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Short communication

Selection of avian influenza A (H9N2) virus with reduced susceptibility to neuraminidase inhibitors oseltamivir and zanamivir

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ABSTRACT

Identification of amino-acid substitutions in the neuraminidase (NA) of low-pathogenic avian influenza (AI) H9N2 viruses is important to study the susceptibility to NA inhibitors (NAI). To identify mutations under NAI selective pressure, the virus was serially passaged with increasing levels of either oseltamivir or zanamivir *in ovo*, and the growth of the viruses in the presence and absence of NAI's compared. Mutations R292 K in the presence of oseltamivir and E119D in presence of zanamivir were observed within passage one and two respectively. The R292 K mutation reduced oseltamivir susceptibility significantly (2,523-fold) and moderately reduced susceptibility to zanamivir. The E119D mutation significantly reduced susceptibility to zanamivir (415-fold) and remained susceptible to oseltamivir. Genetic stability of the mutations assessed by serial passages of the mutant viruses in eggs without drug pressure resulted in the loss of these mutations, making the virus susceptible to both the drugs. Molecular modeling and dynamics simulations revealed that the R292 K mutation disrupted oseltamivir binding similar to other group 2 NAs, while a different mechanism was noted for zanamivir binding for both R292 K and E119D mutations. The study highlights the need for regular susceptibility screening of circulating AI viruses.

Avian influenza (AI) H9N2 viruses play a crucial role at the animal-human interface due to its wide host range, extensive gene re-assortment and potential to cause human infections (Sun and Liu, 2015). Human infections with influenza H9N2 viruses have been reported in China, Hong Kong, Vietnam and Bangladesh (Shanmuganatham et al., 2013; Huang et al., 2015; Shanmuganatham et al., 2016). Presence of antibodies against H9N2 virus in poultry workers proved the virus transmission from poultry to humans (Pawar et al., 2012; Uyeki et al., 2012; Sun and Liu, 2015).

Neuraminidase inhibitors (NAI) are important for treatment or prophylaxis of AI virus infections since vaccines are not available for newly emerging strains. As drug resistant variants may exist naturally or may emerge due to selection, resistance to these drugs has been studied extensively, for human as well as AI H5N1, H7N9 and H4N2 viruses (Gubareva et al., 1997; McKimm-Breschkin et al., 1998; Abed et al., 2006; Oh and Hurt, 2014; McKimm-Breschkin, 2018). Though a study reports susceptibility of H9N2 viruses to oseltamivir and zanamivir (Baranovich et al., 2013), there are no reports on resistance of H9N2 viruses to these drugs in the presence of mutations. The present study was therefore undertaken to identify amino-acid substitutions that can arise in H9N2 virus under NAI selective pressure and their impact on its susceptibility to antiviral drugs. The antigenic as well as phylogenetic analysis of H9N2 viruses of human and avian origin showed that they are similar (Shanmuganatham et al., 2013; Huang et al., 2015), hence the findings of the present study would help understand the probable mutations that might arise if these drugs are used for treatment of human infections with H9N2 viruses.

Various approaches have been used to select NAI-resistant mutants of avian and human influenza viruses. Previous studies using embryonated chicken eggs have demonstrated antiviral efficacy for both oseltamivir and zanamivir with high challenge doses of influenza A virus (Sauerbrei et al., 2006). Thus, embryonated chicken eggs being a natural host system for AI viruses and a convenient and easy to use alternative for testing of antiviral activity to NAIs, were used for selection of the drug resistant variants. Crystal structures of the N2 Neuraminidase (NA) of H9N2 viruses are not available in the protein databases, hence computational studies were further undertaken to gain insight into the molecular mechanism of drug resistance in the mutant H9N2 viruses. The study was approved by the 'Institutional Biosafety Committee' of the ICMR-National Institute of Virology, Pune. The

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experiments were conducted in class II A2 biosafety cabinets in BSL-2 and BSL-3 laboratories.

