DOI:10.1111/j.1750-2659.2009.00072.x
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Original Article

Antiviral resistance in influenza viruses circulating in Central and South America based on the detection of established genetic markers

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Accepted 10 January 2009. Published Online 10 February 2009.

Abstract

Background Recent influenza antiviral resistance studies reveal an alarming increase in both adamantanes and neuraminidase inhibitors (NAIs) resistant viral strains worldwide, particularly in Asia, Europe and the United States.

Objectives In this study, we have evaluated influenza virus resistance in Central and South America.

Methods Influenza viruses, isolated from symptomatic patients throughout Central and South America in 2005–2008 were analyzed for inhibitor resistance. The M2 and NA genes of influenza viruses were sequenced and resistance was inferred by comparison with published sequences and known resistant mutations.

Results Our results indicate that: (i) resistance to adamantanes was seen in the majority (95·5%) of the influenza A/H3N2 isolates but only in one isolate of the influenza A/H1N1 viruses; (ii) resistance to NAIs began to be detected in A/H1N1 isolates from Central America in 2008; and (iii) none of the influenza B viruses analyzed were resistant to NAIs.

Conclusions These findings suggest a limited effectiveness of influenza inhibitors due to the detection of resistance among A/H1 and A/H3 viruses.

Keywords Adamantanes, amantadine, influenza, neuraminidase Inhibitors, oseltamivir, resistance.

Please cite this paper as: García *et al.* (2009) Antiviral resistance in influenza viruses circulating in Central and South America based on the detection of established genetic markers. Influenza and Other Respiratory Viruses 3(2), 69–74.

Introduction

Influenza is a globally important contagion. Worldwide, each year about 20% of children and 5% of adults develop symptomatic influenza A or B.[1] Of the three types of influenza viruses (influenza A, B, and C), only types A and B typically cause widespread outbreaks. Type A influenza viruses are the major cause of influenza in humans and produce approximately half a million fatalities every year.[2]

Influenza viruses have segmented genomes and show great antigenic diversity. Their genome consists of 11 genes encoding for three transcriptases (PB1, PB2, and A), two matrix proteins (M1 and M2), two surface glycoproteins [hemagglutinin (HA) and neuraminidase (NA)], one nucleocapsid protein (NP), and three non-structural proteins (NS1, NS2, and PB1-F2). Based upon their antigenic differences in the HA and NA surface glycoproteins, influenza A viruses have been classified into several subtypes; to date, 16 hemagglutinin subtypes (H1–H16) and nine NA subtypes (N1–N9) have been identified.[3,4] Only three hemagglutinin subtypes (H1, H2, and H3) and two NA subtypes (N1 and N2) have circulated as stable lineages in human populations.

Although vaccination is the primary method used to prevent influenza infections in human populations, this strategy is not always possible in the developing setting. The use of antiviral agents is an alternative approach that can be utilized to abate infection or reduce severity of illness post-infection. These agents can be divided into two classes according to the viral protein they target: the M2 blockers or adamantanes (amantadine and rimantadine), and the NA inhibitors (oseltamivir and zanamivir).

Adamantanes inhibit the viral replication by blocking the proton channel formed by the M2 protein of the influenza A virus. Resistance can be achieved by a single substitution of any of the amino acid residues located at positions 26, 27, 30, 31, or 34 of the transmembrane domain of the M2 protein.[5,6] Recently, the incidence of adamantane resistance among the influenza A/H3N2 viruses has increased from 0·8% in the early 1990s to approximately 12·3% in 2004, reaching as high as 96% in certain regions of China.[7–10] Currently, the proportion of adamantane's resistance among influenza A/H1N1 viruses reaches a global average of only 5·8%.[10] There are three disadvantages in using adamantanes: (i) they have no activity on influenza B viruses because these viruses do not have the M2 protein; (ii) they have adverse side effects; and (iii) drug resistance emerges rapidly during treatment.

Neuraminidase inhibitors (NAIs) inhibit the enzymatic activity of the NA protein preventing the virion's release form the cell surface and thus, its dissemination and infection of adjacent cells. Resistance to NAIs involves a mutation in the active site of the NA protein at different positions depending on the virus subtype,[11–15] altering its sensitivity to inhibition. In contrast to the M2 blockers, the NAIs are licensed for treatment of both influenza A and B, resistance to NAIs is drug specific and they have fewer side effects.

Most influenza antiviral resistance studies have been conducted in East Asia, Australia, Europe and the US and revealed an alarming increase in both M2 and NA inhibitors resistant viral strains.[10,16–18] In Central and South America, viral inhibitors are not a commonly used treatment for influenza infection mostly because of their high cost and availability.[19,20] However, Deyde *et al.*[10] found a dramatic increase in M2 resistant A/H3N2 viruses in Central and South America with 7·2% resistance in 2004 and 96% in 2005. In this study we have analyzed the variants of influenza viruses circulating in this region of the world during the 3 year period July 2005–July 2008, focusing on the detection of the most commonly known mutations conferring antiviral resistance (resistance markers).

Material and methods

Specimen collection, isolation and identification of influenza viruses

Influenza A ($n = 466$) and B ($n = 216$) viruses were collected from nasopharyngeal and throat swab specimens, at hospitals throughout Central and South America (Nicaragua ($n = 29$), Honduras ($n = 17$), El Salvador ($n = 6$), Venezuela ($n = 12$), Paraguay ($n = 8$), Colombia ($n = 12$), Ecuador ($n = 48$), Bolivia ($n = 12$), Peru ($n = 520$), and Argentina ($n = 18$)) from patients that were part of the Naval Medical and Research Center Detachment–Lima (NMRCD-Lima) 'Influenza Surveillance Network'. These patients presented with a febrile respiratory syndrome, they had a temperature of ≥38°C, cough or sore throat and absence of other diagnosis. Once collected, swabs were placed in viral transport media and stored at −80°C until transported on dry ice to the NMRCD in Lima, Peru.

