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Genetic divergence of Chikungunya virus plaque variants from the Comoros Island (2005)

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Abstract Chikungunya virus (CHIKV) from a human sample collected during the 2005 Chikungunya outbreak in the Comoros Island, showed distinct and reproducible large (L2) and small (S7) plaques which were characterized in this study. The parent strain and plaque variants were analysed by in vitro growth kinetics in different cell lines and their genetic similarity assessed by whole genome sequencing, comparative sequence alignment and phylogenetic analysis. *In vitro* growth kinetic assays showed similar growth patterns of both plaque variants in Vero cells but higher viral titres of S7 compared to L2 in C6/36 cells. Amino acids (AA) alignments of the CHIKV plaque variants and S27 African prototype strain, showed 30 AA changes in the non-structural proteins (nsP) and 22 AA changes in the structural proteins. Between L2 and S7, only two AAs differences were observed. A missense substitution (C642Y) of L2 in the nsP2, involving a conservative AA substitution and a nonsense substitution (R524X) of S7 in the nsP3, which has been shown to enhance O'nyong-nyong virus infectivity and dissemination in *Anopheles* mosquitoes. The phenotypic difference observed in plaque size could be attributed to one of these AA substitutions. Phylogenetic analysis showed that the parent strain and its variants clustered closely together with each other and with Indian Ocean CHIKV strains indicating circulation of isolates with close evolutionary relatedness in the same outbreak. These observations pave way for important functional studies to understand the significance of the identified genetic changes in virulence and viral transmission in mosquito and mammalian hosts.

Keywords Chikungunya virus · Variants · Genetic variation · Comoros Island

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Introduction

Chikungunya virus (CHIKV) is a single-stranded, positive sense RNA which belongs to the genus *Alphavirus*, in the family *Togaviridae.* Chikungunya (CHIK) is an arboviral disease that is transmitted mainly by *Aedes* mosquitoes. Due to the ubiquitous distribution of the CHIKV mosquito vector, emergence of new strains with new virulence features has become a global concern. A CHIK outbreak emerged in Lamu, Kenya in 2004 [1], in Comoros Island in 2005 [2] and in 2005–2006, the outbreak spread to other islands in the Indian Ocean including Madagascar, Seychelles, Reunion, Mauritius, and Maldives [3]. The epidemic also spread to India and subsequently to Europe, the United States, South Asia and Southeast Asian countries, where it was imported by infected travellers from India and affected Indian Ocean Islands [4–6]. In Reunion Island, the CHIKV was more virulent than in previous CHIK outbreaks [7]. A novel mutation in the Reunion Island resulted in a strain of CHIKV (E1:A226V) that could be easily transmitted by *Aedes albopictus* [7, 8]. Emergent virus strains with different growth phenotypes can be identified by the appearance of plaque variants from mosquito or human virus isolates. Plaque variants which represent mixed virus populations have been reported in CHIKV [4], Sindbis virus [9], Guaroa virus [10], Bunyamwera, and Ngari viruses [11]. During routine culture and titration of CHIKV (Strain: Com5) from 2005 outbreak in the Comoros Island, two distinct plaque sizes were observed from one isolate. The CHIKV isolate was from the serum of a febrile patient from the Grande Comore Island, within the Comoros Island archipelago during 2005 CHIK outbreak. This led to further investigation to determine if the phenotype in these CHIKV variants could be correlated with genetic changes.

Results and discussion

To examine the phenotype and genetic differences associated with the plaque variants, the different plaques observed were isolated by plaque purification using Vero cells (African green monkey kidney derived cell line, ATCC, CCL81). Out of 10 clones of large plaques and 8 clones of small plaques, L2 and S7 variants were selected from each group for further analysis because they exhibited stable plaque sizes. Plaque assay of Com5 on Vero cells produced distinct and reproducible plaques of two sizes,