Avian influenza H9N2 virus, A/chicken/India/Pune-NIV99321/2009 (H9N2) (hereafter referred to as H9N2-wt) belonging to G1 lineage (Pawar et al., 2012), was inoculated into the allantoic cavity of embryonated chicken eggs and incubated at 37 °C for 72 h (World Health Organization (WHO, 2002). Allantoic fluid was harvested from the eggs and presence of the virus was confirmed by haemagglutination assay (HA assay) using turkey erythrocytes with a virus titer of 2048 HA units. The 50% egg infectious dose (EID_{50}) titer of the virus was determined by inoculating serial ten-fold virus dilutions in six eggs per dilution. Eggs were harvested after incubation and HA assay was performed. The EID_{50} titer was $10^{9.7}$ EID_{50}/ml (Reed and Muench, 1938). Viral RNA was extracted using QIAamp viral RNA mini kit (Qiagen, Germany). RT-PCR was performed using specific primers (Supplementary Table 1) and Super Script III Platinum one-step reverse transcription (RT)-PCR system (Invitrogen) according to the manufacturer's instructions. DNA sequencing was carried out using the ABI Prism dye terminator III cycle sequencing kit (Applied Biosystems) and DyeEx spin kit (Qiagen). The sequence was determined using an automated 3130 XL Genetic Analyzer (Applied Biosystems). Nucleotide sequences were analyzed using 'SeqScape' (v 2.5.0 Applied Biosystems) and edited using 'BioEdit' (v 7.0.9.1, Centers for Disease Control and Prevention, Atlanta, USA). NA gene sequences were analyzed to identify amino-acid substitutions. The hemagglutinin (HA) gene of the H9N2 virus was sequenced to identify probable compensatory mutations (Bloom et al., 2010). Analysis of the NA gene sequences of H9N2-wt virus revealed that the amino acid substitutions known to confer reduced susceptibility to NA inhibitors were absent. The NA and HA gene sequences were submitted to the GenBank (accession numbers MH045822 and MH045823).

The susceptibility of the virus to oseltamivir and zanamivir was tested using a fluorescence-based NA enzyme inhibition assay (Hurt et al., 2004). The fold-changes in IC_{50} were interpreted based on the World Health Organization's Antiviral Working Group criteria (World Health Organization (WHO, 2012). Oseltamivir carboxylate was kindly provided by F. Hoffmann-La Roche Ltd., Basel, Switzerland. The substrate MUNANA was purchased from Sigma Aldrich, catalogue no. M8639). It was found that the virus was sensitive to both the drugs with IC_{50} values of 0.07 nM and 0.09 nM, respectively.

The susceptibility of the H9N2-wt virus to NAIs was assessed in embryonated chicken eggs in the presence and absence of oseltamivir and zanamivir. Toxicity of the NAIs to eggs was evaluated by assessing the histopathological changes in the embryos inoculated with the drug (Tare and Pawar, 2015). No toxicity of the drugs was noted at all tested concentrations to the embryos. Oseltamivir and zanamivir concentrations of 14 μg/ml and above showed complete inhibition of the virus growth and hence used as the initial drug concentration in the *in ovo* antiviral assays. The H9N2-wt virus diluted to 100 EID_{50} titer was inoculated 2 h after inoculation of either oseltamivir or zanamivir at a concentration of 14 μg/ml (final concentration 2.8 μg/0.2 ml) in embryonated chicken eggs. The eggs were incubated at 37 °C and harvested after 72 h. The EID_{50} titers were estimated and the second passage was carried out with 100 EID_{50} virus from the harvested fluids of the first passage with the drug concentration 28 μg/ml (final concentration 5.6 μg/0.2 ml) which was two times higher than that used in the previous passage. NAI susceptibility of the allantoic fluids from both the passages were tested in NAI assays. Sequences of NA and HA genes of viruses from both the passages were analysed to study mutations. The first passage of the H9N2-wt virus grown in the presence of oseltamivir showed the presence of R292 K NA mutation (N2 numbering) (hereafter referred to as H9N2-292K). The R292K mutation reduced oseltamivir susceptibility significantly (2,523-fold) whil moderately reduced susceptibility to zanamivir (Table 1). This finding is in agreement with the report of an R292 K NA mutation, which emerged in an H3N2 virus during the treatment of a human case with oseltamivir (McKimm-Breschkin et al.,2003). Additionally, AI viruses such as H7N9 or H4N6 with the R292K substitution in NA have been reported earlier and some of the H7N9 clinical isolates possessed these mutations naturally (Gillman et al., 2013; Hai et al., 2013; Sleeman et al., 2013).

