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Short Communication

Prevalence and Upcoming Threats of Chikungunya Virus in Pakistani Population

Samia Afzal*, Sadia Zahid, Iram Amin, Muhammad Shahid and Muhammad Idrees

Division of Molecular Virology, National Centre of Excellence in Molecular Biology, 87-West Canal Bank Road Thokar Niaz Baig, Lahore-53700, University of the Punjab, Lahore, Pakistan

Samia Afzal and Sadia Zahid authors contributed equally.

ABSTRACT

Chikungunya virus is *Aedes* mosquitoe transmitted virus that has typical signs and symptoms of high fever, headache, hemorrhage and body rashes. Although in the past decade millions of people in several continents like Asia, Africa and some islands of Indian Ocean have faced the major outbreaks of Chikungunya virus because of its travel associated febrile nature. An inexplicable paralyzing disease has trapped thousands of people in Karachi region of Pakistan and the symptomatology among suspected cases was compatible with Chikungunya fever. Thus Chikungunya outbreak was reported in Pakistan but actually the recent reports about the occurrence of Chikungunya outbreak in the southern region of Pakistan has been highly controversial. In order to further confirm the circulation of the virus and put an end to the speculative claims for the corresponding outbreak in Pakistan, a study was conducted at the Center of Excellence in Molecular Biology in Lahore. The study included 500 suspected serum samples. All clinical specimens were tested for IgM and IgG specific antibodies against Chikungunya virus using a commercial ELISA kit. Twenty seven (5.4%) and thirty five (7%) samples were IgM and IgG positive, respectively. All antibody positive samples and a subset of negative samples (195) were further subjected to PCR for confirmatory purposes. Two different sets of primers were used for the identification of the viral genome. Thirty samples were found PCR positive and a subset of ten samples was direct sequenced. Sequence analysis showed 94% similarity to *Stenotrophomonas maltophilia* which is considered to be the second most prevalent bacterial species in mosquitoe's midgut. This observation may lead towards the confusion that the outbreak is either of Dengue or Chikunguna virus. In conclusion, confirmatory molecular characterization of the viral genome remains controversial and further studies are needed in this respect.

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Authors' Contribution
SA and MI conceived the idea of study and its design. SZ and SA performed the practical work and analyzed the results. IA and MS participated in the sample collection and helped in manuscript writing.

Key words
Chikungunya virus, Aedes mosquitoes, Outbreak, Pakistan, Controversy

Chikungunya virus (CHKV) is *Aedes* mosquitoe transmitted virus that has affected millions of people in several continents like Asia, Africa and some islands of Indian Ocean in the past decade and they have faced major outbreaks of Chikungunya virus (Gallian *et al*., 2017). Chikungunya virus is transmitted primarily by bite of *Aedes aegypti* (yellow fever mosquito) and *Aedes albopictus*mosquitoes (tiger mosquito) (San-Ho *et al*., 2010). CHIKV produces illness in humans that are characterized by headache, fever, nausea, fatigue, rash and severe arthralgia. These symptoms are limited to 1-10 days but arthralgia may last for few weeks to several months. These clinical symptoms may mimic to malaria and dengue fever and therefore chikungunya fever sometimes misdiagnosed as dengue fever (Shahid *et al*., 2019). Until now, no vaccine or anti-viral treatment is available against CHIKV infection, but anti-inflammatory and analgesics medicines are given to patients to reduce pain and swelling (Powers and Logue, 2007; San-Ho *et al*., 2010).

Chikungunya virus (CHIKV) belongs to the genus alphavirus, and family Togaviridae. CHIKV was first identified in Tanzania in 1952-1953 from blood of a febrile patient (Staples and Fischer, 2014). Phylogenetic analysis based on E1 gene has classified it into three major CHIKV genotypes: West African, East/Central/ South African (ECSA), and Asian genotypes. CHIKV has caused sporadic outbreaks in west, central and southern Africa and many regions of world (Powers *et al*., 2000). Virus remains inactive over years during inter epidemic periods and CHIKV maintains itself during this period in a sylvatic cycle in non-human primates (Diallo *et al*., 1999; Powers *et al*., 2000). CHIKV is a single-stranded, positive sense RNA virus, its genome size about 11.8 kb consisting of two open reading frames. Its genome has non-structural

* Corresponding author: samiaraza@live.com
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proteins: nsP1-nsP2-nsP3-nsP4 and structural polyproteins C-E3-E2-6K-E1- poly (A) 3`. Function of nsP1 is RNA-capping enzyme, nsP2 has protease, triphosphatase, NTPase and helicase activities, specific function of nsP3's is currently unknown and nsP4 contains RNA dependant RNA polymerase activity. The capsid protein (C) forms the nucleocapsid that enclosed the viral RNA and E1, E2 as well as E3 proteins encode envelope of the virus (Knipe *et al*., 2001).

