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RESEARCH ARTICLE

Neuraminidase Inhibitors Resistance: The Irrational Use of Oseltamivir Can Lead to Genesis of Mutant Avian Influenza Viruses in The Field

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ABSTRACT

Avian influenza virus subtype H9N2 has been circulating and several outbreaks reported in poultry since 1998 in Pakistan. Drug abuse in poultry is the common practice in low to middle income countries including Pakistan. Neuraminidase Inhibitors are the drugs that target its protein neuraminidase. Identification of amino acid substitution in the neuraminidase of low pathogenic avian Influenza H9N2 viruses is important. In our study, the susceptibility and resistance patterns H9N2 were analyzed towards NAIs. Virus was serially passaged in-ovo under increasing concentrations of Oseltamivir and subjected towards haemagglutination assay, molecular confirmation and sequencing. At four points E119V, D151E, E276D, R371K, significant amino acid substitutions due to increase selective drug pressure were observed. These substitutions are responsible in reducing susceptibility of virus towards NAIs. The compensatory mutations in HA gene were also checked associated with NA gene. At position 234 of HA protein, the mutation was heading from Q towards L from passage 3 and persist till passage 5, that indicates that the virus is gradually modifying its host specificity range. As the virus having L at 234 position indicates its zoonotic potential as it has binding affinity more toward α 2,6 sialic acid receptors. So there is a need of continuous surveillance and monitoring while using NAI drugs against field viruses.

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INTRODUCTION

Avian influenza subtype A/H9N2 is considered the potential pandemic candidate and the most prevalently circulating subtype among the terrestrial poultry globally (WHO, 2018; Khan *et al.,* 2021a). In 1966, H9N2 viruses were isolated from turkeys in the US state of Wisconsin (Peacock *et al.,* 2019). Several cases of H9N2 from poultry to human spillover have been reported round the globe (Ali *et al.,* 2019). Human H9N2 infections are somehow meek but the recently, the condition became worsen with the emergence of COVID-19. In July 2022, more than 100 human cases affected with H9N2 were reported in China immediately after COVID-19 outbreak. This shows H9N2 can also be the possible risk agent in Flurona disease (Bi *et al.,* 2022). Therefore, it is a dire need to control influenza viruses within poultry. In Pakistan, H9N2 was first time documented in 1998 and then it begun to circulate in the field which is a potential threat to the community (Khan *et al.,* 2021b).

Class of drugs termed as neuraminidase inhibitors are used against influenza virus that target neuraminidase to prevent and cure from influenza infections (Huang *et al.,* 2008). Neuraminidase is a transmembrane and an antigenic surface glycoprotein that is flecked on the membrane of the virus. It plays an important role in the release of virion from the infected cell after budding (Bouvier and Palese, 2008; Klenk *et al*., 2008). Considering its structure complexity, Neuraminidase is a tetrameric protein of 240kDa having 470 amino acid residues that are arranged into similar and in four spherical subunits, containing a central catalytic pastime.

Neuraminidase consists of hydrophobic stalk and mushroom like spike, attached to virion surface (Aamir *et al.,* 2007; Collins *et al.,* 2008). It basically restricts the binding of viral hemagglutinin to the host cell sialic acid receptors through its sialidase activity. Hence this protein partake in pathogenicity, viral replication, invasion, budding, release and in short a main constituent of the whole virus life cycle (Eichelberger *et al.,* 2018).

Commonly used neuraminidase inhibitors (NAIs) like oseltamivir, peramivir, and zanamivir inhibit the residues including A118, A292, A371, Y408, D151 at glycosylation sites that enacted in neuraminidase catalytic activity (Singh *et al.,* 2019). Mutations in the crucial, sensitive residues of neuraminidase are evolving the strains that are least susceptible and resistant to these inhibitors (Shahzad *et al.,* 2020).