The first passage of the H9N2-wt virus grown in the presence of zanamivir showed 19-fold increase in zanamivir IC_{50} value (1.73 nM) as compared to the wild type virus, without any mutation in the NA gene. The electropherogram of the NA sequence of the virus from passage 1showed polymorphism (double peak) at residue 119, containing a mixed population of glutamic acid (E) and aspartic acid (D) (data not shown). This could be the probable reason for the increase in IC_{50} value in absence of any mutation in the NA. Further studies using pyrosequencing would confirm the exact proportion of the wild-type and mutant viruses. The second passage of the virus with zanamivir showed the occurrence of E119D NA mutation (hereafter referred to as H9N2-119D). The E119D mutation significantly reduced susceptibility to zanamivir (415-fold) but remained susceptible to oseltamivir (Table 1). Analyses of 2971 H9N2 NA gene sequences available in GenBank, revealed four sequences with E119D substitution known to confer zanamivir resistance (AF156392-1, DQ464353, GQ 335508 and CY 023218). However, it is not clear whether these are naturally occurring mutations or induced by the selection pressure of drugs. A study, in which E119D mutation was generated by reverse genetics in the H3N2 virus, showed a medium level of resistance to zanamivir (Zürcher et al., 2006). The E119D substitution in group 2 NA of other influenza viruses has also been reported earlier (WHO GISRS (2016)).

Compensatory mutations in the HA gene are known to occur in drug resistant influenza viruses (Bloom et al., 2010). In the present study, no mutation was noted in the HA gene of the H9N2-119D virus while L234Q mutation was observed in the H9N2-292K virus (Table 1). The mutation persisted even after five passages of the H9N2-292K virus without drug pressure. This substitution has been reported previously in the HA of H5N1 viruses grown under the selection pressure of oseltamivir (Hurt et al., 2009). Molecular characterization of viral surface proteins of H9N2 human isolates, indicated preferential binding of the HA possessing the L234Q mutation to the human α (2–6) sialic acid receptor (Butt et al., 2010). Further studies are warranted to elucidate the role of this mutation in determining receptor specificity of H9N2 virus.

To test the ability of the mutant viruses (H9N2-292K and H9N2-119D) to grow under the NAI drug pressure, the mutant viruses were grown in eggs, in the presence and absence of NAI drugs. The EID_{50} titers without drug and with each NAI drug (14 μg/ml) were compared. All the experiments were performed in triplicates. The significance of difference between the EID_{50} titers of the viruses was calculated using Student's *t*-test, *p*-values < 0.05 were considered as significant. H9N2-wt virus showed a significant difference in titers in the presence and absence of both the drugs, thus confirming that this virus was sensitive to NAI drugs. For H9N2-292K virus, the difference in titers in case of oseltamivir was not significant confirming that this substitution conferred highly reduced inhibition by oseltamivir; however, a marginal significance of difference when tested with zanamivir was observed. The H9N2-119D virus grown in the presence and absence of oseltamivir revealed a significant difference in EID_{50} titers thus showing inhibition of the virus growth by oseltamivir, but in case of zanamivir the titers showed no significant difference indicating that this substitution highly reduced inhibition of the virus growth by zanamivir (Fig. 1).

To assess the genetic stability of the observed mutations in the absence of drug pressure, H9N2-292K and H9N2-119D viruses diluted to100 EID_{50} titer were inoculated in embryonated chicken eggs. The eggs were incubated at 37 °C and harvested after 72 h, up to five serial passages of the viruses. NA and HA genes of the viruses from each passage were sequenced.

