Microbial Pathogenesis 91 (2016) 35–40

Contents lists available at ScienceDirect

Microbial Pathogenesis

journal homepage: www.elsevier.com/locate/micpath

Amantadine resistance among highly pathogenic avian influenza viruses (H5N1) isolated from India

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ARTICLE INFO

Article history:
Received 17 July 2015
Received in revised form
3 November 2015
Accepted 11 November 2015
Available online 27 November 2015

Keywords:
Avian influenza
Amantadine
Antiviral resistance
Matrix

ABSTRACT

Emergence of antiviral resistance among H5N1 avian influenza viruses is the major challenge in the control of pandemic influenza. Matrix 2 (M2) inhibitors (amantadine and rimantadine) and neuraminidase inhibitors (oseltamivir and zanamivir) are the two classes of antiviral agents that are specifically active against influenza viruses and are used for both treatment and prophylaxis of influenza infections. Amantadine targets the M2 ion channel of influenza A virus and interrupts virus life cycle through blockade of hydrogen ion influx. This prevents uncoating of the virus in infected host cells which impedes the release of ribonucleoprotein required for transcription and replication of virion in the nucleus. The present study was carried out to review the status of amantadine resistance in H5N1 viruses isolated from India and to study their replicative capability. Results of the study revealed resistance to amantadine in antiviral assay among four H5N1 viruses out of which two viruses had Serine 31 Asparagine (AGT—AAT *i.e.*, S31N) mutation and two had Valine 27 Alanine (GTT—GCT *i.e.*, V27A) mutation. The four resistant viruses not only exhibited significant difference in effective concentration 50% (EC_{50}) values of amantadine hydrochloride from that of susceptible viruses ($P < 0.0001$) but also showed significant difference between two different types (S31N and V27A) of mutant viruses ($P < 0.05$). Resistance to amantadine could also be demonstrated in a simple HA test after replication of the viruses in MDCK cells in presence of amantadine. The study identifies the correlation between *in vitro* antiviral assay and presence of established molecular markers of resistance, the retention of replicative capacity in the presence of amantadine hydrochloride by the resistant viruses and the emergence of resistant mutations against amantadine among avian influenza viruses (H5N1) without selective drug pressure.

1. Introduction

Highly Pathogenic Avian Influenza (HPAI) H5N1 primarily affects birds but its potential to cross species barrier raises alarm of risk of pandemics in human population. The virus first infected humans in 1997 during a poultry outbreak in Hong Kong SAR, China [1]. Since 2003 the avian virus has spread from Asia to Europe and Africa with the widespread re-emergence and 63 countries have been affected with avian infections [2]. 844 human cases of HPAI H5N1 have been reported from 16 countries with more than 53% case fatality rate [3]. Antiviral prophylaxis and treatment are of prime importance in pandemic events since effective vaccines are not available against H5N1. The recommended strategy for pandemic preparedness is stockpiling of antiviral drugs against which resistance has not been detected regularly.

Adamantanes or M2 inhibitors and neuraminidase inhibitors are the drugs approved for treatment of human infections with influenza A viruses. Segment 7 or M gene of influenza A virus RNA encodes M2 protein which is a homotetrameric integral membrane protein that possesses ion channel activity [4]. The M2 protein comprises 97 amino acids of which 54 are in the cytoplasmic domain, 24 in the extracellular domain and 19 in the transmembrane domain [5]. The interaction between cytoplasmic domain of M2 and M1 is a prerequisite for genome packaging and formation of virus particles [6]. Transmembrane domain of M2 has proton channel activity that aids in virus disassembly during the initial stages of infection [7]. The adamantanes target the M2 ion channel of influenza A virus. The ion channel activity of M2 is

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http://dx.doi.org/10.1016/j.micpath.2015.11.008
0882-4010/

important during virion uncoating and viral budding [8]. M2 inhibitors interrupt virus life cycle by blocking the influx of hydrogen ions through the transmembrane ion-channel of M2 protein and prevent uncoating of the virus in infected host cells [9]. This prevents the release of ribonucleoprotein (RNPs) and the RNPs fail to enter the nucleus which is necessary for the normal transcription and replication of virion [10].

Since 2007 adamantanes are not recommended for use in H5N1 infected patients due to the uncertainty about the effectiveness and rapid development of resistance [11]. The point mutation in transmembrane region of M2 has been established to be responsible for resistance development. The well characterized mutation for resistance to adamantanes is associated with amino acid substitution at positions 26, 27, 30, 31 and 34 of the transmembrane region of M2 protein [12]. Adamantane resistance mutations among different subtypes of *Influenza A* viruses namely, H1N1, H1N2, H3N2, H4N2, H5N1, H5N2, H6N1, H6N6, H6N8, H9N2 and H11N3 have been reported earlier from Taiwan, Japan, South Korea, Mexico, Canada, Central and South America, United States, Eastern Europe, France, Italy, Brazil, China, Ukraine, Hong Kong, Russia, Saudi Arabia, Indonesia, Malaysia and India [13–18]. Circulating neuraminidase inhibitor (NAI) resistant influenza viruses from Indonesia, Mongolia, Turkey, Vietnam, China, Russia, and Hong Kong [14,19,20] were found to be sensitive to amantadine and hence combination therapy with amantadine and oseltamivir has also been considered in pandemic events caused by oseltamivir resistant strains [20]. H5N1 virus isolates with antiviral resistance have been reported from India having S31N and V27A mutations of matrix 2 protein [18,21].

