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Short communication

Zika virus outbreak in Rajasthan, India in 2018 was caused by a virus endemic to Asia

Pragya D. Yadav[a], Bharati Malhotra[b], Gajanan Sapkal[a], Dimpal A. Nyayanit[a], Gururaj Deshpande[a], Nivedita Gupta[c], Ullas T. Padinjaremattathil[a], Himanshu Sharma[b], Rima R. Sahay[a], Pratibha Sharma[b], Devendra T. Mourya[a,*]

[a] Indian Council of Medical Research- National Institute of Virology, Pune, Maharashtra, India
[b] Sawai Man Singh Medical College, Jaipur, Rajasthan, India
[c] Indian Council of Medical Research, Post Box No. 4911, Ansari Nagar, New Delhi 110029, India

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Article Summary Line: Characterization of Zika virus (ZIKV) outbreak strain from the Jaipur city, Rajasthan state, India, using a next-generation sequencing approach, indicates that the circulating ZIKV belongs to Asian lineage.

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ABSTRACT

Zika virus (ZIKV) infection in human has been reported from Gujarat and Tamil Nadu states during the year 2016 and 2017 respectively. In paucity of complete genome data of ZIKV, the analysis and prediction were not possible. Zika cases were reported in Jaipur city, Rajasthan, India during the period of 21st September 2018 to 29th October 2018. In order to understand the circulating ZIKV strain in Rajasthan state about ten human serum samples from the positive cases of Jaipur city, Rajasthan state considering the locality and clustering variations were sequenced using next-generation sequencing (NGS) platform. Complete genome phylogenetic analysis of Jaipur city sequences with known GenBank ZIKV sequences revealed that the outbreak in Jaipur city was being caused by ZIKV belonging to Asian lineage. Partial genome sequencing revealed the presence of a pre-outbreak strain of ZIKV in Gujarat and current outbreak strain of Asian lineage in Tamil Nadu. Further sequence analysis of the five ZIKV positive samples of Jaipur revealed that the S139N and A188V mutations, linked to enhanced neurovirulence and transmission in animal models, were not found in the current outbreak strain. Whether this strain can cause birth defects and cause large outbreaks is not currently known, but they should be treated as such until more is known. With the identification of ZIKV in Gujarat, Tamil Nadu, and recent outbreaks of ZIKV in Rajasthan and Madhya Pradesh states alarm for India to enhance surveillance in other states and monitor the mutation and evolutionary changes in circulating Zika strains.

1. Introduction

Zika Virus (ZIKV) was first reported from a monkey serum sample in Uganda during 1947 (Dick et al., 1952). ZIKV is a single-stranded, positive-sense RNA that belongs to genus *Flavivirus*, family *Flaviviridae* and is known to be transmitted by *Aedes species* primarily by *Aedes aegypti*. The genome of ZIKV is approximately 10.8 kb in size that encodes for three structural (capsid, membrane precursor, and envelope) and seven non-structural proteins (NS1, NS2A, NS2B, NS3, NS4A, NS4B and NS5) along with 5′-untranslated region (UTR) and 3'-UTR (Wang et al., 2017).

World Health Organization declared ZIKV as a public health emergency of international concern after its outbreak (WHO | World Health Organization [WWW Document], 2018), and subsequent microcephaly cases reported amongst newborn in Brazil in 2016 (de Oliveira et al., 2017). In India, ZIKV circulation was declared with the three positive cases detected from Gujarat state in November 2016 (Sapkal et al., 2018) followed by fourth ZIKV positive case detected from Tamil Nadu state in June 2017 (Bhardwaj et al., 2017). The ubiquitous presence of *Aedes mosquitoes* in India raises the concern about the presence of ZIKV in this vector. In India, *Ae. aegypti* is responsible for transmission of viruses like Dengue virus (DENV), Chikungunya virus (CHIKV), and ZIKV.

As part of the country's preparedness program of ZIKV disease, Indian Council of Medical Research (ICMR), Department of Health Research (DHR), had created ZIKV surveillance network laboratories in India. The permission for sample collection, testing and investigation were approved by the Institutional Human Ethics Committee – ICMR-National Institute of Virology (NIV), Pune and Sawai Man Singh (SMS) Medical College, Jaipur. The IHEC No. NIV/IHEC/2015/3043. An 85-years old female, a resident of Rajasthan, India was admitted to hospital on 11th September 2018 with complaints of one episode of generalized

* Corresponding author at: Indian Council of Medical Research- National Institute of Virology, 20-A, Dr. Ambedkar Road, Pune 411 001, Maharashtra, India.
E-mail address: dtmourya@gmail.com (D.T. Mourya).