The NA substitution 292 K persisted in the first passage and reverted to R292 in the second passage while the NA substitution 119D persisted for the first two passages with a mixed proportion of 119E and 119D in

Table 1
Susceptibility to NAIs of influenza A(H9N2) wild-type virus on serial passages in the presence of oseltamivir and zanamivir.

virus grown in presence of the NAI drug	Passage no.	NAI concentration	Oseltamivir IC_{50} meanIC_{50}nmol/L [a](- fold)	Zanamivir meanIC_{50}nmol/L [a](- fold)	NA mutation	HA mutation
Oseltamivir	1	2.8 µg	176.6 (2523)	1.9 (21)	R292 K	L234Q
Zanamivir	1	2.8 µg	0.17 (2)	1.73 (19)	–	–
	2	5.6 µg	0.24 (3.5)	37.4 (415)	E119D	–

Avian influenza H9N2 virus was serially passaged in embryonated chicken eggs in the presence of increasing concentrations (two fold) of either oseltamivir or zanamivir (NAI concentration). Eggs were harvested after incubation at 37 °C for 72 h. Each passage level was tested for susceptibility to zanamivir and oseltamivir and HA and NA genes were sequenced to look for the generation of any mutations.
IC_{50}, 50% inhibitory concentration determined as per Hurt et al. (2004) (mean of values for assays performed in triplicate).
[a] Fold increase values in comparison with the H9N2-wt virus.

the sequence of the second passage virus. However, at passage three, the virus showed reversion of the substitution to 119E (Table 2). Studies using cell culture-based assays have shown the generation of mutations after several passages of the virus. On the contrary, the mutations in the present study got selected and reverted quickly within one or two passages. One of the explanations for quick selection of the mutations could be the use of embryonated chicken eggs as a host, which is the natural host of AI viruses. It is possible that the quick reversion of these mutations could be a characteristic of H9N2 viruses. Previous studies using cell culture for selection of mutations have shown that the pathogenicity of NAI-resistant influenza viruses and the stability of mutations vary among different viruses (Yen et al., 2005).

NAI assays of the harvested allantoic fluids from the first three passages, were also performed and the IC_{50} values were determined. There was a gradual reduction in the IC_{50} values at each passage and the results of the NAI assays were in agreement with the sequence data (Table 2).

The mechanism of drug resistance in the H9N2 virus due to the observed NA mutations was studied using bioinformatics approaches. Homology models of wild type NA, and mutants NA-292 K and NA-119D with Oseltamivir (wt-OSL, 292 K-OSL and 119D-OSL respectively) and Zanamivir (wt-ZMR, 292 K-ZMR and 119D-ZMR respectively) were generated using the SWISS-MODEL web server (Waterhouse et al., 2018). The corresponding co-crystal structures (PDB ID: 4K1I and 3TIC) of NA (H2N2 subtype) showing ~86% sequence identity with the target H9N2-wt NA were used as templates. Molecular dynamics (MD) simulations of all the complexes (Supplementary Fig. 1) were performed using YASARA package (version 13.6.16) using simulation parameters as described earlier (Bhoye et al., 2016) and the interaction analyses were done using BIOVIA Discovery studio visualizer. Structural comparisons by superimposition of the wt-OSL and 292 K-OSL complexes (Fig. 2a) revealed that the absence of the guanidinium group of R, resulted in an increase in the distance between the residue at 292 and the carboxylate group (~3 Å to ~5 Å), and subsequently in the loss of a hydrogen bond (H-bond) and salt-bridge interaction (Supplementary Fig. 2a and c). However, comparison of the 292 K-ZMR and wt-ZMR complexes showed that the interactions with both the carboxylate as well as the glycerol group of zanamivir were unaffected. The loss of a single interaction with E276 in 292 K-ZMR when compared to 292 K-ZMR explains the slightly weakened interaction with the drug (Fig. 2b; Supplementary Fig. 2b and d). Similar studies undertaken previously for other group 2 NAs such as N9, reported similar findings for the mode of oseltamivir binding (Woods et al., 2013). In contrast in case of zanamivir binding, in both H7N9 and H11N9, lysine at position 292 could bind only to the bulky group and not the carboxylate group, causing a change in the binding of zanamivir in these NAs (Varghese et al., 1995; Varghese et al. (1998); Woods et al., 2013). In case of H9N2-119D mutant, the 119D-ZMR complex showed loss of H-bond between the guanidino group of zanamivir at position 119 coupled with the loss of the crucial interaction of E276 with the bulky group in comparison to the wt-ZMR complex (Fig. 2d; Supplementary Fig. 2b and f), whereas no effect of this mutation on oseltamivir binding was seen as the single salt bridge interaction between the residue at the 119 position with the amine group was maintained (Fig. 2c; Supplementary Fig. 2a and e). The mechanism of resistance to zanamivir due to E119D mutation in pandemic H1N1 influenza viruses has been reported earlier (L'Huillier et al., 2015) as the loss of one of the two salt bridges that coordinate the guanidino group of zanamivir, though no role of E276 was noted. To the best of our knowledge no molecular mechanism has been proposed for the effect of E119D towards oseltamivir binding.

