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Assessing the susceptibility of highly pathogenic avian influenza H5N1 viruses to oseltamivir using embryonated chicken eggs

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***Background & objectives*: The susceptibility of influenza viruses to neuraminidase inhibitors (NAIs) is studied using enzyme-based assays, sequence analysis and *in vitro* and *in vivo* studies. Oseltamivir carboxylate (OC) is the active prodrug of the NAI oseltamivir. There is lack of information on the use of embryonated chicken eggs for studying susceptibility of highly pathogenic avian influenza (HPAI) H5N1 viruses to antiviral drugs. The aim of the present study was to assess the use of 10 day old embryonated chicken eggs for studying antiviral susceptibility of HPAI H5N1 viruses.**

***Methods*: Two HPAI H5N1 viruses isolated from India were used in the study. Fluorescence-based NAI assay was performed to determine antiviral susceptibility of these viruses. *In ovo* antiviral assays were carried out using 10 day old embryonated chicken eggs. The virus dilutions were incubated with 14 µg/ml of OC and inoculated in the allantoic cavity. In the eggs, 50 per cent egg infectious dose (EID_{50}) titres as well as mortality were quantitated.**

***Results*: The two viruses used were susceptible to OC in the NAI assay. It was found that there was a significant drop in EID_{50} titres; however, no significant protection from mortality after OC treatment was observed.**

***Interpretation & conclusions*: By measuring viral titres, the egg model was suitable to study the susceptibility of HPAI viruses to antiviral drugs along with NAI assay. The present study highlights the use of eggs as a model to study susceptibility of HPAI viruses to OC.**

Key words Antivirals - embryonated chicken eggs - H5N1 - highly pathogenic avian influenza - oseltamivir

The emergence of resistance among highly pathogenic avian influenza (HPAI) H5N1 viruses to oseltamivir has been an increasing cause of concern among public health professionals[1]. The virus has been known to infect humans associated with poultry[2] and has caused >159 outbreaks in poultry in different parts of India since 2006, with the most recent outbreaks in Odisha from January to September 2019, in which crows and backyard poultry birds died[3]. There are four neuraminidase inhibitor (NAI) antiviral drugs

namely, oseltamivir phosphate, zanamivir, laninamivir and peramivir, of which oseltamivir phosphate and zanamivir are the two currently licensed NAI drugs used for the treatment and prophylaxis of influenza[4]; peramivir is licensed for use in the United States of America[5]. Recently resistance to the NAIs oseltamivir and zanamivir was reported among H5N1 viruses isolated in India[6]. Due to concerns regarding the emergence of resistance to antivirals and the possibility of human infections, it is imperative to carry out regular antiviral surveillance of HPAI H5N1 viruses.

Advanced molecular-based assays including Sanger sequencing, real-time reverse transcription polymerase chain reaction (RT-PCR), pyrosequencing and next-generation sequencing are used to determine genotypic mutations leading to resistance to adamantanes in influenza viruses. However, for the determination of susceptibility or resistance to NAI drugs, the fluorescence-based NAI assay and neuraminidase (*NA*) gene sequence analysis are considered the gold standard[7]. *In vitro* cell culture susceptibility studies have used Madin Darby Canine Kidney cells, whereas *in vivo* studies include experiments in mice and ferrets. In ferrets, oseltamivir doses of 5.0 mg/kg twice daily had a significant impact on reducing the severity of the disease and survival[8]. Oseltamivir has also been known to prevent mortality in mice infected with lethal doses of HPAI H5N1 virus[9]. For *in vitro* studies, a reduction in viral haemagglutination (HA) titres was considered as a measure of drug susceptibility[10]. In addition to these methods, the use of 10 day old embryonated chicken eggs has been proposed as a model to study antiviral susceptibility of HPAI H5N1 viruses. *In vivo* studies in mouse and ferret model provide more insight into the exact mechanism of drug resistance; however, ferrets are not easily available, expensive and require specialized housing facility. The embryonated chicken eggs, on the other hand, are easily available and cost-effective. The chick embryo is the standard host system for the propagation and isolation of avian influenza viruses[11]. Since the *in ovo* system (using embryonated chicken eggs) is considered to be at the borderline of *in vitro* and *in vivo* studies, both the parameters; embryo mortality, as well as the HA titres of the virus in the allantoic fluids of the eggs, were assessed as indicators of virus susceptibility to the drug.