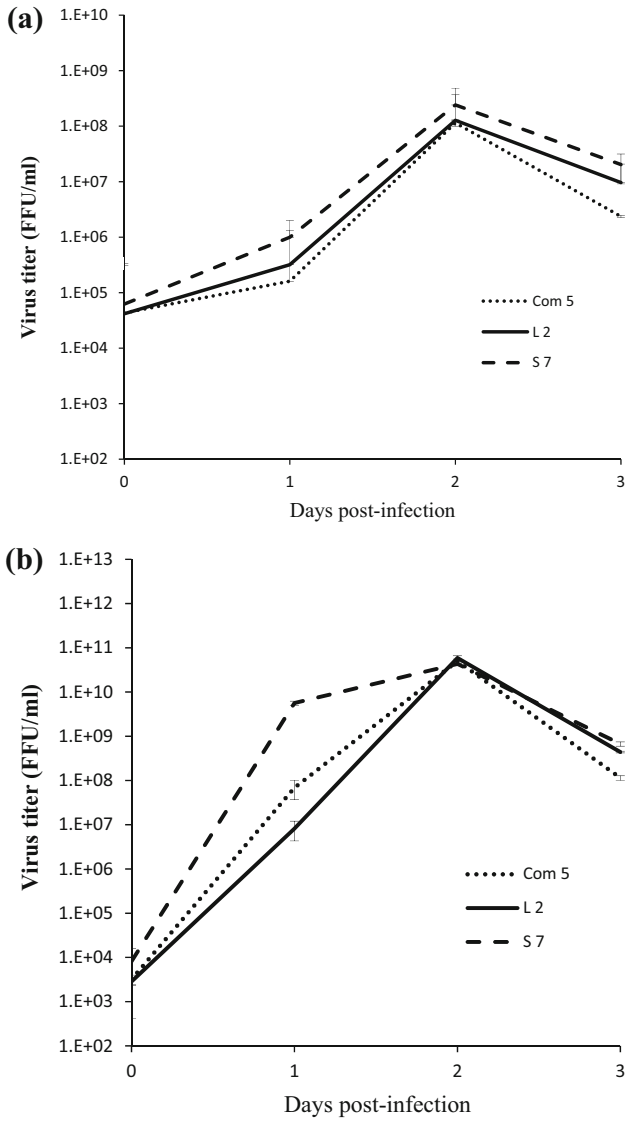

Fig. 1 **a** In vitro growth kinetics of the original CHIKV isolate (Com5) and the two plaque variants (L2 and S7) cultured in Vero, African green monkey kidney derived cells. Equal viral titre of each CHIKV strains was inoculated and cultured for 3 days and viral titres quantified by focus assay. Com5 (*dot*) = original isolate, L2 (*solid*) = large plaque variant and S7 (*long dash*) = small plaque variant. The data indicate an average of three independent experiments ± standard deviation. The difference in viral titre between the Com5, L2 and S7 was not significant at all time points. ($p > 0.05$). **b** In vitro growth kinetics of the original CHIKV isolate (Com5) and the two plaque variants (L2 and S7) cultured on C6/36, *Aedes albopictus* derived cells. Equal viral titre of each CHIKV strains was inoculated and cultured for 3 days and viral titres quantified by focus assay. Com5 (*dot*) = original isolate, L2 (*solid*) = large plaque variant and S7 (*long dash*) = small plaque variant. The data indicate an average of three independent experiments ± standard deviation. The difference in viral titre between the Com5 and the plaque variants (L2 and S7) at 1 DP1 was significant ($p < 0.05$). S7 had the highest viral titre throughout the experimental period

1.5 ± 0.3 mm (L2) and 0.5 ± 0.3 mm (S7) in diameter (Fig. S1). The small plaques predominated in the Com5 virus population, representing 68 % of all plaques.

To examine differences in the growth kinetics of the parent strain and plaque variants, confluent monolayers of C6/36 (mosquito, *Aedes albopictus* derived) cells (28 °C) and Vero cells (37 °C) were inoculated with virus infected culture fluid (ICF) containing equal titres of Com5, L2 and S7 at a multiplicity of infection of 1. One millilitre of ICF was collected from the culture flask at 0 day post infection (DPI) and every 24 h for 3 days and CHIKV titre determined by focus assay [12]. Three independent growth experiments were performed for each isolate. When in vitro growth kinetics of Com5, L2 and S7 were examined, S7 had the highest viral titre in Vero cells (Fig. 1a) and in C6/36 cells at 1-2 DPI (Fig. 1b). The difference in viral titre between Com5, L2 and S7 was insignificant ($p > 0.05$) in Vero cells but significant ($p < 0.05$) at 1 DPI in C6/36 cells. All the three virus isolates had higher viral titres in C6/36 than in Vero cells ($p = 0.03$). The significant difference in viral growth rate among the parent strain and plaque variants in C6/36 cells agrees with previous findings [13–15]. S7 had a slightly higher viral titre in both cell lines, contrary to other studies where the large plaque variants typically replicates faster and to a higher viral titre than small plaques [15]. This suggests that S7 has a growth advantage than L2 in both cell lines.