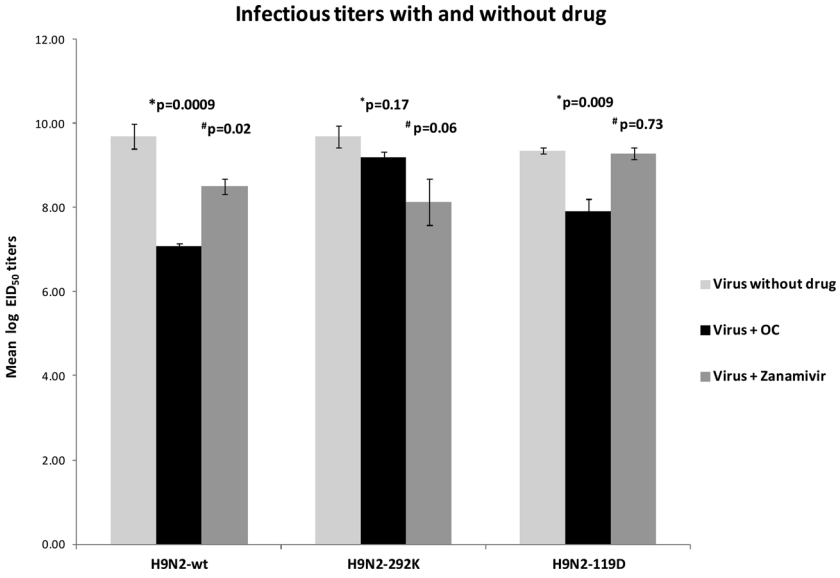

Fig. 1. Comparison of EID_{50} titers of H9N2 viruses grown with and without oseltamivir and zanamivir.
The mean of log EID_{50} titers have been plotted for the viruses (H9N2-wt, H9N2-292k and H9N2 119D) without drug treatment and after treatment with 14 µg/ml (2.8 µg/0.2 ml) each of oseltamivir carboxylate and zanamivir.
The error bars represent the standard error for experiments performed three times.
*Significance of difference between EID_{50} titers with and without oseltamivir for each virus.
Significance of difference between EID_{50} titers with and without zanamivir for each virus.

Table 2
Phenotypic and genetic stability of influenza A(H9N2) viruses carrying R292 K and E119D substitutions during passages in embryonated chicken eggs.

viruses	IC_{50}, Mean ± SD, nmol/L				Observed mutations			
	Oseltamivir				**NA gene**			
	P0[a]	P1[b]	P2[c]	P3[d]	P0[a]	P1[b]	P2[c]	P3[d]
H9N2-292K	176.59±8.13	13.09±2.57	0.08±0.0	0.03±0.03	292 K	292 K	R292	R292
H9N2-119D	0.24±0.18	0.08±0.0	0.02±0.01	0.03±0.03	119D	119D	119D	E119
	Zanamivir				**HA gene**			
	P0[a]	P1[b]	P2[c]	P3[d]	P0[a]	P1[b]	P2[c]	P3[d]
H9N2-292K	1.88±0.21	0.32±0.08	0.17±0.05	0.09± 0.03	L234Q	L234Q	L234Q	L234Q
H9N2-119D	37.36±4.65	5.18±1.09	1.250±.3	0.12±0.04	–	–	–	–

