosquitoes in 6492 pools were processed for both ZIKV and DENV infections. Among these, three and 63 pools respectively were found positive for ZIKV and DENV. ZIKV infections were recorded from Aedes aegypti sampled during 2018 sporadic ZIKA outbreak in Jaipur, Rajasthan, which belonged to the Asian lineage, already circulating in the Country. Both Ae. aegypti and Aedes albopictus were found infected with DENV and were distributed to ten states/ UTs. Both male and female specimens of Ae. albopictus recorded DENV infections indicating trans-ovarial transmission of DENV in the species. **Conclusion** This national vector surveillance study evinced no active transmission of the “American lineage - pandemic ZIKA virus” in India during 2016-2019, although Asian lineage of the virus already circulating in the Country was detected from Ae. aegypti from Jaipur, Rajasthan.

Introduction

World Health Organization (WHO) declared a Global Health emergency during 2016 after the explosive outbreak of ZIKA Virus (ZIKV) in Brazil, owing to its rapid spread and increased neuro-virulence. The pandemic affected 86 Counties across the Globe (https://www.who.int/health-topics/zika-virus-disease#tab=tab_1) affecting a population of about 85000 [1]. In addition, the mutant strain of ZIKV (American Lineage) involved in this pandemic ZIKA caused incredible clinical complications such as microcephaly [2,3,4] and increase in the incidence of Gillian Barre Syndrome [5,6]. World Health Organization called for active surveillance in Countries where no outbreaks were reported initially, including India.

The primary route of transmission of ZIKV is through infected mosquito bites of Aedes mosquitoes such as *Aedes aegypti* [7,8,9] and *Aedes albopictus* [10,11]. In addition, other routes of transmission (congenital, sexual, trans-placental and by contact with body fluids) are also reported [12]. As a preparedness measure, Indian Council of Medical Research (ICMR) initiated a multicentre (seven ICMR institutions coordinated by ICMR-Vector Control Research Centre) countrywide vector surveillance of ZIKV in the high-risk zones distributed to 49 Districts of 14 states during 2016-2019. Initially it was restricted to Kerala, Tamil Nadu and Pondicherry states/Union Territories and further extended to 11 more states viz., Karnataka, Odisha, Madhya Pradesh, Gujarat, Rajasthan, New Delhi, Assam, Arunachal Pradesh, Nagaland, Meghalaya and Andaman & Nicobar Islands of the Country.

ZIKA virus had been an innocuous Flavivirus until the recent major global pandemic, which occurred recently since 2015 [13]. It was first reported from Zika forest in Uganda during 1947 in Rhesus monkeys [14]. Human ZIKV infections was recorded during 1962-63 from Uganda and Republic of Tanzania [15]. Sporadic outbreaks of this disease occurred since 1960s from different Countries of Africa, the Americas, Asia and the Pacific. The first largest outbreak occurred in the Pacific Yap Island during 2007 [16] followed by a larger one in other Pacific islands viz., French Polynesia, Easter islands, The Cook Islands and New Caledonia during 2013 [17]. However, all these outbreaks caused only minor illness among man. Earlier investigations reported the occurrence of Asian strain of the virus from India [18].

On March 2, 2015, Brazil notified the WHO about occurrence of an outbreak of fever with about 7000 cases with mild symptoms during Feb-April. However, this outbreak of fever gradually increased in its distribution to the entire Brazil and got converted to a pandemic affecting 86 Countries in the World. The cause of the Disease was soon attributed to the *Flavivirus* - ZIKA Virus [19] and the vector species was incriminated to be *Aedes* species [20]. The disease manifestations in the outbreak was found associated with Gullain- Barre syndrome during July 2015 and to microcephaly in new born infants during October 2015. Zika virus infection in pregnancy caused many abnormalities in the new borne children. As the outbreak spread to a pandemic in different regions of the Globe, the WHO declared it as a Public Health Global Emergency during February 2016. Countries where ZIKA outbreak was not reported were also advised to carry out active vector-surveillance, as a preparedness measure. Indian Council of Medical Research, the apex body of Medical Research in India, recommended a pilot program of vector-surveillance in high risk Districts during 2016 and which was later extended to 49 high risk Districts distributed to 14 states of the Country during 2017. The program was undertaken as a multicentre mode run by seven ICMR institutions with the ICMR-Vector Control Research Centre as the co-ordinating agency. A uniform Standard Operating Protocol was followed across the Country.

