Persistence of Chikungunya virus in samples stored at different temperatures

Chikungunya virus (CHIKV), a mosquito-borne alphavirus (family Togaviridae), has become a globally major public health problem since 2004 (ref. 1). No vaccines or therapeutics are available except for mosquito control. CHIKV is relatively stable in blood at room temperature and remains viable for at least 8 h (ref. 2). However, no documented data is available regarding survival of CHIKV outside the host, i.e. in serum or infected mosquitoes. Understanding the survival and environmental stability of CHIKV in the above samples is important for effective management during outbreaks and handling clinical samples.

Six ml of serum from a healthy individual spiked with 3.1×10^9 RNA copies of tissue culture grown CHIKV was aliquoted (140 µl each aliquot) and stored at 4°C, 37°C, –20°C and –80°C. *Aedes aegypti* mosquitoes were procured from the insectary maintained at the National Institute of Virology, Pune, inoculated intra-thoracially as described earlier[3] and incubated at 37°C. In brief, 3 to 4-day-old female mosquitoes ($n = 100$) were inoculated intra-thoracically with CHIKV at the rate 0.2 µl virus suspension per mosquito. After infection, the mosquitoes were maintained on 10% glucose solution for 24 h at 28°C, killed instantly by freezing at –80°C and stored at 37°C. Serum samples were retrieved every week while mosquito samples were retrieved weekly till the 8th week and thereafter monthly till the sixth month and stored at –80°C till completion of the experiment. Infectivity/virus titer was detected by $TCID_{50}$ assay in Vero E6 cells as described earlier[3]. Frozen serum samples were thawed quickly, spun at 5000 rpm for 20 min at 4°C and the supernatant was diluted serially (10-fold) in minimum essential medium (MEM) supplemented with 2% fetal bovine serum (FBS). The serially diluted virus was then inoculated over Vero E6 cell line grown to confluent monolayer in 96-well plates (Nunc, Denmark) in quadruplicate. The cultures were incubated at 37°C for 96 h, scored cytopathic effect (CPE) under an inverted microscope, stained with amido black and virus titer determined as described by Reed and Muench[4]. Mosquito samples were triturated using a battery-operated hand-held homogenizer (Sigma, USA) with sterile disposable pestles in 1 ml chilled MEM supplemented with 2% FBS, millipore filtered (0.22 µm), diluted serially and $TCID_{50}$/ml in Vero E6 cell line determined as described above. RNA persistence was detected by RT-PCR and quantitative RT-PCR (qRT-PCR). For RT-PCR and qPCR, RNA was isolated using QIAamp viral RNA mini kit (Qiagen, Hilden, Germany) according to manufacturer's instructions. The RT-PCR targeting *nsp1* gene was carried out using primers (F4 5′ CCG GAG GAT GCA CAG AAG CT 3′ and R4 5′ TCG GAG TCT CTA TTA TTC CT 3′) that produced a 572-bp fragment. The second set of forward primer (F5, 5′GCA GAG GAA GAA CGA GAAG 3′) with the same reverse primer amplified a 135 bp product. Viral load in the serum samples was determined by qPCR targeting the E3 structural protein region using the standard curve method. One step qRT-PCR was performed in 25 µl reaction mixture containing 5 µl RNA, 12.5 µl TaqMan one-step RT-PCR 2X Master Mix, 1 µl 40X (RT + RNAasin) (Applied Biosystems, USA) each 1 µl sense (µM), 1 µl anti-sense (µM) primer and 1 µl TaqMan probe. qRT-PCR was performed in a 96-well format using 7300 real time PCR system and SDS software V 1.0.2 (Applied Biosystems). The amplification programme included: reverse transcription at 48°C for 30 min, initial denaturation at 95°C for 10 min, and 50 cycles of denaturation (95°C for 15 sec) and annealing and extension (60°C for 1 min)[5–7]. Statistical analysis was carried out using software PASW ver. 18. All experiments were repeated three times independently.

