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Short communication

A novel I117T substitution in neuraminidase of highly pathogenic avian influenza H5N1 virus conferring reduced susceptibility to oseltamivir and zanamivir

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ABSTRACT

Occurrence of avian influenza (AI) with Neuraminidase (NA) mutations which confer reduced neuraminidase inhibitor (NAI) susceptibility has remained a cause of concern. The susceptibility to NAIs of 67 highly pathogenic avian influenza H5N1 viruses isolated during 2006–2012 in India was tested in phenotypic fluorescence-based NA inhibition assay, sequence analysis and *in ovo*. One isolate showed a novel NA I117T amino acid substitution (N2 numbering) and eight isolates showed previously known NAI-resistance marker mutations (I117V, E119D, N294S, total 9/67). The overall incidence of resistant variants was 13.4%. The novel I117T substitution reduced oseltamivir susceptibility by 18.6-fold and zanamivir susceptibility by 11.8-fold, compared to the wild type AI H5N1virus, thus showed cross-resistance to both oseltamivir and zanamivir in NA inhibition assays. However, the other two isolates with I117V substitution were sensitive to both the NAIs. In addition, the comparison of growth of the I117T and I117V variants in presence of NAI's in the *in ovo* assays exhibited difference in growth levels. The present study reports the natural occurrence of a novel I117T mutation in AI H5N1 virus conferring cross-resistance to oseltamivir and zanamivir highlighting the urgent need of antiviral surveillance of AI viruses.

1. Introduction

Highly pathogenic avian influenza (HPAI) H5N1 are emerging influenza viruses, having potential to cross the species barrier and cause human infections. Poultry outbreaks of HPAI H5N1 have occurred on a large scale in 68 countries with pandemic potential and sporadic transmission to humans that results in large number of deaths (Durand et al., 2015; OIE, 2019). More than 130 outbreaks of HPAI H5N1 viruses have been reported from India (OIE, 2018). Antiviral drugs are important for treatment or prophylaxis of avian influenza (AI) virus infections since vaccines are not available for newly emerging strains. Two classes of drugs have been approved for clinical use against influenza, the adamantanes and the neuraminidase inhibitors (NAIs). The global circulation of adamantane-resistant virus variants has rendered the NAIs as the drugs of choice (Pizzorno et al., 2011; Govorkova et al., 2013). Until recently, the NAIs (oseltamivir, zanamivir, peramivir and laninamivir) was the only class of antiviral drugs available to treat influenza infections worldwide (McKimm-Breschkin, 2013), which are targeted against the neuraminidase (NA) active site pocket inhibiting viral propagation. There are 19 highly conserved residues in the NA active site of all influenza A and B viruses. These include eight catalytic residues (R118, D151, R152, R224, E276, R292, R371, and Y406) that directly contact the sialic acid (SA) and 11 framework residues (E119, R156, W178, S179, D198, I222, E227, H274, E277, N294, and E425) that support the enzymatic binding pocket (Gubareva et al., 1997). The new influenza antiviral drug Baloxavir marboxil (trade name Xofluza) which is a PA protein inhibitor was approved in 2018 in Japan and the US for the treatment of influenza (Ng, 2019). As drug resistant variants may exist naturally or may emerge due to selection, resistance to these drugs due to amino acid changes in the viral NA has been studied extensively (Gubareva et al., 1997; Oh and Hurt, 2014; Takashita et al., 2015; McKimm-Breschkin et al., 2018).

Several subtype-specific mutations in framework or catalytic residues of NA, such as V116A, I117V, E119A/G, Q136L/K, V149A, D198G, I222M/V, S246N, H274Y, N294S (N2 numbering) that confer resistance to these drugs have been described previously. While

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oseltamivir resistance has been studied extensively there are only a few reports of zanamivir resistance (Le et al., 2005; Hurt et al., 2009; Boltz et al., 2010; Ilyushina et al., 2010; Naughtin et al., 2011; Nguyen et al., 2013).

NAI resistant influenza viruses may differ substantially in replicative capacity and transmissibility (Yen et al., 2005). Therefore, it is essential to investigate the effect of the mutations on virus replication in presence of the NA inhibitors for assessment of risk. Different host systems such as cell culture, ferrets, mice, have been used to compare replicative capacity of the mutant viruses with that of wild-type viruses (Pizzorno et al., 2011; Nguyen et al., 2013; Baek et al., 2015). 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 in the present study for the assessment of growth of the NAI-resistant viruses (Tare and Pawar, 2015; Kode et al., 2019).