Methods

Initially the surveillance program was carried out in proposed high-risk areas in Kerala state, Tamil Nadu and Puducherry (2016-2017) by ICMR-Vector Control Research Centre. Subsequently, it was extended to all the high-risk zones of the Country covering 49 Districts distributed to 14 states / Union Territories of the Country (Fig. 1 & Table 1).

Study Area

Table 1: State-wise distribution of *Aedes* samples processed in the Country-wide ZIKA vector surveillance project 2016-2019.

Sl. No.	State	Districts surveyed	Institution
1	Kerala	Thiruvananthapuram, Kollam, Alappuzha, Kottayam, Pathanamthitta, Ernakulam, Thrissur, Malappuram, Waynadu, Kozhikode, Idukki.	ICMR-VCRC Field Station, Kerala
2.	Tamil Nadu	Kanyakumari, Tirunelveli, Madurai, Theni, Salem, Ramanathapuram, Vellore, Tiruchirappalli, Coimbatore, Pudukkottai, Dindugal, Nilgiris, Krishnagiri, Chennai.	ICMR-VCRC Field Station, Madurai, Tamil Nadu
3.	Puducherry	Puducherry	ICMR-VCRC, Puducherry
4.	Karnataka	Vijayapur, Mangalore, Mysore, Belagavi	ICMR-NITM, Belagavi, Karnataka
5.	Gujarat	Ahmedabad	ICMR-NIMR, New Delhi
6.	Madhya Pradesh	Narasigpur, Jabalpur	ICMR-NIRTH, Jabalpur, MP
7.	Rajasthan	Jaipur	ICMR-NIMR, New Delhi
8.	Delhi	Delhi	ICMR-NIMR, New Delhi
9.	Odisha	Cuttack, Anugual, Jajapur, Keonjhar, Bhadrak, Jagatsighpur	ICMR-RMRC Bhubaneswar, Odisha
10	Assam	Guwahati	ICMR-RMRC, North-East
11.	Arunachal Pradesh	Pasighat	Dibrugarh, Assam
12.	Meghalaya	Tura	"
13.	Nagaland	Dimapur	"
14.	Andaman & Nicobar Islands	South Andamans, North Andamans, Middle Andamans, Nicobar.	ICMR-RMRC, Port Blair, A&N Islands

All the institutions involved were offered with a weeklong training program on the uniform Standard Operating protocol (SOP) to be adopted for the project studies. Regions for sampling of *Aedes* specimens invariably included high-risk zones as international entry sites such as International Airports, Seaports and national ones like Airports, metropolitan cities, urban corporations, major rail stations, major bus terminals etc. *Aedes* mosquitoes were sampled on weekly intervals. The collections were planned in co-operation with local State health Department machinery. However, the modalities varied from state to state. The proposed methodology was as follows:

Adult *Aedes* mosquito collections

Resting mosquitoes indoor were collected using vacuum aspiration in early morning hours (8.00-10.00 hrs). A battery operated prokopack or handheld mechanical aspirators were used to collect mosquitoes resting in and

around houses. The specimens from aspirators were transferred to Barraud cages or to test tubes (one specimen in a test tube).

Mosquitoes attempting to bite was sampled by lure baited Bio Gents (BG) sentinel traps set for about 24 hours in morning hours set indoors and outdoors. The specimens collected in BG sentinel traps were collected next day. Gravid traps collected mosquitoes seeking habitats for egg laying. Gravid traps were set in the morning hours and specimens trapped were recovered daily in the early morning. Sweep nets were used to collect outdoor resting mosquitoes. All collected live *Aedes* mosquitoes were brought to the laboratory, stunned (either keeping in a freezer for 20 minutes or by hand tapping) and identified morphologically using standard taxonomic keys. They were sorted by sex, species, collection method, and collection period and pooled to a maximum no. of 20 identical category specimens and were placed into 2 ml eppendorff tubes with 50µl TRI reagent (MRC, USA) and were stored at 4° Celsius. These tubes were transported to the ICMR institutions once a week, where it was further processed for arbo-viral infection status.

Immature Aedes collections

All indoor and outdoor water-containing receptacles at the selected households were inspected for *Aedes* mosquito larvae and pupae. Live larvae observed in receptacles were collected and reared to adults towards identification. All the pupae found were collected were also reared to adulthood. On emergence, the specimens were sorted according to species and gender and were pooled (maximum 20 specimens) to Eppendorf tubes with 50-µl TRI reagent. These were also stored at 4° C and were transported to ICMR institution for further processing.

