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IN VITRO CHARACTERIZATION OF OSELTAMIVIR RESISTANCE AMONG H5N1 VIRUSES ISOLATED FROM INDIA

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Abstract: Avian influenza is one of the most important zoonotic diseases with its potential ability to cause pandemics. Stockpiling of antiviral drugs is taken as a well recognized strategy for treatment and control of pandemics in most of the countries. There is knowledge gap about the development of antiviral resistance and replication capacity of the resistant strains. Oseltamivir resistance in 9 selected H5N1 viruses was studied by *in vitro* neuraminidase inhibition assay and IC_{50} values were calculated. *In vitro* neuraminidase inhibition assay revealed out of 9 selected H5N1 virus isolates eight were susceptible to oseltamivir carboxylate and one (A/Chicken/West Bengal/81010/2008) was resistant. The IC_{50} values of susceptible virus varied between 0.1 nM to 3.4 nM where as that of resistant virus was (45nM).
Keywords: Oseltamivir, Neuraminidase inhibition assay. Point mutation.

Introduction

Avian influenza is an emerging zoonotic disease with potential of causing great economic loss. Influenza viruses are classified based on two surface glycoproteins expressed on virus particles: Hemagglutinin (HA) and Neuraminidase (NA). There are a total of known 18 HA and 11 NA serologically distinct influenza virus subtypes and any combination of HA and NA is possible (Tong *et al*., 2013). Influenza A viruses infecting poultry are further classified as high pathogenic avian influenza (HPAI) or low pathogenic avian influenza (LPAI) depending on their ability to cause disease in chickens (*OIE*, 2008). The HPAI causes mortality as high as 100% in poultry species. Avian influenza viruses pose a threat to both avian and animal populations because of their capacity to initiate epornitics, to mutate from low to high pathogenicity in poultry, and to be transmitted as strains with the potential to initiate human pandemics (Guan *et al*., 2004). The influenza virus is continually evolving and under immune pressure; it may either evolve through small gradual changes in the virus (antigenic drift) or through abrupt major changes in the virus (antigenic shift) most frequently by genetic reassortments (CDC, 2010). Such changes can result in the emergence of new influenza viruses that can cause pandemics (CDC, 2010). The unpredictable nature of

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influenza presents a challenge for both research and pandemic preparedness planning (Taubenberger *et al.,* 2007). Most of the countries stockpile anti influenza chemotherapeutic agents as a well recognized strategy of pandemic preparedness. Neuraminidase (NA) inhibitors (mainly orally administered oseltamivir) and matrix 2 (M2) ion-channel blockers or adamantanes were used earlier but due to rapid development of resistance against M2 blockers especially under drug pressure and even in the absence of drug pressure limits their use and are nowadays not recommended for use (Deyde *et al*., 2009). Oseltamivir was introduced into clinical practice in various countries between 1999 and 2002. The amino acid substitution that causes oseltamivir-resistance is strongly related to drug usage, and no oseltamivir-resistant strain was found before the use of the drug (McKimm-Breschkin *et al*., 2003). Soon after, studies from other countries in Europe also reported the isolation of oseltamivir resistant viruses, and eventually, oseltamivir resistance was recognized as a global phenomenon (Dharan, 2009). The increasing use of influenza virus neuraminidase (NA) inhibitors (NIs) necessitates the development of reliable methods for assessing the NI susceptibility of clinical isolates. The most obvious requirement to prevent and manage a future influenza pandemic is expansive influenza surveillance. Since anti-influenza therapeutics are urgently needed in events of influenza pandemics (Layne *et al.,* 2009) studies on development antiviral resistance among various subtypes influenza A viruses are requisite. With the escalating use of NA inhibitors, routine monitoring of isolates for drug resistance will require reliable and relatively simple assays. Therefore, surveillance of oseltamivir resistance by Fluorometric Neuraminidase Inhibition assay is essential for influenza pandemic preparedness.

Material and Method

Avian Influenza virus

Representative viruses from various outbreaks during 2012 and 2014 were taken up for the study and obtained from “Avian Influenza Virus Repository” of National Institute of High Security Animal Disease (NIHSAD), Bhopal, Madhya Pradesh. The study was conducted at BSL 3+ containment laboratory, National Institute of High Security Animal Disease, Anand Nagar, Bhopal, 462021, M.P.