Recently, a study from India reported the NAI susceptibility of AI H5N1 viruses with presence of NAI-resistance markers such as E119A, N294S, and I117V + E119A (Sood et al., 2018). Similar to these findings, the AI H5N1 viruses tested in the present study showed presence of E119A, N294S, and I117V markers of NAI-resistance (data not shown). Interestingly, in addition to these known marker mutations, one isolate possessed a novel I117T substitution which conferred cross-resistance to oseltamivir and zanamivir. The antiviral susceptibility of this naturally occurring I117T NA mutation is reported in the present study using NA inhibition assays and *in ovo* assays.

2. Materials and methods

2.1. Viruses

A total of 67 HPAI H5N1 viruses isolated at the ICMR-National Institute of Virology, Pune, India from various poultry outbreaks, from six states namely Maharashtra, Manipur, West Bengal, Tripura, Assam and Jharkhand, during 2006–2012 were used. The viruses belonged to clade 2.2 (n = 43) and 2.3.2.1 (n = 24) and were isolated from avian species, chicken (n = 64), crow (n = 1), pigeon (n = 1) and one from environmental sample. The viruses were propagated in embryonated chicken eggs at 37 °C for 48 h. The allantoic fluids were harvested, and the presence of virus was confirmed by hemagglutination assay (HA assay) using 0.5% turkey red blood cells (World Health Organization (WHO, 2002). All experiments were conducted in biosafety level 3+ laboratory.

2.2. RNA extraction, RT-PCR and sequencing

Viral RNA was extracted using QIAamp viral RNA mini kit (Qiagen, Germany) and RT-PCR was performed using specific primers (primer sequences will be made available upon request) and Super Script III Platinum one-step reverse transcription (RT)-PCR system (Invitrogen) according to 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 sequences were determined using an automated 3130 XL Genetic Analyzer (Applied Biosystems). Nucleotide sequences were analysed 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). The sequences were deposited into the GenBank database under the accession numbers 88765 (MK392480), 816720 (MK392502) and 816855 (MK392528).

2.3. Fluorometric neuraminidase inhibition (NAI) assay

The 50% inhibitory concentration (IC_{50}) of the viruses to NAIs was determined using a fluorescence-based NA enzyme inhibition assay (Hurt et al., 2004). Oseltamivir carboxylate was kindly provided by F. Hoffmann-La Roche Ltd., Basel, Switzerland. Zanamivir (Cipla, India) was procured locally. The substrate MUNANA was purchased from Sigma Aldrich (catalogue no. M8639). Briefly, viruses standardized to equivalent NA enzyme activity in the linear range of the curve were mixed with concentrations of inhibitor ranging from 0.01 nM to 10,000 nM, in 96-well flat-bottom black opaque plates (FluoroNunc plates; Nunc). The virus-inhibitor mixture was incubated at room temperature for 45 min., 50 µl of MUNANA substrate (final concentration 0.1 mM) was added and incubated at 37 °C for 60 min. The reaction was terminated by adding 100 µl stop solution (0.14 M NaOH in 83% ethanol). The concentration of NAI that reduced NA activity by 50% (IC_{50}), was determined using the logistic curve fit program Jasper v 1.2 kindly provided by Centers for Disease Control and Prevention, USA. IC_{50} values were recorded as the mean of 3 independent assays.

2.4. In ovo assays

Further, the infectivity and the growth potential of the viruses having I117T and I117V mutations, was assessed in embryonated chicken eggs, based on the 50% egg infectious dose (EID_{50}) values of the viruses, in the presence and absence of NAIs. Toxicity of the NAIs to eggs was evaluated by assessing the histopathological changes in the embryos inoculated with the drug. 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 the drug concentration 14 µg/ml was used in the *in ovo* assays (Tare and Pawar, 2015; Kode et al., 2019). Briefly, the test viruses were serially diluted tenfold (undiluted to 10^{-9}). Equal volumes (0.2 ml each) of 14 µg/ml drug, either oseltamivir or zanamivir at a concentration of 14 µg/ml (final concentration 2.8 µg/0.2 ml) and each dilution of the virus were mixed and incubated at 37 °C for 1 h. prior to inoculation into embryonated chicken eggs. Allantoic fluids were harvested after incubating the eggs at 37 °C for 48 h. The 50% egg infectious dose (EID_{50}) titers were determined (Reed and Muench, 1938). The significance of difference between the EID_{50} titers of the viruses was calculated using analysis of variance (ANOVA) with Tukey's post-hoc test (IBM SPSS PASW 18 software), p-values < 0.05 were considered as significant. All the experiments were performed in triplicate.