RNA Extraction from mosquito pools

The entire procedure was carried out in BSL-Class 2 facilities. Pooled specimens were homogenized in Eppendorf tubes thoroughly using Kontes pellet pestle motor until no visible parts remained. It was made up to 200µl with TRI reagent (1-2 specimens). (100µl of additional TRI reagent for each additional specimen in the pool and for a pool of 10 specimens required quantity of TRI reagent was 1ml). The homogenate was incubated for 5 min at RT, added 200 µl of chloroform (for 1 ml homogenate) and vortexed for 15 sec. Further, the reaction mixture was incubated at room temperature for 15 min. and centrifuged at 12,000 g for 15 min at 4°C.

The aqueous upper layer was pipetted out and was transferred to a fresh tube. 500 µl of isopropanol was added and stored at RT for 6-7 min. Centrifuged at 12,000g for 8 min (4°C). Discarded the supernatant by tilting the tube gently. Washed pellet twice with ice-cold 75% and RT 100% ethanol. Air dried the pellet at RT and dissolved in 30 ul de-ionized water.

RT- PCR for detection of DENV/ZIKV infections

One-step Roche transcriptor kit was used for the multiplex RT-PCR reactions. Partial envelope gene of ZIKV and CprM gene of DENV were amplified by performing a multiplex PCR [21,22].

The primers used are as follows:

ZIKA Virus infection:

ZIKVENVF (5'-GCTGGDGCRGACACHGGRAC-3')

ZIKVENVR (5'-RTCYACYGCCATYTGGRCTG-3')

Dengue Virus infection

DI 5'-TCAATATGCTGAAACGCGCGAGAAACCG-3'

D2 5'-TTGCACCAACAGTCAATGTCTTCAGGTTC-3'

25 µl PCR reactions were setup, which consisted of 5 µl Buffer, 1 µl each of all the 4 DNA primers cited above, 0.5 µl enzyme mix, template 2 µl and 13.5 µl water supplied with the kit. DENV and ZIKV positive control and a Negative Control were included in all the reactions.

The reaction conditions was an incubation at 50⁰ C for 30 min.; initial denaturation step of 94⁰ C for 5 min. followed by 35 cycles of 94⁰ C for 50 sec., 55⁰ C for 1 min. and 68⁰ C for 80 sec, followed by a final extension of 7 min at 68⁰ C.

The PCR reactions were run on a 1.5% agarose gel to visualize the fragments amplified.

Real Time PCR – ZIKV (Realstar ZIKA Virus RT-PCR kit)

2-5 % of the samples of both *Ae. aegypti* and *Ae. albopictus* specimens distributed to all the Districts surveyed were processed by Real time PCR following Real star ZIKA RT-PCR kit, following their protocol.

30 µl (20 µl master mix +10 µl sample) reactions were setup in 96 well plates. Reporter Dye: used was FAM. Reaction parameters: 55⁰ C for 20 minutes, 95⁰ C for 2 minutes followed by 45 cycles of 95⁰ C for 15 seconds, 55⁰ C for 45 seconds, 72⁰ C for 15 seconds and cooling cycle 37⁰ C for 600 seconds.

Samples were observed as positive, where the CT values did not exceed 32 cycles.

Results

During the period of study, altogether 79492 specimens of *Aedes* mosquitoes were processed across the Country, 1470 specimens of *Aedes vittatus*. (Table 1). These were processed as 6492 pools for RT-PCR. Among these, three pools were found positive for ZIKV infection and 63 pools for DENV infection.

ZIKV infections were recorded from three pools of *Ae. aegypti* specimens collected during the outbreak investigation of ZIKA, which occurred in Jaipur, Rajasthan during 2018. On further analysis the ZIKV involved in the infections were found to be the Asian Lineage of the virus already circulating in the Country. The 1524 bp sequences Envelope gene amplified matched 100% with the human isolates collected from the same area from a parallel ICMR [23]. Rest of the Country did not report any infection of ZIKV in *Aedes* specimens sampled.