The NAIs class of drugs might play important role for influenza prophylaxis, treatment and inhibit the inter and intra-host virus dissemination. However, irrational drug use may lead to genesis of the resistant viruses. Previous reports on amantadine resistance show that mutant viruses can originate due to blind use of anti-viral in the field (Kode *et al.,* 2019). Just like deliberate use of antibiotics have led to the evolution of various antibiotic resistant bacteria, similarly blind use of anti-viral have also played an important role in evolving new variants of H9N2 influenza viruses which are resistant to these drugs (Wise, 2002). The current study was designed to investigate the effect of NAI on influenza virus mutation. Here, we tested indigenous H9N2 viruses for the neuraminidase resistance and induced mutations associated to neuraminidase resistance in response to repeated NAI drug pressure in embryonated chicken eggs. This study was designed to check the presence of significant mutations under increasing drug pressure in NA gene of H9N2 that are involved in making H9N2 resistant towards NAIs and also to check the counter mutations in Haemagglutinin gene of H9N2.

MATERIALS AND METHODS

Virus isolation and confirmation: The avian influenza virus (A/Chicken/Pakistan47/2019/H9N2) was obtained from the Influenza Lab, University of Veterinary and Animal Sciences, Lahore. The virus was propagated in 9 days old embryonated chicken eggs to increase the amount of virus stock and sufficient viral fluid for our experimental study. The harvested avian influenza virus (AIV) was re-confirmed through reverse transcriptase PCR method.

The 50% Embryo Infective Dose (EID_{50}) of virus was calculated by inoculating ten-fold serial dilution of viral harvest in three eggs per dilution. After incubation, allantoic fluid was harvested and Hemagglutination assay was performed. The EID_{50} titer was calculated by Reed and Muench method and found to be $10^{7.16}$/1ml.

RNA extraction: Viral RNA was extracted from the harvested fluid by using commercially available QIAamp viral RNA DSP v mini kit (catalogue number 52906, Qiagen) cDNA was synthesized by using Thermo Scientific Revert Aid First Strand cDNA Synthesis Kit (catalogue number K1622). To produce cDNA against extracted RNA, 5µl of RNA harvest was added in microfuge tube then 1µl of random hexamer primer and 6µl of nuclease free water was added. Then 4µl of 4X reaction buffer, 1.0µl of RiboLock RNase Inhibitor (20U/µl), 2µl of dNTPs, 1µl of RevertAid M- MuLV RT (200U/µl) were added and all the contents of the PCR tube were briefly centrifuged. The reaction was carried out at 25ºC for 5 mins, 42ºC for 60 mins and was terminated by providing incubation at 70ºC for 5 mins. The synthesized cDNA was stored at -20ºC until further use.

PCR and sequencing: After cDNA synthesis, polymerase chain reaction (PCR) was performed in a thermal cycler Applied Biosystems GeneAmp PCR System 2700 in the final volume of 30ul using reported primers (WHO, 2002).

In-ovo repeated treatment of oseltamivir: To investigate the neuraminidase gene associated mutations in response to repeated treatment of oseltamivir in embryonated chicken eggs. Pronto (Oseltamivir) 75mg capsules available from the pharmacy in Lahore were used and stock solution was made by using sterile water for injection as a diluent. From stock of 75mg/5ml, different concentrations of antiviral were made as 8ug/ml, 16ug/ml, 32ug/ml, 64ug/ml, and 128ug/ml. The wild type H9N2 (H9N2-wt) virus diluted to $100EID_{50}$ followed by treatment with oseltamivir with dose rate 1.6ug/0.2mL/inoculum per egg and incubated at 37ºC for 2 hours. The eggs were further incubated at 37ºC and allantoic fluid was harvested after 72 hours. The second passage was carried out with $100EID_{50}$ virus from the harvested fluids of first passage with the dose rate 3.2ug/0.2ml/inoculum per egg. The eggs were incubated and harvested after 72 hours. The passaging of virus under increasing drug pressure was continued as third passage by using $100EID_{50}$ virus titer of second passage with drug concentration as 6.4ug/0.2ml/ inoculum per egg, fourth passage under 12.8ug/0.2ml/ inoculum per egg and fifth passage under oseltamivir concentration as 25.6/0.2ml/ inoculum per egg.

Haemagglutination test: Further comparison was done on the basis of HA test among reference virus (stock virus) and its passaging with antiviral (harvested fluid after NAI exposure) using the standard protocol described in OIE 2012.