The use of embryonated chicken eggs to study the susceptibility of influenza H3N2 virus to NAIs has been reported[12]. The susceptibility of low pathogenic avian influenza viruses to oseltamivir carboxylate (OC), the active metabolite of oseltamivir phosphate, has also been demonstrated[13]. The advantages of the use of embryonated chicken eggs are that these provide a live yet controlled host environment for the optimal interactions of the virus and the drug, and are also a cost-effective option before going for *in vivo* studies. The HPAI H5N1 viruses cause 100 per cent mortality in embryonated chicken eggs, chickens being the compatible host. The objective of the present study was thus to assess embryonated chicken eggs as a model for studying the susceptibility of the HPAI H5N1 viruses to OC.

Material & Methods

The H5N1 viruses used in the study, A/chicken/India/NIV33487/2006 (H5N1-33487) (GenBank accession no. EF362420.1) and A/duck/India/TR-NIV4396/2008 (H5N1-4396) (GenBank accession no. CY046104.1), were isolated from outbreaks of HPAI H5N1 in poultry from the States of Maharashtra and Tripura, India, respectively. The following amino acids were present in the respective positions in the NA of the H5N1 viruses: I97, E99, D179, H255 and N275. Thus, these viruses did not possess any known molecular markers for antiviral resistance[14]. To prepare stocks, the virus isolates were propagated in 10 day old embryonated chicken eggs (Venkateshwara Hatcheries, Pune) as described previously[13]. Briefly, the virus isolates were inoculated in 10 to 11 day old embryonated chicken eggs by the allantoic route. The eggs were incubated for 72 h at 37°C in a humidified incubator (Meta-Lab Scientific Industries, Mumbai) and were observed daily. After completion of the incubation, the embryos were chilled overnight at 4°C. The allantoic fluid was harvested, and HA assay was performed using 0.5 per cent turkey red blood cells[11]. The virus stock was stored at −80°C. HPAI H5N1 viruses were handled in a biosafety level (BSL) 3+ laboratory, and other viruses were handled in a BSL 2 laboratory in a class II A2 biosafety cabinet. The standard sensitive and resistant reference viruses for H5N1 are not available; therefore, the sensitive and resistant H1N1, as well as pH1N1 strains provided by the International Society for Influenza and other Respiratory Virus Diseases[15] (ISIRV)-antiviral group namely, A/Mississippi/3/2001 (H1N1 wild type), A/Mississippi/3/2001 (H1N1 H275Y variant) (GenBank accession no. JF972564.1), A/Perth/265/2009 [pandemic H1N1 (pH1N1) wild

type] (GenBank accession no. HM624082.1) and A/Perth/265/2009 (pH1N1 H275Y variant) which were sensitive and resistant to oseltamivir were used as controls. The experiments were carried out at the Avian Influenza and High Containment Laboratory, ICMR-National Institute of Virology, Pune, India from December 2015 to October 2016.

Fluorescence-based neuraminidase inhibitor (*NAI*) *assay*: For use in the NAI assay, HPAI H5N1 viruses were inactivated using 0.1 per cent formalin (Fisher Scientific, New Hampshire, USA) as described elsewhere[16]. Briefly, 0.1 per cent of formalin (by volume) was added to the virus stock and mixed thoroughly. The mixture was transferred carefully to a fresh, previously labelled container, at the bottom, making sure that there was no fluid sticking to the walls or the brim, and incubated at 37°C for 16 h (overnight). Confirmation of inactivation was carried out by two passages in embryonated chicken eggs and carrying out HA assay for the allantoic fluids. The fluorescence-based NAI assay was carried out as per the method described previously[17]. The appropriate virus dilution of each virus sample to be used in the NAI assay was determined by NA activity titration. For NAI assay, the determined virus dilutions were incubated with serial ten-fold drug dilutions ranging from 30000 to 0.03 nM OC. The artificial fluorogenic substrate, 2'-(4-methyl-umbelliferyl)-α-D-N-acetylneuraminic acid (MUNANA, Sigma-Aldrich, USA) was added. After the specified incubation, the reaction was stopped, and the fluorescence was measured in a fluorometer (VictorX Multilabel plate reader, PerkinElmer, USA) at an excitation wavelength of 365 nm and an emission wavelength of 460 nm. The IC_{50} values were calculated using the curve-fitting software JASPR (v1.2, Centers for Disease Control and Prevention, USA).

