Critical reappraisal of the A226V mutation in Chikungunya outbreaks: possible role in increased pathogenesis?

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SUMMARY

CHIKV is a mosquito-transmitted alphavirus responsible for the first autochthonous Italian outbreak in 2007. We previously analyzed 7 CHIKV isolates (5 imported and 2 autochthonous) with respect to the presence of A226V mutation in E1gp. All the isolates showed this mutation except the one imported from India in 2006. Since this mutation has been associated with enhanced replication and fitness in *A. albopictus* vector, we investigated the possible involvement of A226V mutation in enhanced infection capability in primate cells.

To this aim, Vero E6 and C6/36 cells were infected with two CHIKV isolates, one carrying the A226V mutation and one wild type, using single replication cycle conditions. Progeny virus was measured by both quantitative real time RT-PCR and viral infectivity assay.

No significant differences were observed between the two isolates either in terms of replication kinetic or in virus yield, on both Vero E6 and C6/36 cells. Moreover, experiment of inhibition of virus replication were performed for both isolates on Vero E6 cells using increasing amounts of recombinant IFN-alpha and virus yield was measured. A dose-dependent inhibition of virus yield for both CHIK isolates was observed, with a different sensitivity to IFN-alpha between the isolate carrying the A226V mutation and the wild type one.

Our results suggest i) that A226V mutation does not influence replication ability in both host species, when using single replication cycle conditions; ii) the differences between wild type and mutated strains may be due to different sensitivity and/or activation ability of innate immune mechanisms.

Chikungunya virus (CHIKV) is a mosquito-transmitted alphavirus belonging to *Togaviridae* family. Isolated for the first time from a Tanzanian outbreak in 1952, is geographically distributed in Africa, Asia, Indian Ocean Islands, India, (1,2,6,7,8,9,11,12). CHIKV is also responsible for several imported cases in Southern Europe, giving rise, in 2007, to the first autochthonous European outbreak in Italy (5,10). Several mutations of E1 glycoprotein are considered as molecular signatures of the Indian Ocean outbreak, particularly the A226V mutation (13). We have previously analysed 7 CHIKV isolates, 5 imported to Italy and 2 coming from the Italian outbreak, with respect to the presence of A226V mutation (3). All imported and autochthonous strains showed the A226V mutation with the exception of the isolate imported from India in 2006, suggesting that the acquisition and fixation of the A226V mutation may be a common pathway of CHIKV outbreak explosion, in a parallel interplay with the mosquito vector dynamics.

Since this mutation has been associated with enhanced replication and fitness in *A. albopictus* vector, we investigated the possible involvement of A226V mutation in enhanced pathogenesis, by testing infection capability in primate cells. Two primary CHIKV isolates, one carrying the A226V mutation (ITA1_TAM E1, named A226V) and one with wild type aminoacid (ITA4_MRA E1, named 226WT), were serially adapted on Vero E6 cells (6 passages). Then, the two virus preparations were used to infect either A. *albopictus* mosquito C6/36 and Vero E6 cells, both using single replication cycle conditions (i.e. MOI 10, Figure1A and B, respectively) and multiple replication cycle (i.e. MOI 0.01, data not shown). After 1h of adsorption, cells were treated with trypsin and extensively washed. At the indicated times post-infection, progeny virus was harvested by freezing/thawing three times the cultures. After supernatant clarification, virus yield was measured by both quantitative real time RT-PCR and viral infectivity assay. Quantitative real time RT-PCR targeting nsP1 gene was performed according to (4). Virus titration was performed on VeroE6 cells with limiting dilution assay; the results are expressed as TCID50/ml. Moreover, preliminary experiments of inhibition of virus replication by interferon-α (IFN-α) *in vitro* were performed: Vero E6 cells were treated for 24 h with recombinant IFN-α (2, 20, 200, 2.000, 20.000 UI/ml) and infected with the two CHIKV isolates at MOI 0.01. After 24 h of infection virus yield was measured by both quantitative real time RT-PCR and viral infectivity assay. Results are shown in table 1 and are expressed both as log reduction and percentage of reduction.