Genotypic analysis for the NAI susceptibility: After collecting the allantoic fluids from each passage, they were subjected to molecular assays. RNA was extracted, followed by cDNA preparation, PCR amplification of NA and HA genes and gel electrophoresis. NA gene sequences were analyzed in Bio-Edit® software with CLUSTAL W algorithm to identify amino-acid substitutions (Khan *et al.,* 2023). The hemagglutinin (HA) gene of the H9N2 virus was sequenced by Sangers' sequencing method using specific primers which were used previously for PCR to identify probable compensatory mutations.

RESULTS

Molecular analysis of our study in Bio-Edit showed amino acid substitutions at five positions in Neuraminidase protein under the effect of increasing drug pressure. This indicates that significant mutations have occurred at residues including E119D, D151E, E276D, N294S and R371 as presented in table 1. The compensatory mutations in HA gene were also observed as L234Q, G274V, G377V and are presented in table 2.

The result showed significant mutations had occurred under increasing drug pressure which made the virus less susceptible and resistant to the Neuraminidase Inhibitor used in our study. which gives titer of virus even under increasing drug pressure. This claims that virus is present in every passage which gives an incidence that mutations observed are significant in resistance of virus towards NAIs.

Mutation analysis by using BIOEDIT: After sequencing of the NA and HA genes, alignment and mutation analysis was made by using BIOEDIT software and its CLUSTAL W application. Results obtained as at five positions in Neuraminidase protein undergo significant mutations under increasing drug pressure. These residues are E119D, D151E, E276D, N294S and R371K. Yellow highlighted points (Fig. 1, 2, 3) show mutations in NA gene from the reference virus wt-type H9N2 strain. A row and Passages 1, 2 show agglutination upto 11th well while under more drug pressure Passages 3, 4 and 5 show titer upto 10th well. 12th column showing bead formation indicates intact RBCs (Fig. 4).

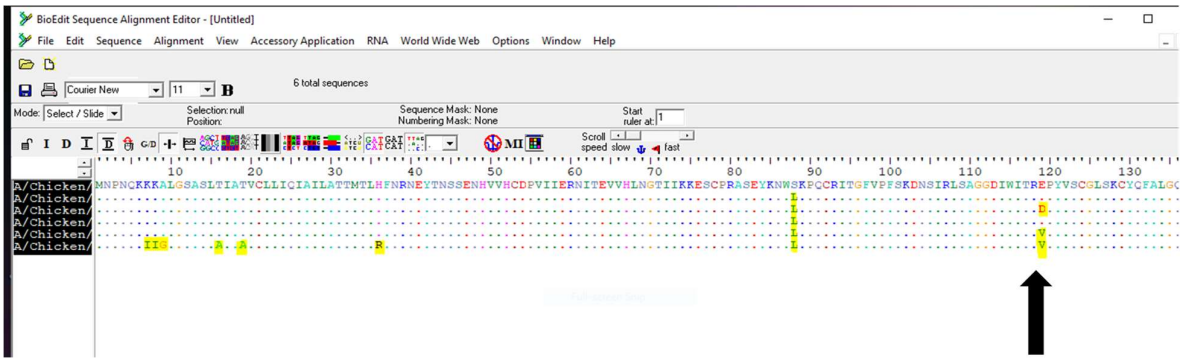

Fig. 1: Comparison of reference virus with 5 passages. Yellow highlighted points show mutations from the reference virus. At position 119 passage 2 show mutation from E to D and the E to V till passage 5. This was the mutation that is induced due to selective drug pressure.

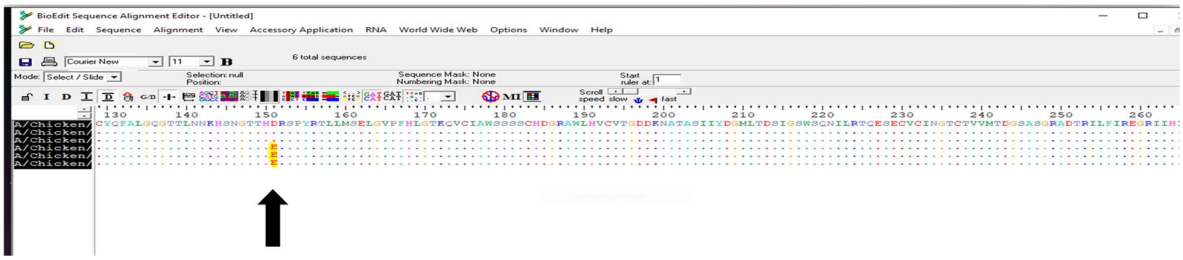

Fig. 2: Showing mutation at 151 position from D151E from passage 3 till passage 5 due to selective pressure of antiviral.