Antiviral drug: For *in ovo* antiviral assays, OC stock of 56 µg/ml concentration (F. Hoffmann-La Roche Ltd., Basel, Switzerland) was prepared in phosphate-buffered saline (PBS, *p*H 7.2) from which 14 µg/ml drug suspension was prepared. This concentration of the administered drug led to a final resultant concentration of 0.35 µg/ml in the allantoic fluid of the eggs (taking into consideration its double dilution after mixing in an equal volume of virus suspension and further dilution in 10 ml of allantoic fluid in the egg). This concentration mimics the peak plasma concentration of OC in humans after the prescribed 75 mg dose of oseltamivir phosphate[18]. In a previous study, 1.75, 3.5, 7, 14, 28, 56 and 112 µg/ml doses were tested against 100 EID_{50} (50% egg infectious dose) virus. OC concentrations of 14 µg/ml and above showed complete inhibition of the virus. Therefore, 14 µg/ml of OC was chosen for the *in ovo* antiviral assays.

In ovo antiviral assays: *In ovo* antiviral assays were carried out using 10 to 11 day old embryonated chicken eggs using the *in vitro* drug treatment method described previously[13]. Serial ten-fold dilutions of the virus (prepared in PBS) were mixed with an equal volume of 14 µg/ml of OC and incubated at 37°C for one hour. After incubation, 0.4 ml of this mixture was inoculated via the allantoic route, into 10 eggs per dilution. The untreated virus controls of corresponding dilutions were also inoculated into 10 eggs each. The eggs were incubated at 37°C for 72 h in a humidified incubator. The viability of the embryos was checked daily by candling. Eggs showing mortality were immediately transferred to 4°C. After completion of incubation, all eggs were chilled overnight at 4°C. The allantoic fluid was harvested from each egg, and HA assay was performed. EID_{50} which is defined as the reciprocal of the dilution of virus per unit volume that results in positive HA titres in 50 per cent of the inoculated eggs and 50 per cent egg lethal dose (ELD_{50}, defined as the reciprocal of the dilution of virus that results in death in 50% of the inoculated eggs) were calculated using the Reed and Muench method[19]. For $EID_{50,}$ eggs showing HA titres ≥2 HA units were considered positive, whereas for ELD_{50}, egg mortality within the 72 h after inoculation was the criterion for positivity.

Statistical analysis: Statistical analyses were carried out using Microsoft Excel 2007 (Microsoft Corporation, USA). Student's *t* test was performed to compare the log EID_{50}, ELD_{50} as well as log HA titres of the treated as well as untreated groups.

Results

Fluorescence-based NAI assay: The H5N1 viruses, H5N1-33487 and H5N1-4396, had mean IC_{50} values of 0.16 and 0.75 nM, respectively, which were in the normal inhibition range, indicating that the two HPAI H5N1 virus isolates were sensitive to OC. The control pH1N1 and H1N1 wild-type reference standard viruses showed mean IC_{50} values of 0.07±0.03 and 0.16±0.04 nM, respectively, whereas pH1N1 and H1N1 variant controls showed mean IC_{50} values of 46.45±1.32 and 42.92±3.06 nM, respectively. All values mentioned

