High throughput profiling identified PA-L106R amino acid substitution in A(H1N1) pdm09 influenza virus that confers reduced susceptibility to baloxavir *in vitro*

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Background

Baloxavir acid (BXA) is a pan-influenza antiviral that inhibits the endonuclease activity of polymerase acid (PA) protein and blocks viral mRNA synthesis. Specific PA substitutions have been identified to confer reduced susceptibility to BXA with variable changes in viral fitness, a factor that determines the transmissibility of the resistant variants.

Objectives

To systematically identify amino acid changes in the PA protein of A(H1N1)pdm09 that confer reduced susceptibility to BXA *in vitro* using the deep mutational scanning method.

Methods

Random mutagenesis was performed at PA residues 1-240 of the A(H1N1)pdm09 virus. Pools of recombinant PA mutant virus were serially passaged five times in vitro under increasing concentration of BXA. Pair-end next generation sequencing was applied to profile mutations detected at low frequency. The relative fitness scores of individual mutations were profiled by normalizing their relative frequency to the wild-type virus at the first passage. Mutations that emerged during serial passage were introduced into recombinant A(H1N1)pdm09 virus for validation.

Results

The I38T/M change known to confer 7- to 124-fold reduced susceptibility to BXA were invariably detected from six experimental replicates during serial passages. In addition, a novel mutation L106R was identified at P3 at the frequency of 9%. Recombinant A(H1N1) pdm09 virus with L106R substitution was observed to confer 13- to 16-fold reduced susceptibility to BXA and around 0.5 $\log_{10}$ reduction in peak viral load in MDCK cells.

Assessing genetic diversity of plasmid libraries and relative fitness of the high confidence single mutants at passage 1

Table. 1. Relative mutation frequency of all single mutants and diversity of high confidence single mutant in wild-type plasmids and mutant libraries after filtering with 0.04% cutoff.

WT plasmid (non-mutagenized)		
Libraries	Frequency mean ± standard deviation (%)	Mutant count with frequency>0.04%
Lib1 (aa 0-80)	0.01 ± 0.03	20/719 (2.7%)
Lib2 (aa 81-160)	0.01 ± 0.03	10/720 (1.3%)
Lib3 (aa161-240)	0.01 ± 0.02	14/720 (1.9%)
Mutagenized library		
Libraries	Frequency mean ± standard deviation (%)	Mutant count with frequency>0.04%
Lib1 (aa 0-80)	0.04 ± 0.04	347/719 (48.2%)
Lib2 (aa 81-160)	0.05 ± 0.04	341/720 (47.3%)
Lib3 (aa161-240)	0.04 ± 0.04	316/720 (43.8%)