Continuous surveillance of antiviral resistance is essential for pandemic preparedness. The growing public health concern about the emergence of resistant strains and reassortants with resistant genes which are capable of interspecies transmission hinder successful prophylaxis and therapy prompted us to study the amantadine susceptibility among H5N1 avian influenza viruses isolated from India during 2006–2013 based on antiviral bioassay and identification of established genetic markers.

2. Material and methods

2.1. Viruses, cells and eggs

Representative viruses were selected from various outbreaks in the country based on location and species involved. Twelve HPAI H5N1 virus isolates were identified and obtained from 'Avian Influenza Virus Repository' of ICAR-National Institute of High Security Animal Diseases, Bhopal, Madhya Pradesh, India (Table 1). The viruses were propagated in 10 day old Specific Pathogen Free (SPF) Embryonated Chicken Eggs (ECE) procured from Venkateswara Hatcheries, Pune, Maharashtra, India, as per WHO manual for the laboratory diagnosis and virological surveillance of influenza [22]. The harvested allantoic fluids from eggs were aliquoted and preserved at −80 °C till further use. Madin Darby canine kidney (MDCK) cells were obtained from American Type Culture Collection (ATCC), Manassas, VA and propagated in Eagle's Minimum Essential Media (Gibco, U.S.A.) containing 10% foetal bovine serum (Gibco, U.S.A.).

Virus stocks: MDCK adapted viruses were aliquoted and stored at −80 °C till further use.

2.2. Antiviral stock solution

Amantadine hydrochloride (Sigma, MO, USA) was dissolved in 1× Phosphate Buffered Saline to obtain 10 mg/ml final concentration and sterilized by filtration using 0.22μ filter, aliquoted and stored at 4 °C.

2.3. Virus adaptation to MDCK cell line

After initial passage in SPF ECEs, the virus isolates (allantoic fluid) were adapted to MDCK cell line at passage 56 in Eagle's Minimum Essential Media containing 10% foetal bovine serum. MDCK adapted viruses were titrated by 50% Tissue Culture Infective Dose ($TCID_{50}$). The viruses showing less than 10^4 $TCID_{50}$/ml were given second passage in MDCK cells. The passage was restricted to two passages to obtain a minimum titre of 10^4 $TCID_{50}$/ml.

2.4. Antiviral assay

Three independent antiviral assays were performed for each virus in confluent monolayer of MDCK cells in 96 well plate in triplicate by MTT (3-(4, 5- dimethyl thiazol-2yl)-2, 5-diphenyl tetrazolium bromide (MTT) or thiazolyl blue) assay as described by Smee et al. [23] and haemagglutination (HA) reduction assay [13]. The monolayer was infected with 100 $TCID_{50}$ of virus and the virus infected cells were treated with amantadine hydrochloride (Sigma, MO, USA) at varying concentrations ranging from 0 to 300 μg/ml (prepared from stock solution of 5 mg/ml in 1× PBS). For HA reduction assay, cell culture supernatants were removed from the plates after 48 h and subjected to HA assay. For MTT assay, the same plates were stained with 25 μl MTT dye (working solution having concentration of 0.5 mg/ml; Molecular Probes, U.S.A) and further the formazan crystals developed were dissolved using DMSO (Sigma, MO, USA) and the absorbance was measured at 550 nm in ELISA reader. Mean absorbance values of the triplicates were calculated for cell control and for test viruses at varying drug concentrations for each experiment. The absorbance developed in test wells was compared with control wells to calculate percent

Table 1
H5N1 viruses selected for the study with their EC_{50} values.

S. No.	Name of selected H5N1 viruses	Place of isolation	EC_{50} best fit Values (nM)
1.	A/chicken/7972/Navapur/India/2006	Nardurbar, Maharashtra	370.7
2	A/chicken/West Bengal/106181/2008	Maldah, West Bengal	2066
3	A/goose/Tripura/103596/2008	Darjeeling, West Bengal	1554
4	A/chicken/India/82544/2008	Tripura	654.2
5	A/chicken/India/82616/2008	Birbhum, West Bengal	1946
6	A/chicken/India/85459/2008	Hooghly, West Bengal	3498
7	A/chicken/West Bengal/155505/2009	South24-Paraganas, West Bengal	553.5
8	A/chicken/West Bengal/81010/2008	Coochbehar, West Bengal	4212
9	A/chicken/West Bengal/239022/2010	Murshidabad, West Bengal	987,054
10	A/chicken/India/241272/2010	Murshidabad, West Bengal	370,571
11	A/chicken/India/07TI01/2013	Durg, Chhattisgarh	47,811
12	A/chicken/India/08CA03/2013	Bastar, Chhattisgarh	12,058