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Table 1
Details of the ZIKV samples collected along with copy number, percentage of NGS read mapped, and Genbank accession numbers from Jaipur city, Rajasthan state, India.

Sample ID	Host	Source type of samples	Collection date	Location	Result of Real-time RT-PCR for ZIKV [Ct value]	RNA copy number./ml[a]	Number of NGS reads	Percentage of ZIKV reads mapped (%)	Sequenced genome size	GenBank Accession No.
17488	Human	Serum	18-Sep-2018	India: Jaipur City,	24	5.4×10^8	1103/363,672	0.30	~10.3 Kb	MK238035
20364			10-Oct-2018	Rajasthan State	24	5.4×10^8	1311/3,201,470	0.04	~6.4Kb	MK238036
20366			10-Oct-2018		24	5.4×10^8	31,126/1,574,810	1.98	~10.8Kb	MK238037
21993			15-Oct-2018		26	1.1×10^8	3098/4,76,930	0.65	~10.2Kb	MK238038
20616			11-Oct-2018		26	1.1×10^8	558/1,322,950	0.04	~6.4Kb	MK238039
21046			12-Oct-2018		24	5.4×10^8	No viral reads	No viral reads	Not Applicable	Not Applicable
22463			16-Oct-2018		28	2.3×10^7				
20293			10-Oct-2018		29	1.0×10^7				
21045			12-Oct-2018		29	1.0×10^7				
22948			NA		26	1.1×10^8				
22894			18-Oct-2018		32	1.05×10^6				

NA- not available.
[a] Reference: Lanciotti et al. Genetic and Serologic Properties of Zika Virus Associated with an Epidemic, Yap State, Micronesia, 2007.Emer. Inf. Dis. 2008;14(8);1232–39.

convulsions with the semi-unconscious state on the day of admission. Relatives reported no other symptoms of fever, rash, myalgia or arthralgia. She was a known case of kyphoscoliosis, hypertension (on regular anti-hypertensive drugs) and had one episode of the cerebrovascular accident one and half year back. She had no travel history before the episode and did not report fever in the past three months. There was no history of blood transfusion. No details of treatment history, biochemical or hematological parameters were mentioned in the clinical referral sheet provided by the hospital. No details of any brain imaging were provided. On the admission, she reported of having one episode of generalized tonic-clonic seizures with altered sensorium and breathlessness. The resident doctor on duty noted warm body on examination and investigated for fever profile. During September 2018, clinical samples (serum and urine) of a ZIKV suspected case from Rajasthan, India were referred to ICMR-NIV, Pune by SMS Medical College, Jaipur city, Rajasthan, India for the confirmation.

Serum and urine specimens were examined for DENV, CHIKV, and ZIKV using CDC Trioplex Real-Time RT-PCR reagents (Santiago et al., 2018). The ZIKV was detected in serum (Ct = 24) and a urine sample (Ct = 28) of the referred index case. The patient recovered completely without any sequel. None of the other family members reported any illness of similar kind or any febrile episodes. A repeat sample of blood and urine was collected from the index case on 26th September (14th day after onset of illness) for confirming evidence of Zika viral disease. The serum sample was tested negative, however urine sample found to be positive (Ct = 34). This confirmed the fifth ZIKV case to be reported from India, which was further substantiated by the more positive cases of ZIKV in Jaipur city, Rajasthan, India and led to the declaration of the outbreak. Detection of ZIKV raised the alarm, and ZIKV surveillance was carried near the vicinity of the index case (Shastri Nagar and Rajpoot Hostel, Sindhi camp). Total 159 positive cases are confirmed and reported from Rajasthan state ("Weekly Outbreaks: Integrated Disease Surveillance Programme (IDSP), 44th week," 2018) (https://idsp.nic.in/WriteReadData/l892s/442018.pdf.). Out of these ten samples of positive cases were considered for next-generation sequencing (NGS) that had a Ct value ranging from 24 to 32.