3. Results and discussion

The NA gene sequences were analyzed for the amino acid substitutions such as V116A, I117V, E119A/G, Q136L/K, V149A, D198G, I222M/V, S246N, H274Y, N294S (N2 numbering) which are known to be associated with the reduced/highly reduced NAI susceptibility in group N1 neuraminidases (WHO, 2018). Nine out of 67 AI H5N1 viral NA sequences showed presence of markers of NAI-resistance (data not shown). A novel I117T substitution was observed in one isolate, A/chicken/India/WB-NIV88765/2008 (hereafter referred to as 88765) and an I117V substitution, which is a known NAI-resistance marker, was observed in the other two isolates, A/chicken/India/Assam-NIV816720/2008 and A/chicken/India/Assam-NIV816855/2008 (hereafter referred to as 816720 and 816855 respectively). All the three variant viruses belonged to the HA clade 2.2. The susceptibility of these three viruses to NAIs was further studied.

The overall incidence of resistant variants was 13.4% which was relatively high compared to 0.8% and 4%, previously reported in poultry (Govorkova et al., 2013; Nguyen et al., 2013). The possible reasons for this could be the smaller sample size and also due to the fact that all the viruses included in the present study belonged to clade 2.2 virus populations from poultry outbreaks.

Susceptibility of all the AI H5N1 viruses to oseltamivir and zanamivir was determined using phenotypic fluorescence-based assay and viruses with potential resistance to NA inhibitors were identified. The

Table 1
NAI susceptibility of the variant AI H5N1 viruses in NA inhibition assays.

Influenza A(H5N1) viruses	NA substitution	Mean IC_{50} ± SD, nM (fold increase)[a]	
		oseltamivir	zanamivir
A/chicken/India/WB-NIV 88765/2008	I117T	1.86 ± 0.92 (18.6)	1.3 ± 0.65(11.8)
A/chicken/India/Assam-NIV 816720/2008	I117V	0.82 ± 0.16 (8.2)	0.4 ± 0.22(3.8)
A/chicken/India/Assam-NIV 816855/2008	I117V	0.9 ± 0.04 (9)	0.22 ± 0.1(2)
A/chicken/India/NIV33487/2006 [b]	–	0.25 ± 0.1(1.5)	0.28 ± 0.33(2)

IC_{50}, 50% inhibitory concentration; (mean of values for assays performed in triplicate).
[a] Fold increase values in comparison with the median IC_{50} of the clade 2.2 viruses.
[b] Wild type AI H5N1 virus.