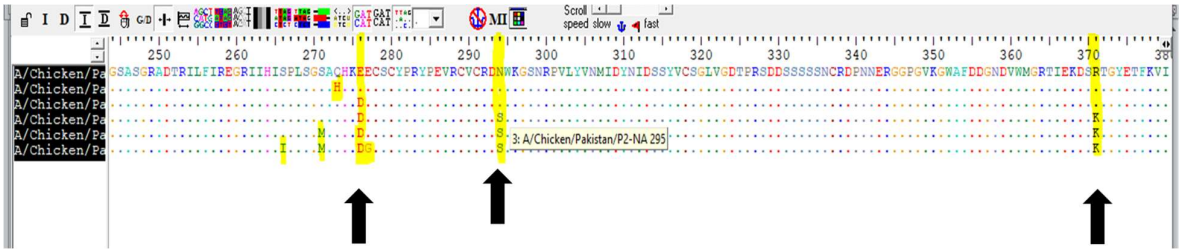

Fig. 3: At 276 position virus was mutated from E to D at passage 2 and persists till passage 5. At position 294, virus mutated from N to S from passage 3 till passage 5 and at 371 position, it was mutated as R371K from passage 3 till passage 5 under increasing drug pressure.

Table 1: Comparison of mutations among reference virus and passages under drug pressure in NA gene.

	Amino Acids Positions						
	7 , 8	9	16, 19	36	88	119	151
A/Chicken/Pakistan47/2019/H9N2) (H9N2-wt)	K	A	T	H	S	E	D
Passage 1 (1.6ug / 0.2mL/inoculum per egg)	K	A	T	H	L	E	D
Passage 2 (3.2ug / 0.2mL/inoculum per egg)	K	A	T	H	L	D	D
Passage 3 (6.4ug / 0.2mL/inoculum per egg)	K	A	T	H	L	E	E
Passage 4 (12.8ug / 0.2mL/inoculum per egg)	K	A	T	H	L	V	E
Passage 5 (25.6ug / 0.2mL/inoculum per egg)	I	G	A	R	L	V	E

	Amino Acids Positions						
	266	271	273	276	277	294	371
A/Chicken/Pakistan47/2019/H9N2) (H9N2-wt)	S	S	Q	E	E	N	R
Passage 1 (1.6ug / 0.2mL/inoculum per egg)	S	S	H	E	E	N	R
Passage 2 (3.2ug / 0.2mL/inoculum per egg)	S	S	Q	D	E	N	R
Passage 3 (6.4ug / 0.2mL/inoculum per egg)	S	S	Q	D	E	S	K
Passage 4 (12.8ug / 0.2mL/inoculum per egg)	S	M	Q	D	E	S	K
Passage 5 (25.6ug / 0.2mL/inoculum per egg)	I	M	Q	D	G	S	K

