Temperature sensitivity and environmental stability of Chandipura virus

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Abstract Chandipura virus (CHPV), a negative-stranded RNA virus of family *Rhabdoviridae* is endemic in Central India since 1965. The virus gained public health importance when it was held responsible for massive outbreak in 2003–2004 in Maharashtra, Telengana and Gujarat with case fatality rates ranging from 55 to 75% among children. We studied the stability of the virus as well as RNA persistence in samples stored at different temperatures for different periods. CHPV remained infective in sand flies and cell culture supernatants at 4 °C for 8 weeks. At 37 °C CHPV remained viable for 18 days when stored in infected cell supernatant (Minimum essential medium supplemented with 10% fetal bovine serum). However, in infected sand flies stored at 37 °C, the virus lost virulence within a week. CHPV RNA, though lost virulence, could be detected in virus exposed sand flies stored at 37 °C for 13 weeks by real time RT-PCR. Retaining virulence at 37 °C for 18 days in serum containing medium is a matter of concern for laboratories and hospital settings where clinical samples are handled. RNA persistence for prolonged periods in dead sand flies might help in surveillance studies of CHPV in sand flies and will help in resource constraint nations where cold chain management is a concern.

Keywords Chandipura virus · Environmental stability · Real time RT-PCR · RNA persistence · Sand flies

Introduction

Chandipura virus (CHPV), a negative-stranded RNA virus, belonging to genus *Vesiculovirus* of family *Rhabdoviridae*, was first isolated in India from human serum collected from adults with febrile illness in 1965 [1]. It is a bullet shaped, enveloped virus with a genome size of approx. 11 KB. The virus gained importance as a pathogen of public health importance in 2003–2004 when it caused massive outbreaks with high mortality among children in central India comprising parts of Maharashtra, Telengana and Gujarat [2, 12, 16]. CHPV induced death among children within 24 h of exhibition of symptoms and the case fatality ratio ranged from 55 to 77% [2, 12]. The symptoms included sudden onset of high grade fever, convulsions, altered sensorium, vomiting followed by Grade IV coma [2, 12, 16]. However, the exact mechanism of action of the virus is still not clear, though it is presumed to be neuro-degeneration [5, 8]. Sand flies belonging to *Phlebotomus* spp. are incriminated as the vector of CHPV due to their prevalence in outbreak areas, virus isolation and their ability to transmit the virus vertically and venereally [9]. Recent studies have shown that sand flies belonging to *Sergentomyia* spp. also harbor the virus though their role in virus transmission is not clearly understood [14, 16]. Central India has become endemic to the virus as sporadic cases continued to be reported every year especially during post monsoon periods [16, 17]. Geographic expansion of the virus has seen recently as evidenced by the presence of IgM antibodies in humans in Odisha, Uttar Pradesh etc [3].

CHPV has been isolated from clinical samples viz., human and animal serum and also from sand flies during outbreak periods [9, 14, 16]. Antibodies to CHPV or a virus antigenically close to CHPV has been detected in almost all

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parts of the country since early 1950s [9]. During non outbreak periods, the virus is thought to be maintained in nature by the sand flies as no other vertebrate host is found to harbor the virus in India [9]. In the present communication, we present data on the duration of survival of the virus in culture medium supplemented with fetal bovine serum (FBS) and in sand flies stored at different temperatures.

Materials and methods

Virus CHPV strain number 034627, isolated in Vero E6 cells from human serum collected during the 2003 outbreak in Andhra Pradesh was used for the present study. The integrity of the isolate was confirmed by serological and molecular techniques including sequence analysis.

Cell lines Vero E6 cell line procured from ATCC was used throughout the study. The cell line was maintained in Minimum Essential Medium (MEM; Invitrogen, USA) supplemented with 10% FBS (Invitrogen, USA). Sub culturing was made two to three times a week giving a split ratio of 1:3.

***Phlebotomus papatasi* sand flies** Sand flies were procured from the laboratory colony maintained at the National Institute of Virology Pune. The flies were maintained on commercially available fish food and maintained at 28 °C and 70–75% relative humidity.

Determination of virus stability in different host systems stored at different temperatures

CHPV survival in cell culture fluid Confluent monolayer of Vero E6 cell cultures were infected with CHPV at 1 MOI (multiplicity of infection) and harvested when > 75% cells showed cytopathic effect (CPE). The cells were freeze-thawed thrice, centrifuged at 5000 rpm at 4 °C, aliquoted the supernatant and stored at – 80 °C. Two aliquots were used to determine virus titer of the stock by CPE method as described earlier (11) and the other aliquots were stored at 4 °C and 37 °C. One aliquot from each batch was removed on alternate days and stored at – 80 °C till completion of the experiment. Virus titer of each sample was determined and compared with the original titer to determine loss in virus titer after exposure to different temperatures.