The first three samples to be confirmed positive was from Gujarat samples during 2016, however complete genome sequences could not be retrieved (Sapkal et al., 2018). Evolutionary analysis of the retrieved sequence of the ZIKV case from Gujarat state belonged to Asian lineage. In 2017, ZIKV was reported from Tamil Nadu state, and again the region sequenced was small. This Ministry of India was specifically interested in sequencing and identifying ZIKV strain in the recent outbreak in Jaipur city, Rajasthan. Also, we were looking for an answer to three questions (i) currently there are two known, accepted lineages for ZIKV, which are Asian and African (Beaver et al., 2018). We ought to determine which lineage of ZIKV that is circulating in India? (ii) Whether ZIKV sequence from Gujarat, India retrieved during 2017 was similar to this sequence from Jaipur city Rajasthan in 2018? and (iii) Are there any amino acid (aa) difference between the two sequences?

RNA library was prepared using 300 ul serum, and 1 ml urine samples using QIAmp viral RNA extraction kit (Qiagen, USA). The concentration of the eluted RNA was determined using Qubit fluorometer (Thermofisher, USA). The steps involved in the RNA library preparation involves fragmentation, amplification, and quantification of the RNA (Yadav et al., 2018). These RNA libraries were normalized and loaded on the Illumina Miniseq NGS platform. The reads generated by the machine were imported in the CLC Genomics Workbench software 11.0.1 (CLC, Qiagen) for analysis. Reference-based mapping was performed to retrieve the genome of the ZIKV since the virus was known. A strain of Malaysia (KX694533: ~10.7 kb) was used as the reference sequence for the analysis, which leads to the retrieval of a nearly complete sequence of the ZIKV [MK238035 (~10.3 kb)] and a complete sequence 10.8 kb for MK238037 [Ct value =24]), and partial sequences were retrieved for three more human serum sample [MK238036, MK238038, and MK238039 (6467 bp, 10,240 bp,

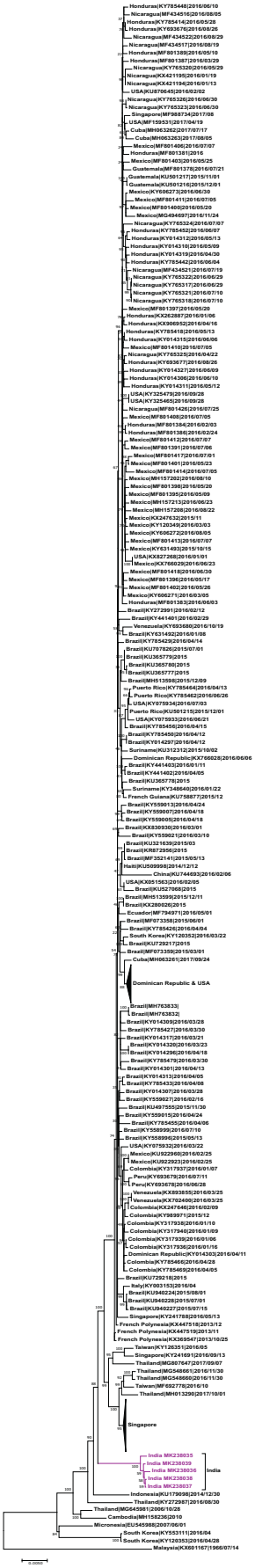

Fig. 1. A) Phylogenetic tree of the nearly complete genome of 330 ZIKV retrieved from the reference database and the serum sample of Jaipur, Rajasthan state, India. The evolutionary distance was generated using the general time reversible model with invariable sites. A bootstrap replication of 1000 cycles was performed to access the statistical robustness. Indian strains are marked in red color. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

6467 bp)] having Ct value in the range of 24–26 (Table 1). As we have no access to the ZIKV positive samples from Bhopal, India; the study will further describe ZIKV sequences from Rajasthan, India. NCBI virus variation database was used to download nearly complete genome sequences of ZIKV belonging to different lineages. The travel sequences with no specific origin were excluded from the phylogenetic analysis. Fig. 1 depicts the Maximum-Likelihood tree derived from the coding region of the different ZIKV strains. A bootstrap of 1000 replications was performed for assessing the robustness of the generated tree. It was observed that ZIKV sequence from Jaipur city, Rajasthan outbreak in 2018 clustered with the Asian lineage.

Till date, no complete ZIKV genome has been reported from India, so the comparison of known sequences of Tamil Nadu and Gujarat ZIKV was not possible. Phylogenetic comparison of the current ZIKV outbreak strain with other reference ZIKV sequences determined that ZIKV from current outbreak in Rajasthan, India clusters as a separate group. It was observed that the most recent ancestorial strain to India was from Indonesia. A phylogenetic tree was also constructed from the partial sequences of the ZIKV available in GenBank from Gujarat and Tamil Nadu states [2017] to identify the clustering pattern of the Indian sequences. It was observed that Indian sequences clustered in two different groups; first the ZIKV sequence from Gujarat [2017] formed a cluster with the *Aedes aegypti* isolate of 1966 from Malaysia, whereas; the outbreak in Jaipur Rajasthan [2018] and Tamil Nadu [2017], ZIKV sequences branched to form a separate cluster compared to other Asian ZIKV sequences (Supplementary Fig. 1). Although the sequences used in the comparison were smaller in size, they clustered in the separate group indicating that they are different from each other.