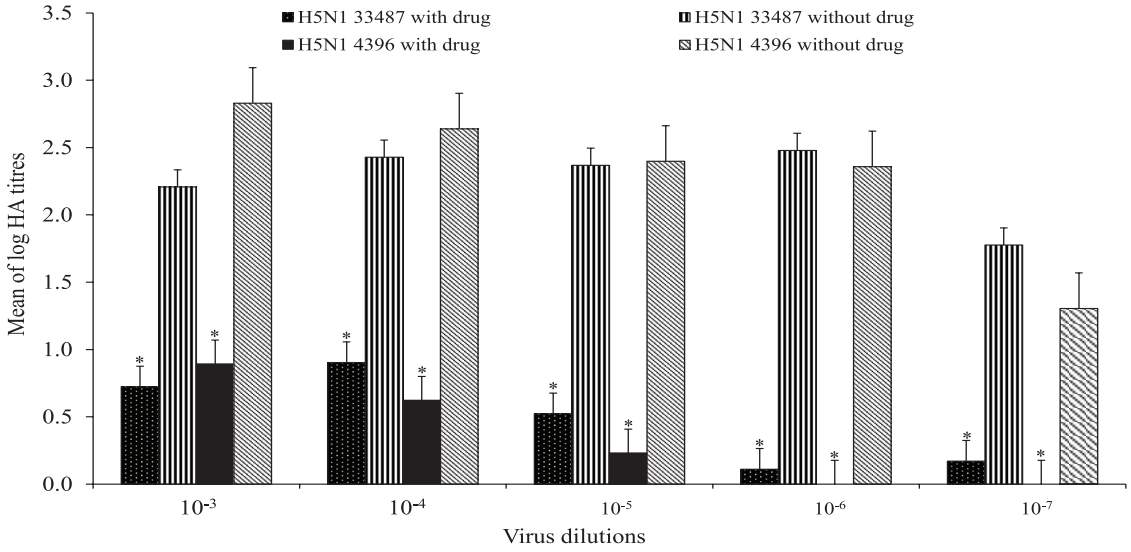

Figure. Comparison of mean log haemagglutination titres of H5N1 viruses with and without drug. *P<0.05 compared to respective without drug values. Comparison of mean log haemagglutination titres of A/chicken/India/NIV33487/2006 and A/duck/India/TR-NIV4396/2008 with and without treatment with 14 µg/ml oseltamivir carboxylate, plotted against virus dilutions. Values are mean of log haemagglutination titres from triplicate experiments, with ten eggs per dilution in each experiment. Standard error bars have been shown.

here are the means with standard deviations of at least four replicates.

In ovo antiviral assay: Both H5N1-33487 and H5N1-4396 viruses showed a significant drop (P<0.05) in HA titres after OC treatment, as compared to untreated controls (without drug). A significant reduction in the HA titres of treated viruses for every individual dilution ranging from 10^{-3} to 10^{-7} was observed as compared to respective controls (Figure). There was also a one-log drop in EID_{50} titres, but no significant reduction in ELD_{50} (Table). The H1N1 wild-type and pH1N1 wild-type control reference viruses showed the EID_{50} titres of $10^{6.0}$ and $10^{3.7}$, respectively, in the absence of OC, as compared to the treated groups, which had nil EID_{50} titres for both. H1N1 variant and pH1N1 variant control viruses showed the EID_{50} titres of $10^{4.8}$ and $10^{6.0}$, respectively, in the presence of OC, while the titres of $10^{4.7}$ and $10^{6.0}$ in the absence of OC. Thus, there was no significant drop in EID_{50} titres of the variant control viruses. There was no mortality in eggs inoculated with any of the control viruses.

Table. Inhibitory concentration$_{50}$ (IC_{50}) values, 50% egg infectious dose (EID_{50}) and 50% egg lethal dose (ELD_{50}) of viruses

Viruses	Mean IC_{50} values (in nM)	EID_{50}[b]		ELD_{50}[c]	
		With drug[d]	Without drug[e]	With drug[d]	Without drug[e]
A/chicken/India/NIV33487/2006 (H5N1)	0.16	$10^{4.80*}$	$10^{7.52}$	$10^{7.04}$	$10^{7.49}$
A/duck/India/TR-NIV4396/2008 (H5N1)	0.75	$10^{4.96*}$	$10^{7.02}$	$10^{6.77}$	$10^{6.84}$
A/Mississippi/3/2001 (H1N1) Wild type	0.16	$10^{6.0*}$	0	No mortality[f]	
A/Mississippi/3/2001 (H1N1) Variant	42.92	$10^{4.8}$	$10^{4.7}$		
A/Perth/265/2009 (pH 1N1) Wild type	0.07	$10^{3.7*}$	0		
A/Perth/265/2009 (pH 1N1) Variant	46.45	$10^{6.0}$	$10^{6.0}$		

*P<0.05 compared to respective without drug values.
[a]Mean IC_{50} values in the fluorescence-based neuraminidase inhibitor assay of at least three replicates; [b]EID_{50} per 0.2 ml, presence of HA titre ≥2 was a measure for positivity; [c]ELD_{50} per 0.2 ml, presence of mortality in eggs was a measure for positivity. No significant difference in ELD_{50} between with drug and without drug groups was observed.
[d]Mean of EID_{50}/ELD_{50} in the presence of 14 µg/ml oseltamivir carboxylate, carried out in three replicates each; [e]Mean of EID_{50}/ELD_{50} in the absence of 14 µg/ml oseltamivir carboxylate, carried out in three replicates each; [f]Since there was no mortality observed in the eggs inoculated with the H1N1 and pH1N1 viruses, the ELD_{50} values were not calculated