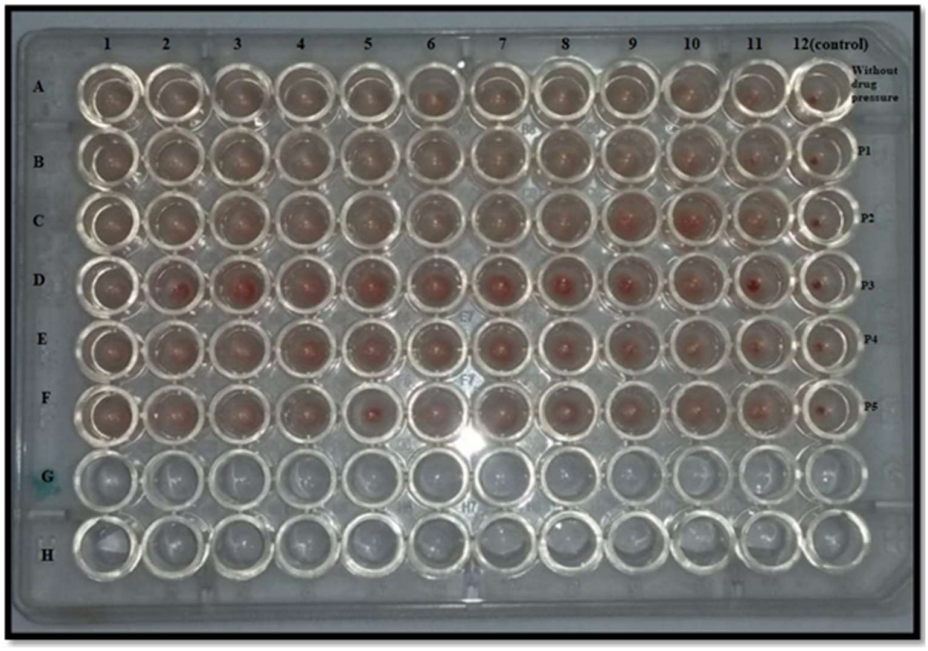

Fig. 4: Comparison of HA results of the reference virus (without antiviral pressure) with the 5 passages (under increasing drug pressure). Reference virus in A row and Passages 1, 2 show agglutination upto 11th well while under more drug pressure Passages 3, 4 and 5 show titer upto 10th well. 12th column showing bead formation indicates intact RBCs.

Table 2: Compensatory mutations in HA gene.

	Amino Acids Positions		
A/Chicken/Pakistan47/2019/H9N2) (H9N2-wt)	234	274	377
Passage 1 (1.6ug / 0.2mL/inoculum per egg)	L	G	G
Passage 2 (3.2ug / 0.2mL/inoculum per egg)	L	G	G
Passage 3 (6.4ug / 0.2mL/inoculum per egg)	Q	G	V
Passage 4 (12.8ug / 0.2mL/inoculum per egg)	Q	G	V
Passage 5 (25.6ug / 0.2mL/inoculum per egg)	Q	V	V

DISCUSSION

This study was designed by keeping in mind the main objectives i.e., H9N2 prevalence in poultry, drugs used against it, identification of any amino acid substitution in neuraminidase by increasing drug pressure and the importance of that mutation in terms of virus susceptibility.

A study conducted in India showed that influenza virus was mutated at residue R292K under oseltamivir pressure and E119D under zanamavir at passage 2 under 14ug/ml (2.8ug/0.2ml) that might reduce virus susceptibility to NAIs by 415 fold (Kode *et al.,* 2019). By comparing its results to the present designed study, residue 292 remained unmutated as R292 but with the E119D, four more residues were mutated, under only oseltamivir increasing concentrations. These are the significant mutations that enhance virus resistance towards neuraminidase Inhibitors. In accordance with the study conducted by Wu *et al.* (2018), residues 119, 151 and 371 are the binding domains of neuraminidase inhibitors and are functional residues , also our study correlated as under increasing drug pressure, these binding residues were mutated and these significant mutations made mutated virus resistant to the neuraminidase inhibitors.

Mutation observed in NA gene from passage 3 till passage 5 under Tamiflu pressure at position 294 as Asn294Ser is in correspondence with the study conducted on crystal structures of oseltamivir-resistant Influenza virus neuraminidase mutants by Collins *et al. (*2008).

In the present study, mutations in HA gene was observed as L234Q, G274V, G377V. L234Q is the compensatory mutation that was observed with the above discussed mutations in NA gene (Bloom *et al.,* 2010). Leucine at 234 also indicates its potency to cause human infections but it moved toward Q that is a typical virus signature (Butt *et al.,* 2010) at passage 3 and persists till passage 5 that makes it favorable for the α2,3 sialic acid receptors (Avian receptors). That might be due to propagation of virus up to 5 passages in-ovo.

Conclusion: The H9N2 AI virus mutates under drug pressure and that mutation is causing resistance to NAI in influenza virus. This resistance if left unchecked can lead to the spread of mutant viruses in the field. Hence, rational use of anti-viral along with continuous surveillance is necessary in order to control NAI resistant AI virus.

Authors contribution: RI conceptualized and designed the study. MF, SJ and KK assisted in conducting the experiments and analyzed the data. RI performed the experiments and interpreted the results. RI, KK and IM wrote the manuscript. NR edited the figures, tables and references in the manuscript.

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