Our current analysis demonstrates ZIKV sequence detected in Gujarat, India [2017] seems to be an old Asian ZIKV strain. The current strain detected in Rajasthan [2018] seems to be evolved from another ZIKV, strain ancestor. Rapid diversification of these strains to other Asian ZIKV strains cannot be denied. We hypothesize that the presences of two different strains of Asian lineage co-exist in the nearby regions of India and also speculate ZIKV introduction from Africa to the Indonesian region and subsequently to India (Pettersson et al., 2018). ZIKV surveillance, public awareness, and vector control need to be prioritized in India. The abundance of the *Aedes* vector is responsible for the transmission of ZIKV and probability of future outbreaks in the country cannot be overlooked.

It was observed that the nucleotide and amino acid (aa) divergence between the ZIKV Indian sequences included in the study was 0.2–0.3% and 0.1–0.3% respectively (data not shown). Specific changes were observed between the genes regions of the Rajasthan and Gujarat sequences which are as mentioned (capsid: T106A, T108A, G114S, T119S; GlyM: D140M, A144T, M153 V; E gene: T570A, E619G, NS2B: I1425M). The partial ZIKV sequences from Tamil Nadu had 100% aa similarity to the outbreak Jaipur strain, but the unavailability of the complete genome which is the limitation for the analysis. The higher similarity was due to a very small region of 83 aa residues, and hence no changes were noted. The mutations linked to enhanced neurovirulence (S139N) (Yuan et al., 2017) and higher transmissibility (A188V) (Liu et al., 2017) in animal models was not observed for the Indian ZIKV (Rajasthan, Gujarat and Tamil Nadu) sequences. A word of caution should be maintained on the claims of these mutations on the development of microcephaly in humans, given no direct clinical evidence of their effect.

2. Conclusion

The ZIKV strain from the Jaipur, Rajasthan outbreak belongs to Asian lineage. It appears that there are two different strains [Rajasthan and Tamil Nadu ZIKV as the current Asian outbreak strain and Gujarat as pre-outbreak Asian strain] circulating in the country. The mean evolutionary rate of Asian ZIKV is 1.2×10^{-3} substitutions per site per year (Faria et al., 2016). The mutation (S139 N) leading to increased infectivity of ZIKV in human and mouse neural progenitor cells causing microcephaly is not present in the outbreak strain circulating in Rajasthan state. The presence of a reverse mutation (N139S) has been demonstrated to have milder neurovirulence (Yuan et al., 2017). In one of the reports from Thailand by Wongsurawa et al. Asian ZIKV sequences did not contain S139N substitution but was reported to cause microcephaly (Wongsurawat et al., 2018). Further ZIKV strains without S139N mutations were also found to cross the mice placental barrier (Jaeger et al., 2018). Rosenfeld demonstrated that all the ZIKV strains are neurotropic (Rosenfeld et al., 2017). Liu et al. determined the NS1 gene to be lined with ZIKV infectivity and proposed spontaneous accumulation of mutation to be resulting in the increased NS1 gene activity (Liu et al., 2017).

These observations reveal that once the ZIKV establishes in the Indian ecosystem, its chances of mutation cannot be neglected. Despite the absences of the proposed mutation on the transmission and microcephaly, chances of not finding such a clinical condition cannot be guaranteed. Retrospective analysis on the 73 referred human samples from Rajasthan state received for viral hemorrhagic fever diagnosis during year September 2016 on negative Crimean Congo hemorrhagic fever cases identified a Zika case (NIV unpublished data). This was confirmed by real-time RT-PCR (Ct = 35) and IgM antibody, establishing that the virus was present in Ajmer, Rajasthan before the current outbreak. Hence awareness about the ZIKV needs to be geared up to withstand for its future outbreaks.

Supplementary data to this article can be found online at https://doi.org/10.1016/j.meegid.2019.01.026.

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Conflict of interest

Authors have no conflict of interest.

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Accession numbers

Accession numbers of the sequences submitted in this study: MK238035- MK238039.

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