Figure I shows replication kinetics experiments performed on insect cells (A) and primate cells (B) using MOI 10 for both the isolates. Results are expressed as viral RNA (log copies/mL; continous lines) titers and infectivity (log 50% tissue culture infectious dose [TCID50/mL]; dotted lines).

Under single replication cycle conditions virus yield is about 10 times higher in mosquito cells (Figure IA) as compared to primate cells (Figure IB).

No significant differences are observed between the two isolates in terms of replication kinetic and virus yield on Vero E6 (Figure IB) and C6/36 cells (Figure IA) both using MOI 10 and MOI 0.01 (data not shown).

The time course curve using MOI 10 indicates that replication kinetics peak at 24h post-infection, remaining at plateau level thereafter (Figure I).The time course curve using multiple replication cycle indicates that replication kinetics peak at 48h post-infection, remaining at plateau level thereafter (data not shown).

A dose-dependent reduction of virus yield in Vero E6 cells by IFN-α, assessed by infectivity and viral RNA titration, is observed for both the isolates (Table 1).

Preliminary results show different sensitivity to IFN-α between the two isolates: the one carrying the A226V mutation seems to be more inhibited from recombinant IFN-α with respect to the wild type.

The presence of A226V mutation seems not to influence the replication kinetics in both host species, both using single and multiple replication cycle conditions.

The difference between the two isolates, one carrying the A226V mutation (A226V) and one with wild type aminoacid (226WT), in terms of virus yield, is not significant either at MOI 10 or at MOI 0.01.

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The differences between wild type and mutated strains may be due to different sensitivity and/or activation ability of innate immune mechanisms. Further characterization is in progress.

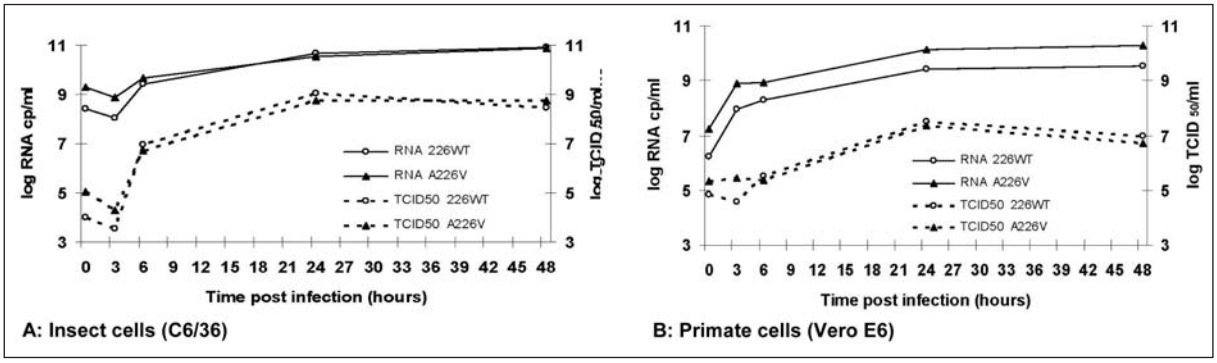

Figure 1. *Influence of A226V on CHIK replication using MOI 10*

Table 1. *Inhibition of virus replication by recombinant INF-α*

	Log reduction	% reduction	Log reduction	% reduction
IFN-α UI/ml	**RNA 226WT**	**RNA 226WT**	**TCID 50 226WT**	**TCID50 226WT**
0	0	0.00	0	0.00
2	0.2	38.48	0.3	54.81
20	0.5	69.14	0.6	75.73
200	1.9	98.64	1.7	97.86
2000	3.4	99.96	3.5	99.97
20000	4.5	100.00	5.0	100.00
IFN-α UI/ml	**RNA A226V**	**RNA A226V**	**TCID 50 A226V**	**TCID50 A226V**
0	0	0.00	0	0.00
2	0.3	47.68	0.3	51.02
20	1.4	95.95	1.3	95.10
200	2.7	99.79	2.0	99.00
2000	4.3	100.00	4.7	100.00
20000	4.6	100.00	5.5	100.00

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