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Isolation of Chandipura virus (*Vesiculovirus: Rhabdoviridae*) from *Sergentomyia* species of sandflies from Nagpur, Maharashtra, India

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***Background & objectives*: An outbreak of acute encephalitis syndrome was reported from Vidarbha region of Maharashtra State, India, during July 2012. Anti-IgM antibodies against Chandipura virus (CHPV) were detected in clinical samples. Sandfly collections were done to determine their role in CHPV transmission.**

***Methods*: Twenty nine pools of *Sergentomyia* spp. comprising 625 specimens were processed for virus isolation in Vero E6 cell line. Diagnostic RT-PCR targeting N-gene was carried out with the sample that showed cytopathic effects (CPE). The PCR product was sequenced, analysed and the sequences were deposited in Genbank database.**

***Results*: CPE in Vero E6 cell line infected with three pools was detected at 48 h post infection. However, virus could be isolated only from one pool. RT-PCR studies demonstrated 527 nucleotide product that confirmed the agent as CHPV. Sequence analysis of the new isolate showed difference in 10-12 nucleotides in comparison to earlier isolates.**

***Interpretation & conclusions*: This is perhaps the first isolation of CHPV from *Sergentomyia* spp. in India and virus isolation during transmission season suggests their probable role in CHPV transmission. Further studies need to be done to confirm the precise role of *Sargentomyia* spp. in CHPV transmission.**

Key words Acute encephalitis syndrome - Chandipura virus - *Sergentomyia* species - virus isolation - Vero E6 cell line

Chandipura virus (CHPV) belongs to genus *Vesiculovirus*, family *Rhabdoviridae* and was discovered during an outbreak of dengue-chikungunya-like illness in Nagpur district, Maharashtra, India, in 1965 from a patient with febrile illness[1]. Prevalence of CHPV in India was established by sporadic cases reported from different parts of the country, virus isolations from humans and sandflies as well as presence of antibodies in humans and vertebrates[2]. However, CHPV, as a virus of public health importance was realized only when an outbreak of encephalitis with high case fatality rate (CFR) among children was reported from central India in 2003[3,4]. The outbreak was characterized by high morbidity followed by rapid deterioration of cases and death in three States of India, *viz*. Andhra Pradesh, Maharashtra, and Gujarat with CFR ranging from 55 to 76 per cent. Though the outbreak was contained, sporadic cases are reported every year from certain districts of Andhra Pradesh and Vidarbha region of Maharashtra[5,6].

Sandflies are incriminated as the vector of CHPV due to their presence in outbreak areas and repeated virus isolations[3]. CHPV has been isolated from *Phlebotomine* sandflies from India and from Africa during arbovirus investigations[7].

During July 2012, an outbreak of acute encephalitis syndrome (AES) with high case fatality was reported from several districts of Vidarbha region of Maharashtra *viz*. Nagpur, Bhandara, Chandrapur, Wardha, *etc*. Nine of the 18 serum samples collected from AES patients were tested positive for CHPV IgM antibody (NIV unpublished data). Though two AES cases with Japanese encephalitis aetiology were reported by the initial investigation, vector mosquito population was found to be below the threshold of virus transmission. Both the cases recovered subsequently and hence virus could not be isolated either from the human serum or from mosquitoes. Here we describe the isolation and characterization of a CHPV isolate obtained from field collected *Sergentomyia* spp. during the recent July 2012 AES outbreak in Maharashtra.

Material & Methods

Sandfly collection was done in 13 villages/localities in the four districts of Maharashtra to determine their role in CHPV transmission (Table). Collection was made using hand held mouth aspirators from indoor and outdoor resting places. Oral consent from house owners was obtained to inspect their houses and peri-domestic areas for sandfly collection. Emphasis was given to collect sandflies from households, from where cases were reported. Majority of the houses had un-plastered brick/mud walls which are ideal for sandfly breeding. Collections were made from the damp/dark places of living rooms, kitchen, bathrooms, toilets and cattle sheds attached to the houses. The adult sandflies were transported alive to National Institute of Virology (NIV), Pune, and identified following the keys provided by Lewis[8]. Pools were prepared according to genera, gender and locality.

Individual pools of sandflies were triturated in a small volume (0.5-1 ml) of chilled minimum essential medium (MEM, Sigma, USA), with pre-chilled, sterile mortars and pestles as described by Sudeep *et al*[9]. The suspension was centrifuged at 2790 *g* for 30 min at 4°C, collected supernatant, Millipore filtered (0.22 um) and inoculated on confluent monolayer of Vero E6 cells grown in 24-well plates (Nunc, Denmark) in duplicate (100 µl per well). Virus adsorption was carried out at 37°C for 2 h with intermittent rocking of the plates at every 15 min. The cultures were fed with maintenance medium (MEM supplemented with 2 per cent foetal bovine serum), incubated at 37°C and observed for cytopathic effects (CPE). The samples that did not show CPE, were re-passaged in the cell line twice and screened for CPE.