The genomes of Com5, L2 and S7 were sequenced to assess if any genetic changes could be responsible for the observed plaque size difference between the CHIKV variants, this was done using the 454 Genome sequencer (Roche Branford, CT, USA) according to manufacturer's instructions. The partial genomic sequences excluding the 3′ and 5′ UTR region were obtained and deposited in Genbank with the following accession numbers: Com5 (KP702297), L2(KF283986) and S7 (KF283987) [http://blast.ncbi.nlm.nih.gov/Blast.cgi]. When the RNA sequences were aligned, the overlapping regions of the two plaque variants were 99.93 % similar. Phylogenetic analysis was performed using nucleotide sequences of L2 and S7 and other complete genome sequences of CHIKV strains available in the GenBank. L2 and S7 clustered closely with ComJ, Indian Ocean Islands, India and Sri Lanka CHIKV isolates and they all belong to the East Central South African (ECSA) genotype (Fig. S2), reflecting a close genetic and geographic relationship of strains in the same outbreak.

Alignment of amino acid (AA) sequences of L2 and S7 using S27 prototype strain as reference showed that these variants were identical except for two AA substitutions. The first was a missense AA substitution at position 642 located in the nsP2 region where L2 had a Tyrosine (Y642) and S7 a Cysteine (C642) (Table 1). When we examined this substitution in Kenyan and other Indian Ocean CHIKV

Table 1 Comparisons of amino acid sequences of the non-structural proteins of Com5, L2 and S7 and selected CHIKV Indian Ocean isolates using S27 prototype strain as a reference sequence

Non-structural protein	nsP1								nsP2					nsP3												nsP4						
Polypeptide position	171	172	234	383	384	481	488	507	589	909	**1177**	1178	1328	1508	1550	1659	1664	1670	1685	1709	1715	1794	1795	1804	**1857**	1938	2117	2363	2377	2418	2467
Amino acid position	171	172	234	383	384	481	488	507	54	374	**642**	643	793	175	217	326	331	337	352	376	382	461	462	471	**524**	75	254	500	514	555	604
Tanzania/S27/53	R	L	E	M	I	T	Q	L	S	H	**C**	S	A	V	Y	P	V	T	K	I	A	L	S	P	**R**	T	T	Q	I	V	V
Kenya/Lamu33/04 Kenya/KPA15/04 Comoros/Com 25/05 Comoros/Com 125/05 Reunion/LR-2006/06 India/DRDE-06 /06 India/DRDE-07/07	R	V	K	L	L	I	R	R	N	Y	**Y**	N	V	I	H	S	A	I	E	T	T	P	N	S	**X**	A	A	L	T	I	I
Comoros/Com J/05 **Comoros /L2/05**	Q	–	–	–	–	–	–	–	–	–	**–**	–	–	–	–	–	V	–	–	–	–	–	–	–	**R**	–	–	–	–	–	–
Comoros /S7/05	Q	–	–	–	–	–	–	–	–	–	**C**	–	–	–	–	–	–	–	–	–	–	–	–	–	**–**	–	–	–	–	–	–
Sri-Lanka/SL-CKI/07	Q	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	**–**	–	–	–	–	–	–

Isolates and the two amino acid substitutions of the present study are indicated in bold. (–) denotes similarity to amino acids of the Lamu33 strain at corresponding positions

Table 2 Comparisons of amino acid sequences of structural proteins of Com5, L2 and S7 and selected CHIKV Indian Ocean isolates using S27 prototype strain as a reference sequence

Structural protein	C	E3	E2															6K		E1		
Polypeptide position	63	284	382	399	404	485	489	506	519	536	592	624	637	669	700	711	756	802	1035	1078	1093
Amino acid position	63	23	57	74	79	160	164	181	194	211	267	299	312	344	375	386	8	54	226	269	284
Tanzania/S27/53	K	I	G	I	G	N	A	L	S	I	M	S	T	A	S	V	V	I	A	M	D
Kenya/Lamu33/04 Kenya/KPA15/04 **Comoros/L2/05** **Comoros /S7/05** Comoros /Com J/05 Comoros /Com 25/05 Comoros/Com 125/05 India/DRDE-06 /06 Sri-Lanka/SL-CKI/07	R	T	K	M	E	T	T	M	G	T	R	N	M	T	T	A	I	V	A	V	E
Reunion/LR-2006/06 India/DRDE-07/07	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	**V**	–	–