Optimization of virus dilution for Neuraminidase Activity

NA activity was determined by using 2’-(4-methylumbelliferyl)-α-D-N-acetylneuraminic acid(MUN; Sigma Chemical Co., St. Louis, Mo.) as a substrate. Viruses used in the NAI assay were grown in embryonated chicken eggs and were obtained from the allantoic fluid

after centrifugation at 2,000 × *g* for 10 min. The NA activity of each virus was determined before it was used in NA inhibition tests.

Neuraminidase Inhibition Assay Procedure

Fluorescence-based NA inhibition assay was used to determine the 50% inhibitory concentration (IC_{50}) of viruses to the NA inhibitor compound oseltamivir carboxylate. A series of 10-fold dilutions of the NA inhibitor oseltamivir carboxylate was prepared in assay buffer to achieve concentrations ranging from 0.00002 to 20 µM. A 50µl aliquot of each virus was mixed with an equal volume of the prepared concentration of oseltamivir carboxylate in black microtitre plate. Following a 45 minute incubation period at room temperature, 50 µl of MUNANA substrate was added to the virus/inhibitor mix. Following incubation at 37 ◦C for 60 minute, the reaction was terminated by the addition of 100µl of stop solution. Fluorometric quantification of 4-methylumbelliferone was determined with a Fluorometer (promega) spectrophotometer (excitation wavelength, 360 nm; emission wavelength, 448 nm and the 50% inhibitory concentration (IC_{50}) was calculated.

Result and Discussion

Antiviral agents play a major role in the control of influenza outbreaks and are also expected to confer significant prophylactic and therapeutic benefits during an influenza pandemic. The NA inhibitors are an important component of influenza pandemic preparedness. Antiviral resistance among highly pathogenic influenza A (H5N1) viruses isolated worldwide in 2002-2012 shows need for continued monitoring. In preparation for a highly pathogenic avian influenza H5N1 outbreak, many countries have stockpiled oseltamivir. However, the isolation of oseltamivir-resistant H5N1 viruses necessitates the development of novel antivirals that are effective against oseltamivir-resistant viruses. A total of 9 influenza A viruses isolated during the period of 2012-2014 were selected and assayed for sensitivity to the NAI oseltamivir. Neuraminidase Inhibition assay showed that eight avian H5N1 viruses were susceptible to NA inhibitor oseltamivir except A/Chicken/West Bengal/81010/2008 virus. The IC_{50} values of oseltamivir for 8 sensitive viruses oscillated between 0.1 nM to 3.4 nM which is in consensus with what had been reported earlier (Govorkova *et al*., 2009). Oseltamivir resistance was described as at least 10 fold increase in IC_{50} value than that of the wild-type (WT) virus (Mishin *et al*., 2005). A/Chicken/West Bengal/81010/2008 isolate demonstrated a significantly raised IC_{50} (45 nM) that was approximately 450-fold higher than the other A (H5N1) isolates tested. This study has demonstrated that avian H5N1 viruses are highly susceptible to NA inhibitor oseltamivir except A/Chicken/West Bengal/81010/2008

isolate in NA inhibition assay. A/Chicken/West Bengal/81010/2008 isolate in the current study showed increase in IC_{50} value than sensitive virus and was confirmed to possess resistance to the oseltamivir through Neuraminidase inhibition assay. This data demonstrate the significance of continued characterization of all H5N1 isolates for susceptibility to NA inhibitors in order to identify novel NA markers of altered susceptibility. The obtained results are essential for planning appropriate management strategies for a future H5N1 pandemic. Future efforts should focus on refining the design of current NAIs, developing new classes of anti-influenza agents, and exploring possible combination therapy regimens. Meanwhile, the emergence of resistant variants among H5N1-infected patients receiving NAI treatment should be closely monitored.

Conclusion

Emergence of novel mutation in H5N1 avian isolates alarms the threat to human population since the potential of H5N1 viruses to cross species barrier has been established earlier. Hence continuous surveillance study both *in vitro* for antiviral resistance to oseltamivir must be an urgent & mandatory requirement for a country like India with poultry population more than 37 billion to keep the country prepared in the pandemic eventuality with the dreaded disease, Avian Influenza.

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