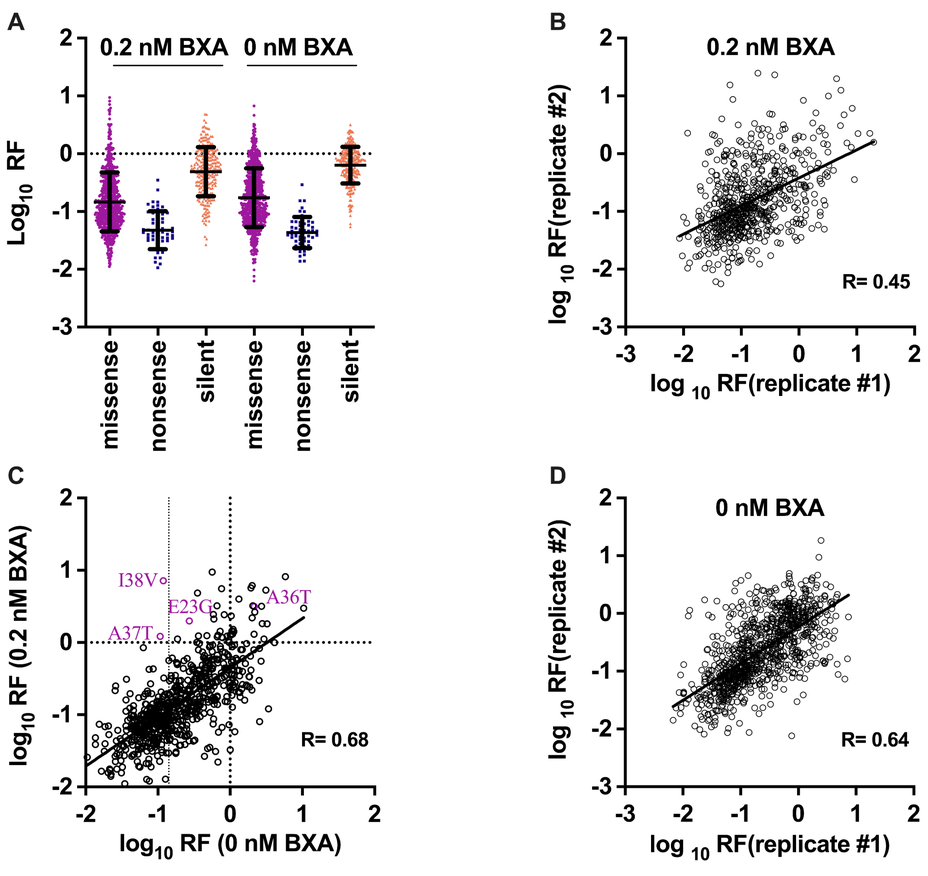

Fig 2. Relative fitness of high confident single mutants at passage 1. (A) Selection efficiency was evaluated by examining the fitness distribution of high confident mutants (relative frequency >0.04% in plasmid library) that were classified into missense, nonsense, silent mutations. Assay reproducibility in the presence (B) or absence (C) of BXA was evaluated before profiling of mutational effect in the presence or absence of BXA (I).

Identifying mutant viruses emerged under BXA selection

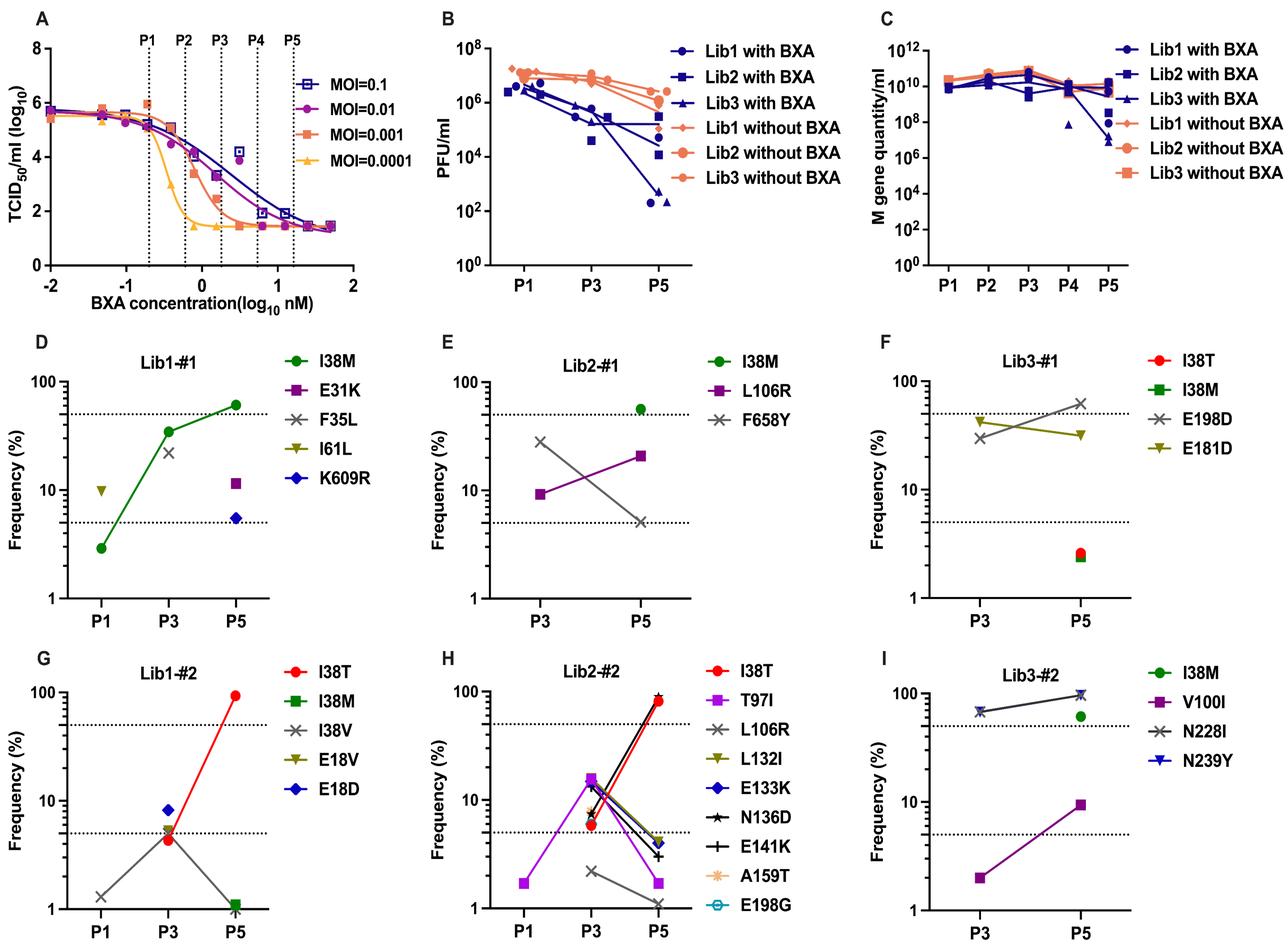

Fig 2. (A) EC_{50} of A(H1N1)pdm09 virus under different MOI, the dished line indicated BXA concentration used for serial passage of mutant virus library in MDCK cells, infectious virus titer (B) and M gene quantify (C) was determined during serial passage; (D-I): identification of PA mutations (>5%) emerged with increasing concentration of BXA during serial passage in MDCK cells. Lib1-3: mutant virus library 1-3.