CHIKV remained infective for 6 months in human serum and in mosquitoes at –20°C and –80°C. However, at 37°C and 4°C, CHIKV remained infective for 2 days and 4 weeks respectively (Figure 1). RT-PCR studies detected stable CHIKV RNA in sub-zero temperature samples for six months while at 37°C and 4°C, RNA detection was possible only for 4 and 18 weeks respectively (Figure 2). On the contrary, qRT-PCR could detect CHIKV-RNA in samples for at least six months irrespective of their storage temperatures barring a slight reduction in RNA copies in samples stored at higher temperatures (Figure 1). The overall mean log10 viral load with standard deviation was found to be 5.65 ± 1.6, 8.50 ± 1.7, 9.45 ± 0.3 and 9.54 ± 0.2 for 37°C, 4°C, –20°C and –80°C respectively. The paired-*t* test showed no significant difference in mean log10 viral load for –20°C and –80°C temperature samples while significant decrease was detected in mean log10 viral load of 4°C and 37°C. The weekly viral load for –20°C and –80°C samples was not found statistically different. On the other hand, the mean log10 viral load for 4°C and 37°C showed significant decrease from the 13th and second week onwards. In parenterally inoculated mosquitoes, infectivity was lost within one week of storage at 37°C while CHIKV RNA was detected by qRT-PCR for six months. This is in contrast to earlier studies conducted with RT-PCR where RNA persistence was demonstrated only up to the 12th week in samples stored at 28°C (ref. 8).

Stability of a virus in serum is presumably related to its envelope, which protects the viral genome from dehydration and drying[9]. It is influenced by several biological and environmental factors, viz. virus concentration, temperature, humidity and the maintenance medium. The rate at which infectivity is lost is an important determinant of the potential risk of transmission. High temperatures adversely affect viral persistence due to several mechanisms, i.e. protein denaturation, RNA damage and influence on microbial or enzymatic activity[10]. Documented data shows that viral RNA could be maintained in serum or plasma at various temperatures for prolonged periods for HIV, HCV, HBV, influenza, etc.[11–14].

CHIKV is vectored by *Aedes aegypti* and *Aedes albopictus* mosquitoes and it has been shown that virus contaminated blood on mosquito proboscis-induced infection in mice even after 8 h (ref. 15). Since both the species feed during day time, it is imperative that mechanical transmission plays an important role during outbreaks of CHIKV contributing to explosive outbreaks. In the present study,