Dengue infections were recorded from both *Ae. aegypti* as well as from *Ae. albopictus* specimens, from Kerala (1), Puducherry (1), Karnataka (3), Andaman Nicobar islands (4), Arunachal Pradesh (29), Assam (19), Nagaland (5) and Meghalaya (1). Dengue infections in *Ae. aegypti* pools were distributed to 8 states surveyed. *Ae. albopictus* was found naturally infected with DENV in Andaman Nicobar islands, Arunachal Pradesh and from Assam. The Minimum Infection Rate (MIR) computed for the Country from this study is 0.793. Also, both male and female specimens of *Ae. albopictus* was found infected in this study indicating trans-ovarial transmission of DENV in *Ae. albopictus*.

Besides, the infection status the study also provided an insight into the prevalence of these two major *Aedes* vector species in the Country. The state wise distribution of *Aedes* specimens samples are provided in Table 1. Maximum number of the *Aedes* specimens 27674 (34.81%) were collected from Kerala state, since the collection procedure could be amalgamated with the existing vector surveillance system and the Health infrastructure in the state was pro-active. The specimens sampled during routine surveillance activities and the specimens were sent every week to the Kottayam field station of ICMR-VCRC, which was also the co-ordinating centre for the multicentre program in the Country. Altogether, 8425, 18370 & 879 specimens of *Ae. aegypti*, *Ae. albopictus* and *Ae. vittatus* specimens were processed as 2422 pools from Kerala during the study period. However, no ZIKV infections were recorded from the State; even though the population of the state is very nomadic and about 10.0% of the population is migrants from other states of the Country. Only a pool of specimens of *Ae. aegypti* from Thiruvananthapuram District was found to be positive for DENV in this Dengue endemic state.

Largest number of *Ae. aegypti* specimens were collected from Tamil Nadu (9881), followed by Kerala State (8425) and Delhi (6736). *Ae. albopictus* was found the predominant species in Arunachal Pradesh (94.05%), Odisha (89.51%), Andaman & Nicobar islands (80.70%) and Kerala (68.56%), while in other states *Ae. aegypti* was found to be the predominant species (Fig 2). Interestingly, natural infections with DENV was recorded in both *Ae. aegypti* as well as *Ae. albopictus* in the study. This study clearly evinced the role of both the species as major vectors of Dengue in the Country [24].

Real time RT-PCR was performed using Real star ZIKA Virus RT-PCR kit among 5% of pools distributed to all the Districts surveyed. Totally, 1400 specimens in 174 pools were processed by Real time PCR. The infection of ZIKV recorded in 3 pools of *Ae. aegypti* from Rajasthan also were subjected to Real time PCR for confirmation of the infection. Also, analysis of sequences of a larger fragment of the Envelope gene of the virus indicated the lineage of the virus isolate belonged to the Asian lineage [23].

Table 2. Statewise details of *Aedes* samples processed for ZIKV/DENV infection status 2016-2019

Sl. No.	ICMR institution involved	States/ Union Territories	No. processed			Total Aedes specimens processed	No. of PCR pools	No Positive for ZIKV	No Positive for DENV
			<i>Ae. aegypti</i>	<i>Ae. albopictus</i>	<i>Ae. vittatus</i>				
1	ICMR-VCRC-Puducherry (Co-ordinating institute)	Kerala	8425	18370	879	27674	2422	0	1
		Puducherry	3225	749	302	4276	366	0	1
		Tamil Nadu	9881	882	0	10763	554	0	0
		Total	21531	20001	1181	42713	3342	0	2
2	ICMR-NIMR, New Delhi	Delhi	6736	0	0	6736	938	0	0
		Gujarat	57	0	0	57	57	0	0
		Rajasthan	246	0	0	246	65	3	0
		Madhya Pradesh	64	0	0	64	11	0	0
		Total	7103	0	0	7103	1071	3	0
3	ICMR-NITM, Belagavi	Karnataka	2790	560	260	3650	365	0	3
		Total	2790	560	260	3610	315	0	3
4	ICMR-NIRTH, Jabalpur	Madhya Pradesh	1525	83	9	1617	209	0	0
		Total	1525	83	9	1617	209	0	0
5	ICMR-RMRC, Port Blair	Andaman & Nicobar	2621	10959	0	13580	760	0	4
		Total	2621	10959	0	13580	760	0	4
6	ICMR RMRC (NE), Dibrugarh	Arunachal Pradesh	134	2119	0	2253	168	0	29
		Assam	1484	113	0	1597	134	0	19
		Meghalaya	470	7	0	477	62	0	1
		Nagaland	1858	44	0	1902	151	0	5
		Total	3946	2283	0	6229	515	0	54
7	ICMR-RMRC, Bhubaneswar	Odisha	480	4100	20	4600	230	0	0
		Total	480	4100	20	4600	230	0	0
Grand Total			39996	37986	1470	79492	6492	3	63