Determination of stability of CHPV in sand flies Laboratory reared 2–3 days old adult female *Phlebotomus papatasi* (n = 150) sand flies were inoculated intra-thoracically with ~ 0.1 µl of CHPV suspension as described earlier [7]. In brief, sand flies were immobilized

by keeping the vial containing sand flies in an ice bath for 4–5 min and inoculated with virus suspension through the thoracic region under a dissecting binocular microscope. Inoculation was carried out using specially prepared and calibrated capillary needle and syringe plunger inside a bio-safety level-2 laboratory with containment facility. After inoculation, the sand flies were secured in sand fly maintenance jars (plastic jars having plaster of Paris bottom), maintained on 10% glucose solution and incubated at 28 °C with 80 ± 5% RH. The sand flies were incubated for 24 h at the above conditions and killed instantly by freezing them at – 80 °C, distributed three flies per vial and kept at 4 °C and 37 °C. Every week, one vial from each storage condition (4 °C and 37 °C) was removed and stored at – 80 °C. After completion of the experiment, sand flies were retrieved from – 80 °C and processed individually as per their storage conditions using CPE method and real time RT-PCR to determine virus titer and RNA copy numbers respectively.

Determination of virus titer using CPE method The sand fly suspension was clarified by centrifugation at 4 °C for 30 min at 5000 rotations per minute (rpm), filter sterilized (Millipore, pore size = 0.22 µm), diluted serially (10-fold) and titrated in Vero E6 cells in quadruplicate as described earlier (11), [15]. Virus titers were determined as described by Reed and Muench [13]. The experiment was carried out in triplicate and analyzed for determination of growth kinetics.

RNA isolation and real time PCR

Viral RNA was extracted from sand fly suspensions (prepared as above) of each week's sample using silicon based spin columns (QI Amp viral RNA minikit, QIAGEN, Valencia, CA) as per manufacturer's instructions. Five µl of extracted RNA was used for real time RT-PCR assay using real-time one step RT-PCR master mix as described by Kumar et al [6]. In brief, 25 µl of reaction volume contained 12.5 µl of 2X Master Mix, 0.6 µl of 40 × multiScribe RT and RNase inhibitor mix, 500 nM of PF1 forward primer and 300 nM of PR1 reverse primer (PP1 TaqMan MGB probe). DNase/RNase free water was used to adjust the volume to 25 µl. Real-time one step RT-PCR was performed in a 96 well format using Step one plus real time PCR system (Applied Biosystems) and SDS software version 1.3.1. The real-time one step RT-PCR employed the following thermal cycler settings: 30 min at 50 °C and 5 min at 95 °C, followed by 45 cycles of 15 s at 95 °C and 1 min at 60 °C. Each real-time one step RT-PCR assay had at least two non template controls in which RNA was substituted by DNase/RNase free water.

Results

CHPV stability at 4 °C

In cell culture medium (MEM supplemented with 2% FBS), CHPV titer remained unchanged for 10 days post infection (PI). A slight decline of approx. 1 log titer was observed on the subsequent days till the 20th day PI (Fig. 1). Stability of CHPV at 4 °C in MEM supplemented with 10% FBS was not studied.

In virus exposed and killed sand flies, CHPV remained infective for 8 weeks at 4 °C (Fig. 2). Virus titer remained stable for 5 weeks and showed a decline in virus titer (2 logs) on 6th week post storage. No virus could be detected on the 9th week suggestive of total degeneration.

CHPV stability at 37 °C

In MEM supplemented with 10% FBS, virus remained virulent till 18th day PI. No change in virus titer was observed for the first 2 days PI. However, a gradual but steady decline in virus titer was observed on subsequent days PI till the 14th day PI reaching a titer of 2 log₁₀ TCID₅₀/ml (Fig. 1). A few infective virus particles (virus induced plaques) could still be observed in Vero E6 cell cultures infected with samples of 16th and 18th day PI. Conversely, in MEM supplemented with 2% FBS, the loss in virus titer was drastic as approx 3 log virus was lost within the first 2 days of storage (Fig. 1). On subsequent days PI, a gradual decline (approx. 1 log/day) in virus titer was observed till the 14th day PI. No infective virus particle (plaques) could be seen from 16th day PI onwards.

In infected sand flies, however, no infective virus could be detected after 1 week of storage (Fig. 2). Attempts were

Fig. 1 CHPV stability in MEM with different FBS concentrations and temperatures

Fig. 2 Persistence of CHPV in dried sand flies stored at different temperatures

not made to detect the presence of virus in Infected sand flies during day 1 to day 7.