Validation of identified PA amino acid changes *in vitro*

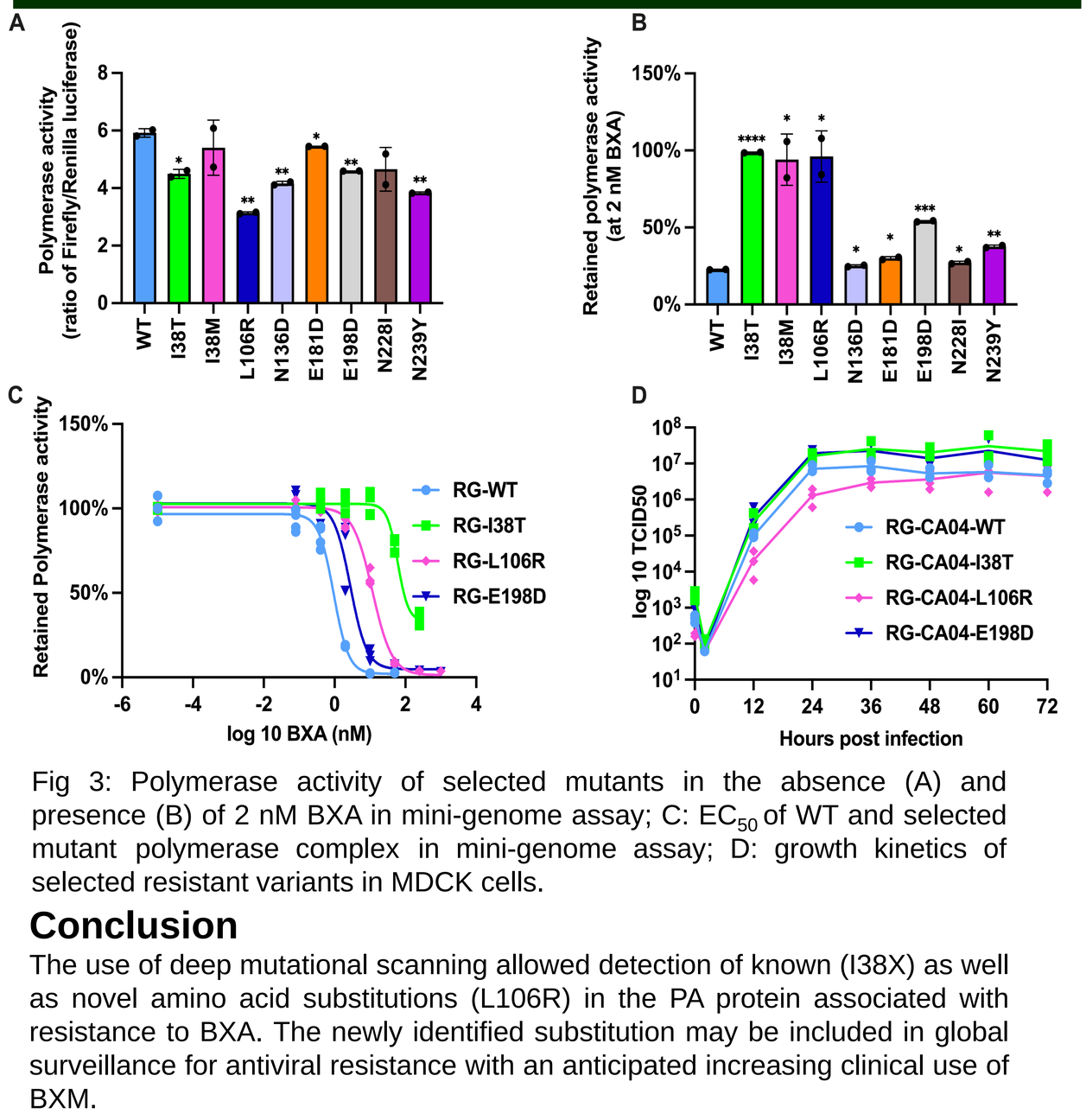

Fig 3: Polymerase activity of selected mutants in the absence (A) and presence (B) of 2 nM BXA in mini-genome assay; C: EC_{50} of WT and selected mutant polymerase complex in mini-genome assay; D: growth kinetics of selected resistant variants in MDCK cells.

Conclusion

The use of deep mutational scanning allowed detection of known (I38X) as well as novel amino acid substitutions (L106R) in the PA protein associated with resistance to BXA. The newly identified substitution may be included in global surveillance for antiviral resistance with an anticipated increasing clinical use of BXM.

Acknowledgements

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