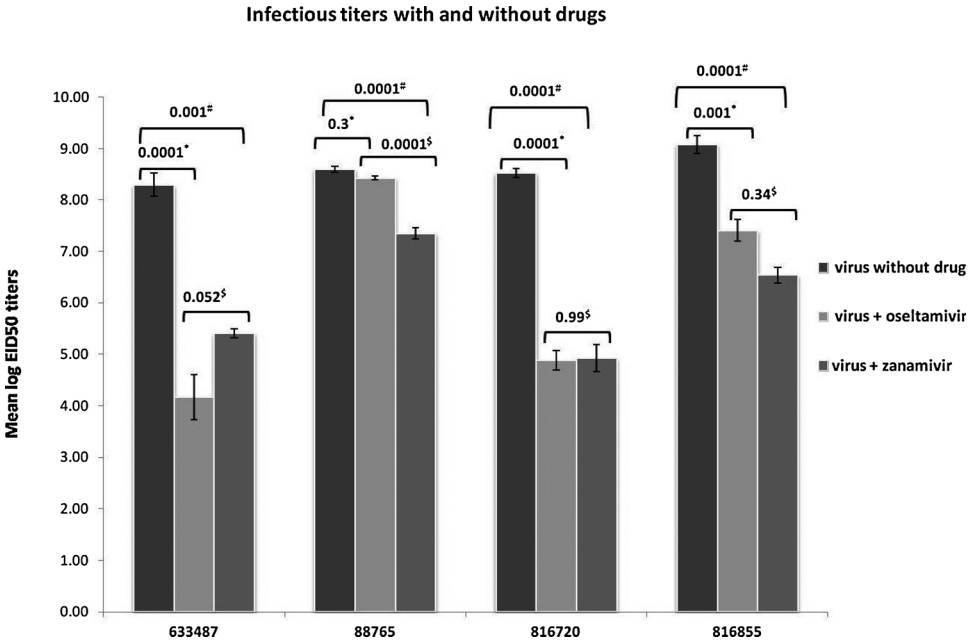

Fig. 1. Comparison of EID_{50} titers of AI H5N1 viruses grown with and without oseltamivir and zanamivir.
The mean of log EID_{50} titers have been plotted for the viruses (633487, 88765, 816720, 816855) grown in absence of drug and in presence of 14 μg/ml each of oseltamivir carboxylate and zanamivir. The error bars represent the standard error for experiments performed three times.
*p values of "ANOVA" test comparing mean log EID_{50} titers of each virus without and with oseltamivir; #p values of "ANOVA" test comparing mean log EID_{50} titers of each virus without and with zanamivir; and $p values of "ANOVA" test comparing mean log EID_{50} titers of each virus with oseltamivir and with zanamivir.

IC_{50} values were analyzed clade-wise to enable better correlation with the NA sequences. The mean IC_{50} values of clade 2.2 viruses for oseltamivir ranged between 0.01–0.97 nM and those for zanamivir ranged between 0.03–0.65 nM. The median IC_{50} values for oseltamivir and zanamivir were 0.1 and 0.11 respectively. The fold-changes in IC_{50} were interpreted by comparing it with the median IC_{50} values, based on the World Health Organization's Antiviral Working Group criteria. Influenza A viruses with < 10-fold change in IC_{50} were characterized as exhibiting normal inhibition, while those with 10- to 100-fold and > 100-fold change as exhibiting reduced and highly reduced inhibition, respectively (World Health Organization (WHO, 2012).

The AI H5N1 88765 (I117T) virus showed reduced inhibition by oseltamivir and a marginally reduced inhibition by zanamivir with 18.6-fold and 11.8-fold increase in the IC_{50} value respectively, compared to median IC_{50} value of wild-type clade 2.2 viruses. Whereas, both the AI H5N1 816720 and 816855 (I117V) viruses showed normal inhibition by oseltamivir as well as zanamivir, however, these two viruses showed slightly elevated IC_{50} values than the other clade 2.2 wild-type viruses tested (Table 1).

The amino acid substitution at the NA residue 117 from isoleucine to valine (I117V) resulting in slight reduction in oseltamivir susceptibility has been reported previously (Le et al., 2005; Ilyushina et al., 2010; Takano et al., 2013). In addition, the mutation I117V has shown synergistic effect on IC_{50} values when present in combination with some other mutations known to cause antiviral resistance (Hurt et al., 2007; Sood et al., 2018). There are only a few reports of natural occurrence of zanamivir resistance and some studies have reported NA mutations such as E119A/D or Q136K which cause zanamivir resistance, but most of these studies have used recombinant viruses to elucidate resistance (Ilyushina et al., 2010; Little et al., 2015; Baek et al., 2015). To the best of our knowledge, naturally occurring I117T NA mutation in HPAI H5N1 viruses resulting in cross-resistance to oseltamivir and zanamivir has not been reported previously.

It was noteworthy that the single replacement of isoleucine at the position 117 by threonine in place of valine resulted in resistance to oseltamivir as well as zanamivir. It would be interesting to study further the mechanism of cross-resistance arising due to I117T mutation. Whether the polar side chain of threonine in place of non-polar valine, has any specific role to play in the mechanism of cross-resistance needs to be understood.

In the *in ovo* assays, the significance of difference between the EID_{50} titers in presence and absence of the drug revealed the extent of susceptibility of the viruses to the antiviral drugs.

The EID_{50} titers of the AI H5N1 88765 virus (I117T) grown in presence and absence of oseltamivir did not exhibit significant difference, indicating that the virus exhibited reduced susceptibility which is also in agreement with the findings of the fluorometric NA inhibition assays. However, a significant reduction in EID_{50} titer in presence of zanamivir was observed for this virus. This could be probably because the reduction in zanamivir susceptibility observed in the NAI assays was marginal (11.8 fold). The I117V variants showed statistically significant reduction in EID_{50} titers in presence of oseltamivir as well as zanamivir thus the results were in accordance with the NA inhibition assay findings (Table 1, Fig. 1).

In conclusion, this is the first report of natural occurrence of a novel I117T amino acid substitution in neuraminidase of AI H5N1 virus

conferring reduced susceptibility to oseltamivir and zanamivir. The present study highlights need of monitoring molecular markers along with NAI assay and virus growth for antiviral surveillance.

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Conflict of interest

No conflict of interests declared.

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