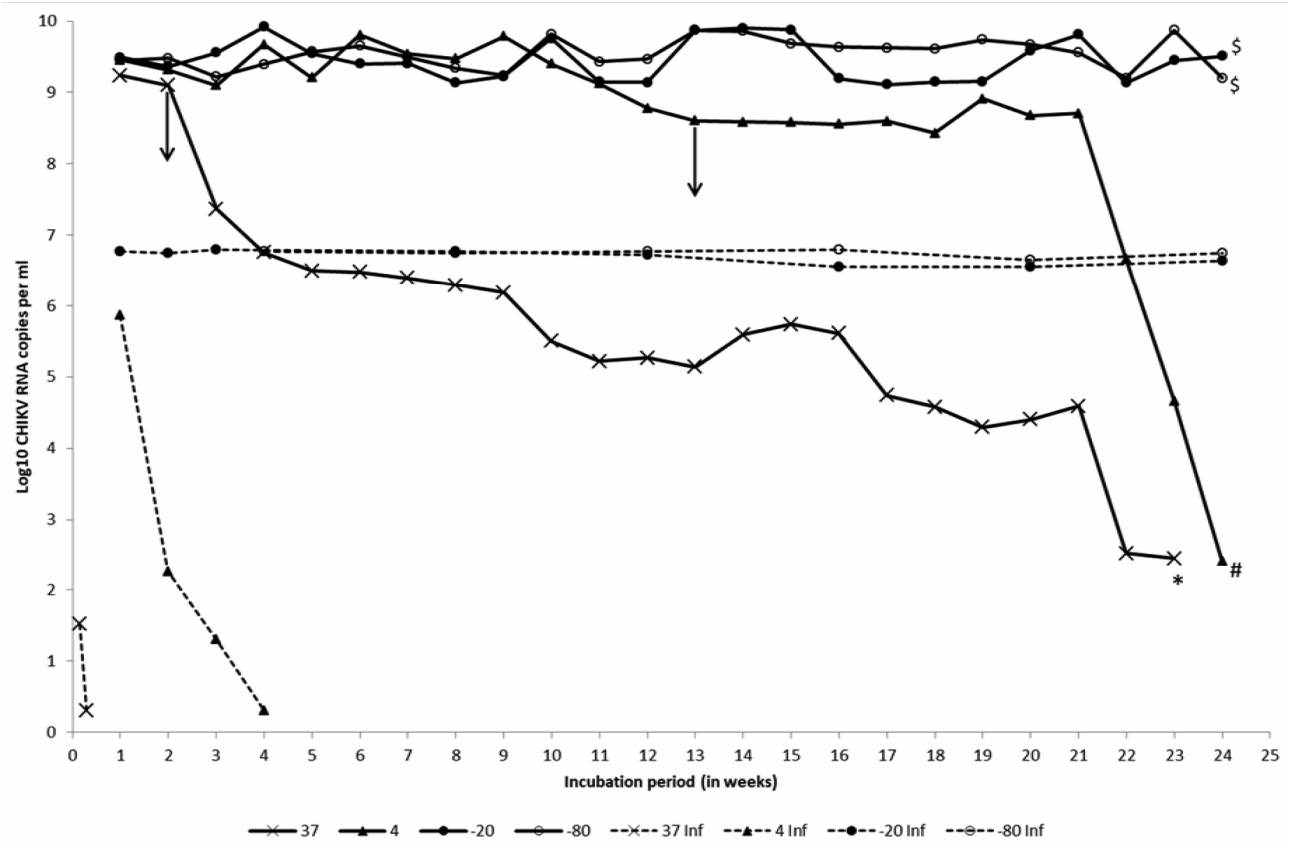

Figure 1. Quantitative RT-PCR and infectivity (Inf) data showing persistence of CHIKV RNA in serum samples stored at different temperatures (in °C). Paired *t* test showed no significant difference between overall mean log10 viral load for –20°C and –80°C temperatures whereas there was significant decrease in mean log10 viral load of 4°C, 37°C as compared to both the subzero temperatures. The weekly mean log10 viral load for 4°C and 37°C showed significant decrease from 13th and second week onwards respectively as compared to both the sub-zero temperatures, as indicated by downward arrows (pairwise comparison of weekly means for different temperature settings by *t* test). $, *, #, Same symbol indicates 'No significant difference' while different symbol indicates 'Significant difference' between the overall mean log10 viral load.

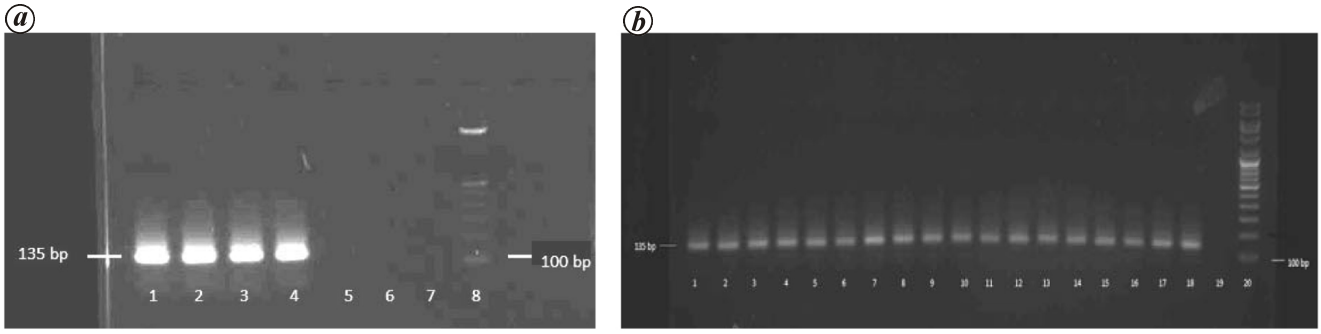

Figure 2. ***a***, RT-PCR studies showing persistence of CHIKV RNA up to 4 weeks in serum samples stored at 37°C. Lanes 1 to 4, Samples from weeks 1 to 4 showing positive results; Lanes 5 to 7, Samples from weeks 5 to 7 showing negative results; Lane 8, Molecular marker. ***b***, RT-PCR studies showing persistence of CHIKV RNA up to 18 weeks in serum samples stored at 4°C. Lanes 1–18, Samples from weeks 1 to 18 showing positive results; Lane 19, Sample of week 19 showing negative results; Lane 20: Molecular marker.

our focus was based on this hypothesis to determine the survivability and persistence of CHIKV RNA in contaminated blood and mosquito specimens. Our studies show that CHIKV remained viable for a substantial period of time even at 37°C in blood samples posing a threat to clinicians and laboratory personnel who handle patients and clinical samples. The results point towards the need to decontaminate needles and other sharps used for invasive procedures.