In September 2016, an inexplicable paralyzing disease has trapped thousands of people in Pakistan, especially in Malir Karachi region and the symptomatology among suspected cases was compatible with Chikungunya fever. Because of travel associated febrile nature of Chikungunya virus, NIH Pakistan issued an alert to the government after the recent outbreak hit the India in 2016 (Mallhi *et al*., 2017). On the basis of media reports, Chikungunya outbreak was reported in Pakistan but actually the recent reports about the occurrence of disease in the southern region of Pakistan has been highly controversial. The purpose of the current study was to confirm the reality of Chikungunya outbreak in Pakistan on molecular basis.

Materials and methods

The study included 500 suspected serum samples collected by the Institute of Public Health (IPH) and Genome centre for Molecular based research and Diagnostic (GCMD). All clinical specimens were tested for IgM and IgG specific antibodies against Chikungunya virus using a commercial ELISA kit (Euroimmun, Luebeck, Germany). All antibody positive samples were further subjected to RT-PCR for confirmatory purposes.

Viral RNA was extracted from serum using Viral Nucleic Acid extraction Kit (Favorgen Biotech Corporation, Australia). cDNA was synthesized from RNA of Chikungunya virus in 30 cycles using 10 µl of RNA with a reaction mixture of 10 µl containing 4 µl 5X first strand buffer (FSB), 0.5 µl 0.1 M Dithiothreitol (DTT), 2 µl 10 mM dNTPs, 1 µl 20 pM antisense primers, 1.3 µl dH2O, 0.2 µl RNA inhibitor (RMI) and 1 µl of Moloney Murine Leukiemia Virus (MMLV) Reverse Transcriptase (RTse) (Invitrogen Biotechnology, USA). The 20 µl reaction mixture was incubated at 37°C for 50 min. followed by 2 min. heat inactivation of M-MLV at 95°C. The samples were then incubated for 2 min. at 22°C.

For PCR amplification, two different sets of primers were used for the identification of the viral genome by using already reported primer sequences for Chikungunya detection. First primer set is of E2 region with product size of (1st round 427bp, 2nd round 172bp) (Pfeffer *et al*., 2002) and second primer set is of NSP1 region with product size 239bp (Kosasih *et al*., 2013) as shown in Table I.

Complementary DNA (cDNA) was used as template for amplification, 2µl of cDNA with 8 µl of master mix. PCR mix was made by mixing 1 µl 10X PCR buffer (with ammonium sulphate), 2.4µl MgCl2, 1 µl 500 µM dNTPs, 1 µl pM forward and reverse primer each, 2.4 µl d H2O and 0.2 µl of 5 U of Taq DNA polymerase (Invitrogen Biotechnology, USA). The thermal profile for first round using outer sense and antisense was initial denaturation at 94ºC for 2 min followed by 30 cycles of denaturation at 94ºC for 45 sec, annealing at 64ºC for 35 sec, extension at 72ºC for 2 min and final extension at 72ºC for 10 min. Same thermal profile was used for second round using type specific primers. Only the annealing was done at 56ºC for 45 sec. in 30 cycles. The PCR product was visualized by2% agarose gel, stained with ethidium bromide, visualized under UV light.

A subset of 10 samples was directed to sequencing of chikungunya virus isolates. Briefly, the PCR product was run on 1.2% agarose gel. The specific bands were excised and purified using the QIA quick Gel Extraction Kit (Qiagen, Germany). This gel purified amplicons were used for sequencing PCR reactions. Sequencing PCR products analysed on automated genetic analyzer according to the manufacturer's instructions (Big Dye Deoxy Terminators; Applied Biosystems, Weiterstadt, Germany). The identity of the sequences was confirmed by *Basic Local Alignment Search Tool* (BLAST). Sequences that were unique (obtained for the first time) were submitted to GenBank Data Base. The BioEdit v7.0.5 software was used for the translation, the alignment of amino acid and protein sequences.

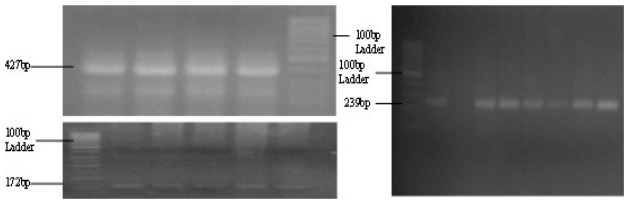

Fig. 1. PCR amplification of E2 region through nested PCR (1st Round 427bp, 2nd Round 172bp) and NSP1 region of 239bp in Chikungunya virus.