CHPV RNA persistence in dried sand flies stored at different temperatures

Persistence of CHPV RNA in dried sand flies could be detected for 13 week post storage in samples stored at 4 °C and 37 °C (Fig. 3). In samples stored at 4 °C, no significant decrease in RNA copies could be detected while in samples stored at 37 °C, the ct value remained almost constant till 9th week suggestive of persistence of CHPV. However, a steady increase was observed on subsequent weeks, reaching a peak at 13th week showing decline in RNA copy number. No RNA could be detected from 14th week onwards.

Fig. 3 Detection of CHPV in infected sand flies using real time RT-PCR (error bars represent standard deviation). In this assay, a positive reaction is detected by accumulation of a fluorescent signal. The Ct (cycle threshold) value is defined as the number of cycles required for the fluorescent signal to cross the threshold (i.e., exceeds background level). The figure represents significant increase in Ct value when samples were stored at 37 °C as compared to 4 °C suggesting decrease in the quantity of nucleic acids

Discussion

CHPV, an arbovirus transmitted by *Phlebotomine* sand flies, has assumed the status as a pathogen of public health importance in India since 2003 [5, 16, 19]. The virus persists in parts of Gujarat, Telangana and Maharashtra with recurring outbreaks and has become a health concern to children within the age group of 0–15 [8, 17]. It is difficult to explain why this part of the country is affected by Chandipura virus despite having *Phlebotomine* sand flies in almost all parts of the country. It could be either the climatological factors or human genetic factor or a combination of both and needs specific studies to understand the phenomenon. Geographic expansion of the virus to neighboring states has also been reported recently as sporadic outbreaks were reported from several districts of Gujarat, viz., Ahmedabad, Kheda, etc in addition to Vadodara, Panchmahal districts from where outbreaks were reported in 2004 (NIV Annual Report 2016). Similarly, IgM antibodies to CHPV in four adult patients in a tribal village in Kandhamal district in Odisha state were detected during investigation of an outbreak of encephalitis like illness [3]. IgM antibodies to CHPV have also been detected in human serum collected from Gaya, Uttar Pradesh and Rajasthan (NIV Annual Report 2017) suggestive of expanded virus activity.

Recent studies have shown that several viruses remain stable at higher temperatures and retain their virulence at least for a few days. In a recent study, Parashar et al. [11] have shown that chikungunya virus (CHIKV) remain infective for 2 days in whole blood stored at 37 °C. The investigators using real time RT-PCR also found that CHIKV RNA persisted in blood samples stored at 37 °C for ≥ 6 months. Similar results were also been reported by Muller et al. [10] with Zika virus, when they demonstrated infectivity of the virus for 3 days in dry conditions.

In the present study, we have observed that CHPV is highly stable at 37 °C as infective virus particles could be detected (as observed by exhibition of CPE in Vero E6 cells) upto 18th and 14th day PI in MEM supplemented with 10% and 2% FBS respectively (Fig. 1). This clearly shows that CHPV can remain viable in blood and body fluids of patients and when exposed, may cause infection to others especially those who handle patients or blood samples. In clinical or research laboratories where testing of multiple viruses are conducted, CHPV might lead to laboratory contaminations due to its ability to survive for longer periods. It is also been observed that CHPV did not get inactivated when exposed under UV light for 30 min (Sudeep unpublished data). In a subsequent study, Sudeep and co-workers observed that CHPV remained viable for

48 h at 42 °C in MEM supplemented with 10% FBS (Unpublished data).

Since no vertebrate hosts have been identified as a reservoir to maintain the virus in nature, sand flies are presumed to maintain the virus during non epidemic periods. This has been substantiated by experimental studies in *Phlebotomus papatasi* and *Phlebotomus argentipes* sand flies which exhibited transovarial transmission or vertical transmission [7, 18].

CHPV was considered as an innocuous virus till the 2003 outbreak and minimum biosafety protocols were followed while handling samples in the laboratory. With the present data, it has been shown that the virus remains viable in serum and infected sand flies for a substantial period of time. It is really a matter of bio-safety concern especially to those handling samples in diagnostic laboratories or during patient management. It is also important to note that CHPV primarily infects children under 15 years of age. However, recent studies have shown the presence of IgM antibodies in adults suggestive of subclinical infection [3]. During the case studies, CHPV has turned out to be a neurotrophic virus causing encephalitis and death [4]. The new findings provide valuable insights about the survival of the virus at high temperatures which warrants the need for not only proper handling of patients/clinical samples with adequate precautionary measures but also proper decontamination of work areas and equipments to avoid accidental infection.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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