IC_{50}, 50% inhibitory concentration.
SD, standard deviation; mean of IC_{50} values for assays performed in triplicate.
[a]IC_{50} of the virus with NA gene substitutions conferring resistance to oseltamivir and zanamivir (H9N2-292K, H9N2-119D).
[b,c,d]Serial passages (passage 1- passage 3) of viruses with amino acid substitutions in embryonated chicken eggs, without drug pressure.

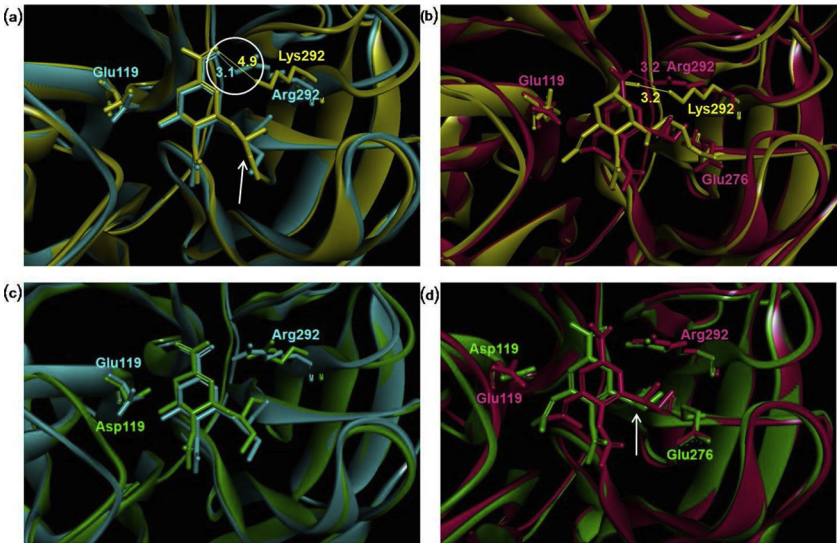

Fig. 2. Superimposition of representative structures of the NA-drug complexes. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)
The NA structures of H9N2-292K and H9N2-119D with Oseltamivir (292 K-OSL and 119D-OSL respectively) and Zanamivir (292 K-ZMR and 119D-ZMR respectively) were generated by replacing the amino acid at the residue positions 292 (arginine to lysine) and 119 (glutamic acid to aspartic acid) in the respective model using BIOVIA Discovery studio (BIOVIA, USA). (a) wt-OSL and 292 K-OSL (b) wt-ZMR and 292 K-ZMR (c) wt-OSL and 119D-OSL (d) wt-ZMR and 119D-ZMR. Molecular dynamics simulations were carried out for 25 ns for wt-OSL, 292 K-OSL, 119D-OSL and 30 ns for wt-ZMR, 292 K-ZMR and 119D-ZMR, with snapshots of the trajectories captured at 25 ps time interval. Interacting residues playing crucial roles are indicated by stick representation. Bulky groups of oseltamivir and zanamivir that are displaced are indicated with arrows and distances between critical residues and the drug are indicated. Cyan, magenta, yellow and green colors represent wt-OSL, wt-ZMR, 292 K and 119D structures, respectively.

Overall, the computational structural analyses could elucidate the mechanism of drug resistance, in particular in H9N2 viruses, due to NA substitutions R292 K and E119D.

In conclusion, the present study identified the mutations R292 K and E119D that can arise in H9N2 viruses under the selection pressure of NAIs. The emergence of possible mutations in H9N2 virus leading to reduced susceptibility to the NAIs necessitates continued antiviral surveillance of H9N2 viruses.

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Conflict of interest

No conflict of interests declared.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.virusres.2019.03.019.

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