Results and discussion

Total 500 suspected enrolled samples were used for antibodies detection. Results indicated that 27(5.4%) out of 500 and 35(7%) out of 500 samples were IgM and IgG positive, respectively. These 62 IgM and IgG positive samples were further confirmed by RT-PCR analysis. Out of these 62 samples, 30 were found RT-PCR positive. Results were confirmed by two different sets of primers with product size 172bp for first primer set and 239bp for second primer set (Fig . 1). 10 randomly selected samples

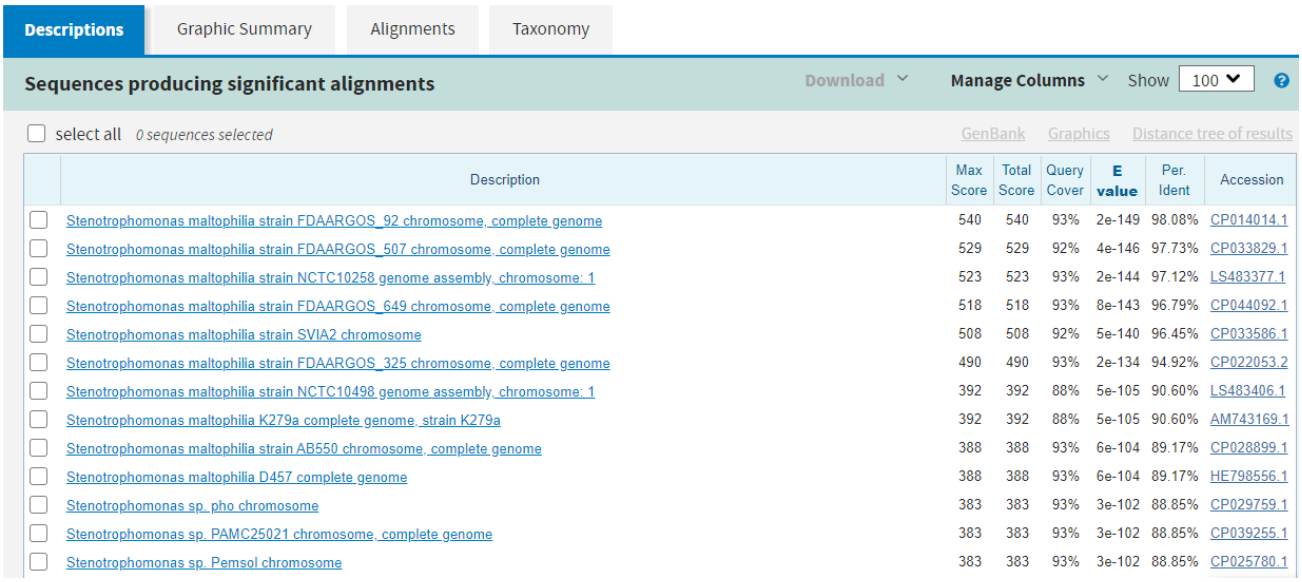

Fig. 2. Sequence homology results showing homology with *Stenotrophomonas maltophilia.*

Table I. Primer sets used for the detection of Chikengunya virus.

Primer sets	Primer name	5'-3' sequence	Product size
1	CHIK-F1	TAATGCTGAACTCGGGGACC	427bp
	CHIK-R1	ACCTGCCACACCCACCATCGAC	
	CHIK-F2	GATCAGGTTAACCGTGCCGACT	172bp
	CHIK-R2	CACTGACACAACTACCACAGTCA	
2	ChikD-F1	GCAGACGCAGAGAGGGCCAG	239bp
	ChikD-R1	CGTGCTGCAAGGTAGTTCTC	
	ChikD-F1	GCTATTTGTAAGAACGTCAG	
	ChikD-R2	TACCGTGCTGCGGTCGGGAA	

were used for genomic sequence analysis of Chikungunya virus and the sequence analysis amazed that it showed 94% similarity to *Stenotrophomonas maltophilia* (Fig . 2) instead of Chikungunya virus.

Previous reported studies showed that Chikungunya infections have normally been connected with the outbreaks (Pulmanausahakul *et al*., 2011) and it has also been observed that sometimes unreported infections being the main cause of an outbreak, as in Malaysia in 2006 (Abu-Bakar *et al*., 2007). The major reason behind the unreported cases of chikungunya is its similarity of clinical symptoms with other infections, like dengue (Lee *et al*., 2012). The rapid diagnostic tests available in most laboratories may sometimes complicate the process of correct diagnosis of Chikungunya, as in the case of IgM antibodies which remain importunate for long time in the patient's blood (Kosasih *et al*., 2012). On the other hand, RT-PCR is an advanced diagnostic test, but it is only available in some state of the art laboratories and sequencing facilities are only present in research institutes.

From our current study it is obvious that *Aedes* mosquitoe's midgut microbiota has an important role in host - pathogen interaction especially the *Stenotrophomonas maltophilia*. This bacteria *Stenotrophomonas maltophilia* is considered to be the second most prevalent bacterial species in mosquitoe's midgut and enhances the vector competency and disease transmission like dengue and Chikungunya fevers. One of our interesting finding is the observation of *Stenotrophomonas maltophilia* bacteria, may leads towards the confusion that the outbreak is either of Dengue or Chikunguna virus (Yadav *et al*., 2015).

Conclusion

In conclusion, our results showed that serological

evidence of acute Chikungunya cases supports the occurrence of clusters of Chikungunya fever in the region, but confirmatory molecular characterization of the viral genome remains controversial. Our findings emphasize on the requirement of affordable and susceptible diagnostic tests for early and accurate diagnosis of Chikungunya virus and in this regard, further vigilant studies are required.

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Statemet of conflict of interest

The authors have declared that they do not have any conflict of interest.

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