Discussion

It was reported earlier that in mice challenged with lethal doses of HPAI H5N1 virus, a daily dose of 1 to 10 mg per kg oseltamivir prevented mortality, reduced virus titres in the lungs and prevented the spread of the virus in the brain[9]. HPAI H5N1 causes severe disease in ferrets, and a significant reduction in morbidity and mortality has been recorded after treatment with oseltamivir[8]. The two HPAI H5N1 viruses used in the present study were representative isolates, which were found to be sensitive to OC. Since HPAI H5N1 viruses possessing molecular markers for resistance to NAIs have been reported in India[6], it is imperative that such models be developed which enable rapid as well functional screening for antiviral resistance.

In a previous study, an influenza H3N2 virus was used to study the susceptibility to NAIs[12] wherein the survival rate of the embryos was used to judge antiviral efficacy. In the present study, it was expected that inhibition of mortality in embryonated eggs would be an appropriate measure to ascertain the susceptibility of HPAI H5N1 viruses to OC. Surprisingly, there was no significant drop in the ELD_{50} after OC treatment. No protection was evident even after high doses of the drug were administered although significant reduction in the HA titres was observed. This disparity between the reduction in HA and EID_{50} titres in contrast with the ELD_{50} could be due to the reason that in eggs inoculated with the higher virus dilutions also, mortality was observed. Such eggs were however, negative for HA. This could be due to the possibility of the presence of virus titres below the limit of detection of the HA assay. Thus, for HPAI H5N1 viruses, in spite of mortality, monitoring a significant drop in HA titres was found more appropriate to assess the antiviral susceptibility. Therefore, it is important to standardize infectious virus titres and select the virus dilutions accordingly in antiviral susceptibility studies. As per our study, the virus dilution corresponding to 100 EID_{50} was found suitable to be used in antiviral experiments.

The eggs used in all the experiments were 10 day old at the time of inoculation and 13 day old at the time of completion of the experiment. Such experiments on less than 14 day old embryonated chicken eggs are considered at the borderline of *in vitro* and *in vivo* studies[20]. It was shown that after amantadine addition in cell cultures infected with susceptible HPAI H5N1 viruses, there was an absolute reduction in HA titres, whereas the amantadine resistant viruses, which possessed the molecular signatures required for resistance, grew to positive HA titres[10]. Thus, parallels could also be drawn between such *in vitro* studies and *in ovo* experiments carried out in the present study. This was due to the observation that a significant reduction in the viral HA titres was seen after treatment with OC for the H5N1 viruses and oseltamivir sensitive wild-type control reference viruses; whereas, there was no significant reduction in variant-type control viruses, which were known resistant, thereby indicating that the embryonated chicken egg model was suitable for carrying out susceptibility studies.

The ISIRV antiviral working group provides standard wild-type and resistant reference viruses with known IC_{50} values range to be used as controls for NA inhibition assays[14]. Due to known susceptibility of the reference viruses, they were used in the *in ovo* antiviral assays to demonstrate the validity as well as relevance of the use of EID_{50} titres in the egg model. In the study by Wang *et al*[20], the antiviral drug ribavirin was administered via the albumen route, while the virus was inoculated via the allantoic route. It was found that ribavirin administered in the albumen of the egg is detectable in the allantoic fluid even after 72 h of administration[20]. In the present study, OC which is the active metabolite of the prodrug oseltamivir phosphate, was administered directly into the allantoic cavity itself from where nutrition to the embryo is provided.

Animal experiments provide reliable data for the mechanisms of drug susceptibility or resistance due to the fact that the results can be monitored over a longer period of time, with simultaneous sampling whereas the experiments in the embryonated chicken eggs have limitations of time and sampling. It has been observed that sometimes substitutions in the viral NA protein do not necessarily lead to an apparent phenotypic change in the susceptibility of the virus to NAIs[14]. Thus, it is necessary to carry out further phenotypic susceptibility analysis of the viruses in addition to sequencing analysis. In conclusion, the present study shows that the fluorescence-based NAI assay in conjunction with *in ovo* antiviral assay may be employed for carrying out susceptibility screening during antiviral surveillance. In view of the emergence of new influenza viruses at the animal-human interface, such studies are necessary to develop antiviral surveillance and pandemic preparedness strategies.

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***Conflicts of Interest*:** None.

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