Discussion

Sporadic cases of ZIKA were reported from Gujarat, Rajasthan, Madhya Pradesh and from Tamil Nadu [23,25] during a parallel investigation in the Country on human infections. Genetic analysis of the ZIKV involved in the infections were traced to the Asian lineage, already reported in the Country. These isolates did not have the crucial mutations 'S139N' proposed to cause microcephaly [25] and 'A188V', which favoured high neuro-virulence in the present pandemic. These sporadic occurrences of cases would have been perceived owing to the active surveillance program initiated, in the wake of the ZIKA pandemic. Also, our team reported natural infection of *Ae. aegypti* with ZIKV in Rajasthan, while carrying out an outbreak investigation in Jaipur where 128 confirmed cases of ZIKA were reported during 2018 [23]. This also belonged to the Asian lineage of ZIKV (GenBank Accession Number MK238037). Thus, the present countrywide investigation carried out as a preparedness measure in the Country did not detect occurrence of natural transmission of the virus involved in the pandemic 2015 (American Lineage) outbreak in India.

Asian lineage of the virus had been reported to be silently circulating in the Country [18]. The sporadic cases of ZIKV recorded in Tamil Nadu (2016), Gujarat (2017), Madhya Pradesh and Rajasthan (2018) indicated that very low level

of the Asian lineage ZIKV is circulating in the Country [26,27]. This would have been brought to lime light owing to the active surveillance studies carried out in India (both human as well as the vector species), in the wake of the ZIKA global pandemic. However, it is alarming to note that the recently evolved 'American lineage' of the virus also originated from the 'Asian Lineage' of ZIKV by undergoing few mutations, cited above. Hence, routine and systematic surveillance on the evolutionary trends of ZIKV along with other arbo-viruses remains inevitable to prevent impending outbreaks of emerging and re-emerging arbo-viruses, causing tremendous mortality and morbidity to human population.

Dengue viruses are widely circulating in the Country. The present investigation recorded DENV infections in the vector species in about 10 states among the 14 states surveyed. Also, infections were recorded in both *Ae. aegypti* and *Ae. albopictus*, clearly evincing the role of both the species as major vectors of Dengue in the Country. A systematic surveillance program for Dengue is also almost lacking in the Country. Routine health surveillance programs for vector population in the Country is mostly active only during an outbreak situation and later. Pre-epidemic surveillance and inter epidemic programs are virtually inactive.

Both ZIKA virus as well as Dengue Virus is circulating in the Country, the former with sporadic infections and latter with frequent outbreaks distributed to different regions.

Even though the current study and the parallel human surveillance study did not indicate indigenous transmission of the American strain of the pandemic ZIKA virus in the Country, ZIKA virus Asian strain was demonstrated to be silently circulating in the Country. As the current pandemic strain is genetically related to Asian lineage, the study warrants a routine surveillance of the host as well as the vector species, so as prevent severe outbreaks similar to one that reported recently. We advocate a year round systematic surveillance program on emerging and re-emerging arbo-viral infections such as Zika, Dengue and Chikungunya in the human host as well as the vector species to tackle and prevent impending outbreaks of arbo-viral diseases in the Country.

Declarations

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- 2) VIR/9/2017/ECD-1 dated 18-08-2017

Ethical approval statement

Institutional Human Ethics Committee approval not applicable for the study as no human samples were involved.

Conflict of Interest Statement

The authors declare no conflicts of interest.

Authors' contributions

Pradeep Kumar, N., the principal investigator of the multi-centric project planned and executed the study and wrote the manuscript with input from all authors. All other authors carried out their respective studies regionally and

submitted reports on their study to P.K.N. Also, all other authors have read and provided inputs to the manuscript.

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Figures

Figure 1

Distribution of study Districts, where *Aedes* samples were screened for ZIKV infection, Note: The designations employed and the presentation of the material on this map do not imply the expression of any opinion whatsoever on the part of Research Square concerning the legal status of any country, territory, city or area or of its authorities, or concerning the delimitation of its frontiers or boundaries. This map has been provided by the authors.

Figure 2

Species composition of Aedine vector species (*Ae. aegypti* and *Ae. albopictus*) in different states of India