Recent advancements have significantly improved the precision and reproducibility of quantitative viral DNA/RNA detection in serum, plasma and other clinical samples[16]. Accurate viral load determination is important because it provides an indirect means of viral replication *in vivo* and can help predict an individual's response to therapy/clinical outcome. Quantitative PCR is a precise and sensitive method which measures viral genomes more effectively than

other molecular tools[6]. In the present study, we have successfully demonstrated the superiority of qRT-PCR over RT-PCR by detecting CHIKV-RNA in dead mosquitoes and serum samples stored at higher temperatures for longer period. QRT-PCR could find application in diagnosis of CHIKV during outbreaks as well as field based surveillance of CHIKV in mosquitoes/blood samples. This will be a boon for resource constraint countries where cold chain maintenance is a concern. Our findings have strongly shown that CHIKV remained viable for a substantial period of time even at 37°C in blood samples posing a threat to clinicians and laboratory personnel who handle patients and clinical samples.

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

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ACKNOWLEDGEMENTS. The authors acknowledge financial support provided by the Indian Council of Medical Research, Ministry of Health and Family Welfare, Government of India.

Received 29 January 2018; revised accepted 20 April 2018

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Total electron content and epicentral distance of 2015 M_w 7.8 Nepal earthquake revealed by continuous observations data

A large magnitude (M_w 7.8) earthquake occurred on 25 April 2015 (06:11 UTC) at 28.1473°N and 84.7079°E, 34 km east-southeast of Lamjung, Nepal. The devastating event was accompanied by two large aftershocks of M_w 6.6 (on 25 April 2015, 06:45 UTC) and M_w 6.7 (on 26 April 2015 at 09:10 UTC). According to the USGS earthquake catalogue, 65 aftershocks were recorded within a period of three days from the main event; the strongest aftershock had occurred on 12 May 2015 at 07:05 UTC.

Here we report the ionosphere total electron content (TEC) anomaly prior to the main shock on 25 April 2015, observed from the data of 14 Global Navigation Satellite System (GNSS) stations (plate boundary observatories) at Nepal maintained by UNAVCO, USA. Ionosphere TEC and its deviation from the average concentration have been studied for a number of earthquakes worldwide using GNSS data. These studies are limited to statistical analysis for TEC variations prior to events from GNSS observations close to the epicentre, or at least falling within the earthquake preparatory zone[1,2]. In this study, data from 14 continuously operating stations were used in the estimation of vertical TEC using the program GPS-TEC[3–5]. Time-series analysis of TEC for a period of 30 days was carried out and anomalies were detected using 15 days running average plus/minus two times 15 days running standard deviation. The TEC values crossing these limits were considered as anomalies. The TEC values corresponding to the anomaly time for all 14 stations were detected and interpolated to plot in two-dimensions to observe the TEC spatial pattern (Figure 1).

The link between ionospheric TEC anomalies and earthquake occurrences has been reported in many studies. This is basically governed by the lithosphere–atmosphere–ionosphere coupling mechanism. The ionosphere records the earthquake due to change in global electric circuit produced by the cluster of ions in the atmosphere emanating due to the development of stress in the crustal region prior to an earthquake[6,7].

The TEC time-series analysis was carried out using GNSS observation stations located at 30–300 km areal distance from the epicentre of the 2015 M_w 7.8 Gorkha earthquake. Analysis with 15 days mean ± 2 standard deviation limit, reveals negative (low) TEC anomaly on 11 April 2015, whereas positive (high) anomaly on 24 April 2015 was measured at station BESI (Besihari, Lumjung) located at 30 km distance from the