The sample, which showed CPE was amplified in Vero E6 cells in 25 cm^2 bottles (Nunc, Denmark) and harvested when >75 per cent cells showed CPE. Three cycles of freeze-thawing were done and the suspension was clarified as described earlier, aliquoted

Table. Details of sandflies collected from Vidarbha region for virus isolation

District	Village/locality	No. of male sandflies	No. of female sandflies	Total number
Nagpur	Ramtake	01	01	02
Nagpur	Chachar	11	02*	13
Nagpur	Pardi	142	57	199
Bhandara	Kharbi	40	37	77
Bhandara	Lakhari	06	12	18
Bhandara	Panchshil wad	02	03	05
Bhandara	Virshi	22	14	36
Bhandara	Chinchtola	146	37	183
Bhandara	Salai	42	26	68
Bhandara	Gondegaon	00	00	00
Chandrapur	Sindiwahi	02	03	05
Chandrapur	Chandrapur	04	00	04
Wardha	Gunjkheda	07	08	15
Total		425	200	625

*Virus isolation obtained

and stored at -80°C. RNA from one of the aliquots was extracted using QIAamp Viral RNA Mini Kit (Qiagen, Hilden, Germany) as per manufacturer's instructions and reverse transcriptase-PCR (RT-PCR) conducted targeting a 527 nucleotide fragment of N-gene as described earlier[6]. Cycling conditions used were 1 cycle at 94°C for 5 min; then 35 cycles each at 94°C (1 min), 50°C (1 min), and 68°C (1.5 min); followed by final extension of 7 min at 68°C. Amplified fragments were visualized by ethidium bromide-agarose gel staining. PCR products were purified by using QIA quick PCR Purification Kit (Qiagen, Hilden, Germany) and sequenced using BigDye Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems, Foster City, CA, USA) in an automatic sequencer (ABI PRISM 3100 Genetic Analyzer, Applied Biosystems, USA). Using ClustalX, version 1.83, multiple alignments of nucleotide sequences were performed.

Results & Discussion

Collections yielded 625 (425 male and 200 female) sandflies belonging to *Sergentomyia* spp. and 17 to *Phlebotomus* spp. (Table). Twenty nine pools of the former were prepared according to sex and locality and processed for virus isolation. *Phlebotomus* sandflies could not be processed for virus isolation as none of them could be brought alive to the laboratory.

In the first passage, CPE in Vero E6 cells was observed with three pools at 48 h post-infection (PI). However, in the 2nd passage, only one sample exhibited CPE and the other two failed. Distinct CPE was observed at 7 h PI in Vero E6 cell line. The isolate was obtained from a pool comprising only two female sandflies collected from Chachar village in Nagpur district (Table). RT-PCR studies targeting the N-gene confirmed the agent as CHPV as a 527 bp band corresponding to the N-gene was observed. A distinct band identical to positive control could be detected in the study (Fig.). Sequencing of the PCR product showed 10-12 nuecleotide changes in the new isolate in comparison to earlier CHPV sequences demonstrating it as a new isolate. The sequences were deposited in Genbank database and have been accepted as a new CHPV isolate (GenBank accession number KF570390).

The first isolation of CHPV from sandflies was reported from *Phlebotomus* spp. collected from Aurangabad district of Maharashtra[10]. Isolation of CHPV was also reported from the same group of sandflies from Africa subsequently[7]. However, CHPV has not been isolated from *Sergentomyia* spp. in India though detection of CHPV RNA was reported earlier[6,11].

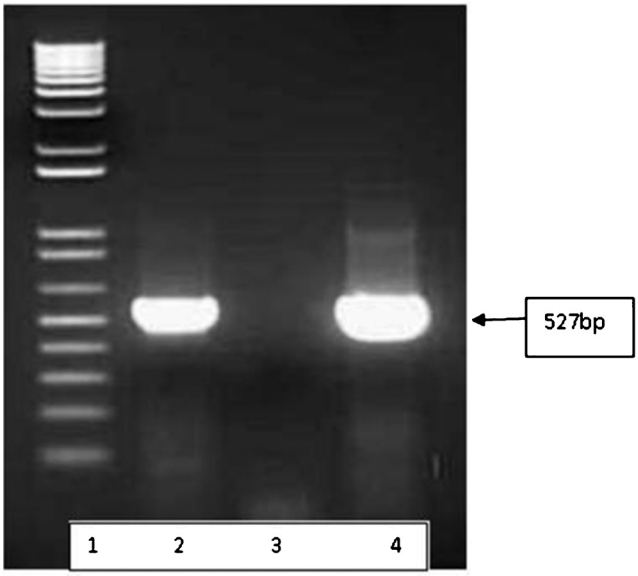

Fig. CHPV diagnostic RT-PCR amplified product of 527 bp from a pool of sandflies. Lane 1: Marker; Lane 2: New CHPV isolate from sandflies; Lane 3: Negative control (normal sandfly suspension); Lane 4: Positive control (laboratory strain of CHPV).

Members of the genus *Sergentomyia* are outdoor breeding sandflies that generally breed in mud burrows, termite mounds, tree holes, *etc*., and rarely feed on humans. Presence of *Sergentomyia* spp. inside houses in large numbers in the study area is interesting as the flies appear to have become anthropophilic. Earlier studies conducted in six districts of Vidarbha region by NIV team also reported the predominance of *Sergentomyia* spp. of sandflies in the area[6]. This is in contrast to studies conducted by NIV in the 1960s and 1970s, which showed high prevalence of *Phlebotomus papatasi* and *Ph. argentipes*. Both the species were abundant in the domestic environments[10]. The recent studies, however, have recorded a drastic reduction of *Phlebotomus* spp. in the domestic environments and are replaced with *Sergentomyia* spp[6]. The reduction in numbers of the former could be probably due to high insecticide application inside houses for mosquito control.

This is perhaps the first isolation of CHPV from *Sergentomyia* spp. of sandflies in India. Sequence analysis has shown a few nucleotide changes in the new isolate in comparison to earlier ones. A detailed study needs to be carried out to assess the pathogenicity

of the new isolate. Similarly, high prevalence of *Sergentomyia* spp. of sandflies in residential areas and isolation of CHPV warrant an in depth investigation to determine virus susceptibility and vector competence of these sandflies. Studies also needs to be initiated not only to determine the precise role of *Sergentomyia* spp. in transmitting CHPV and other sandfly borne viruses, but also to understand vector competence of different species within the genera.

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