Plaque variants of the present study and other CHIKV isolates are indicated in bold and are named in this order: country of origin/strain name/year of sample collection. (–) denotes similarity to amino acids of the Lamu33 strain at corresponding positions. Selected isolates are Indian Ocean outbreak isolates that occurred before and after the Comoros Island isolates that are under study. Accession numbers of the selected isolates shown in Table 2 are S27 (AF369024.2), Lamu33 (HQ456255.1), KPA15 (HQ456254.1), Com J (HQ456252.1), COM25 (HQ456251.1), COM125 (HQ456253.1), DRDE-06 (EF210157.2), SL-CKI(HM045801), LR2006-OPYI (DQ443544.2), DRDE-07 (EU372006.1)

isolates from the same outbreak, the Cysteine (C642) was observed in S7 and S27 prototype strain only and not in any other isolate. Asparagine has also been observed in the same position, in 06.27 strain (accession number AM258993.1) from the CHIKV outbreak in Reunion Island in 2005 [16]. This AA substitution is located in a region that codes for the nsP2 protease, but outside the active site (478–480) of the enzyme. The three AAs observed at this position represent conservative mutations unlikely to affect enzyme function.

In the second AA substitution at position 524 in the nsP3 region, L2 had an Arginine (R524) and S7 a stop codon (X524). Arginine (R524) was unique to L2 and the S27 prototype strain and the stop codon (CGA)/(X524)) was present in all other isolates (Table 1). The opal stop codon is located between the nsP3 and nsP4. The nsP3 is essential for minus strand and sub-genomic 26S mRNA synthesis while nsP4 codes for RNA-dependent RNA polymerase of alphaviruses and its expression is tightly regulated by a read-through stop codon at the end of nsP3 [17]. The presence of a stop codon upstream of nsP4 in O'nyong-nyong virus (ONNV) genome causes more efficient infection and earlier dissemination of the virus in its vector *Anopheles gambiae* mosquitoes, relative to viruses encoding an Arginine (R524) at the same position [18], therefore, we hypothesize that the opal stop codon could improve Chikungunya virus infectivity in its vector *Aedes* mosquito. CHIKV and ONNV are both alphaviruses in the Semliki Forest antigenic complex [19], are antigenically very closely related and have different mosquito vectors. The E1: A226V mutation was present in the Reunion Island isolate and one Indian (DRDE-07) isolate (Table 2). However, the variants in this study did not have this mutation.

To monitor CHIKV evolution overtime, whole genome alignment of AA sequences of the plaque variants using the S27 strain as the reference was done. In total, 30 AA changes in the nsP sequences (Table 1) and 22 AA changes in the structural protein (Table 2) were observed. Notably, within the Comoros Island, 2 variants were circulating (nsP1:R171 and Q171) around the same period. Arginine (R) was present in other Comoros Island isolates (Com 25 and COM125 strains), S27 strain and the Indian Ocean CHIK outbreaks between 2004 and 2006, while, Glutamine (Q) was present in the Com5, L2, S7 and Com J. This substitution (R171Q) was also observed in a traveller diagnosed with CHIK returning to Japan from Sri Lanka in 2006 [15]. Since the Comoros Island isolates (L2, S7, Com5 and ComJ) and Sri Lankan isolates (strains: SL11131 and SL-CKI) share two AA substitutions (171Q and A226) and belong to the ECSA, it is likely that these isolates originated from Kenya (Lamu33), then spread to Comoros Island, India and then Sri Lanka in 2005–2006 (Fig. S2), unlike the majority of other strains which could have spread from Kenya (KPA15-Mombasa), then to Comoros Island, Reunion, Mauritius, Seychelles in 2005–2006, India and Sri Lanka in 2006 [20]. It is possible that different CHIKV variants could have

spread to Sri Lanka, some with R171 and 226V and others with 171Q and A226 AA substitutions through different pathways.

In conclusion, since the complete genome sequences of plaque variants were not obtained in this study, we cannot eliminate the potential role that 3′ and 5′ UTR region could also play in gene regulation. Given that the E1:A226V mutation allowed better adaptation of CHIKV to *Aedes albopictus* in a previous study (10), we recommend further research on the functional significance of the AA substitutions observed in this study by reverse genetics, so as to correlate the AA changes and the biological characteristics on virulence in mammalian hosts and mosquito species, to allow for monitoring of potentially virulent emergent CHIKV strains during outbreaks.

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Author contributions CW, SI contributed in the laboratory work, data analysis and drafted the manuscript. CR, GM participated in sequencing the variants and data analysis; JK and JO contributed in study design and data analysis. RS and LM contributed to study design, data analysis, overall supervision, implementation and management of the project. All authors reviewed and approved the final manuscript.

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Compliance with ethical standards

Conflict of interests The authors hereby declare that they have no competing interests.

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