Virus isolation was carried out by inoculation in Madin-Darby canine kidney (MDCK) cell line without fetal bovine serum. After 7 days, viral identification was performed by indirect immunofluorescence. Viral isolates obtained from cell culture were used for subtyping the influenza viruses using hemagglutinin type-specific anti-sera (D3 ultra DFA respiratory virus screening & ID kit, Diagnostic hybrids).

RNA Extraction and RT-PCR

Viral RNA extraction was performed in a biosafety level-3 laboratory. Nucleic acid was extracted with the use of viral RNA kit (QIAamp, Qiagen®, Valencia, CA) and tested by reverse-transcriptase-polymerase-chain reaction (RT-PCR). Neuraminidase and matrix protein 2 genes were amplified by RT-PCR with the following specific primers: bases 617–995 (338 pdb fragment) of the influenza A matrix protein 2, M2-For3 (5′-CTAGTCAGGCCAGGCAAATG-3′) and M2-Rev (5′-ACTGTCGTCAGCATCCACAG-3′);[21] bases 449–1218 (769 pdb fragment) of the influenza A neuraminidase 1, AN1A (5′-AGGACAGAAGCCCTTATAGG-3′) and AN1DII (5′-TTAGCTCAGGATGTTGAACG -3′); bases 299–997 (698 pdb fragment) of the influenza A neuraminidase 2, AN2A (5′-ATTACAGGATTTGCACCTTT-3′) and H3N2-NA-2R (5′-GGGTGTGTCTCCAACAAGTCTGAGC-AC-3′); bases 352–641 (289 pdb fragment) of the influenza B neuraminidase, NA-RES-F (5′-GCTCTAACCCATTATG-CAG-3′) and NA-RES-R (5′-CTTTCTTGTGTTCTTAG-GATG-3′).

Sequencing and phylogenetic analysis

For direct sequencing of viral nucleic acids from clinical specimens, genes fragments were amplified and sequenced with the use of Big Dye terminator cycle sequencing kit (version 3.1; Applied Biosystems) on a Genetic Analyser system (version 3130xL; Applied Biosystems).

Gene sequences were assembled aligned and edited using Sequencher (version 4.7; Gene Codes Corporation) and BioEdit (version 7.0.0; Isis Pharmaceuticals, Inc.) softwares.

Susceptibility to amantadine

Amantadine sensitivity was determined by plaque assay on MDCK cells. Briefly, 24-well microplates containing MDCK cell monolayers were inoculated with virus diluted in minimal essential medium (MEM) (Gibco) to give 20–30 plaques per well. Cells were incubated for 1 hour at 37°C and then overlaid with MEM containing 3% carboxymethyl cellulose, 1 μg/ml L-1-(tosylamido-2-phenyl)-ethyl chlorom-

ethyl ketone and amantadine (1-aminoadamantane hydrochloride, Sigma-Aldrich) at different concentrations (from 0·1 to 1000 μg/ml). After 3 days of incubation at 37°C, plaques were visualized by staining with naphtol blue solution containing naphtol blue black (1 g/l), sodium acetate (13·6 g/l), and glacial acetic acid (6%). The stain was poured off from the microplates wells and the cell monolayers were gentle washed and allowed to air-dry and the number of lytic plaques were counted. The percentage of plaque reduction in the amantadine-treated infected cells relative to the untreated controls was calculated for each drug concentration. The drug concentration resulting in a 50% reduction of plaque number (IC_{50}) was determined.

Results and discussion

Adamantanes resistance among influenza a viruses

There is great controversy about the use of adamantanes for prophylaxis and therapy because their principal attraction is their lower cost and worldwide availability, but drug resistance emerges rapidly during treatment. The latter is of great concern because of the possibility of a highly virulent strain of influenza virus A causing the next influenza pandemic. Being that resistance can be achieved by a single substitution at the transmembrane domain of the M2 protein, we analyzed the M2 protein sequence of 466 influenza A viruses: 167 of which were influenza A/H1N1 subtype and 298 were influenza A/H3N2 subtype.

A total of 166 out of the 167 (99·5%) influenza A/H1N1 viruses analyzed were found to be susceptible to adamantanes. Only one isolate (0·5% of the total A/H1N1 samples) had a substitution in position 26 (L26F) which confers resistance to M2 blockers (Figure 1). In contrast, 285 out of 298 (95·5%) of the A/H3N2 viruses were found to have the specific S31N substitution which is well known to confer resistance to adamantanes. No other mutations conferring resistance were detected on the analyzed M2 gene.

Resistance to M2 blockers conferred by the S31N or by the L26F substitutions was verified by plaque assay. Ten percent of the samples were tested with increasing amounts of amantadine to determine the IC_{50}. The IC_{50} for all the sensitive samples tested oscillated between 0·1 and 0·5 μg/ml in agreement to what had been reported by others,[8,9,22] while the IC_{50} for the resistant samples (containing the S31N substitution or the influenza B samples (used as negative controls) that are not sensitive to adamantanes) oscillated around 1000 μg/ml or higher (Table 1), thus, showing a decrease in sensitivity of more than 2000 times between the resistant variants and the sensitive variants.

Resistance to neuraminidase inhibitors

Oseltamivir is a licensed NAI for treatment of influenza A and B that has fewer side effects than adamantanes. Influenza A/H1N1 viruses have been demonstrated to be resistant to oseltamivir when the residue Histidine 274 (position 275 of the N1 protein) on the NA gene is replaced by a tyrosine residue, other substitutions of the same residue are also possible. By sequence analysis and comparison with published sequences we found that seven of the 167 (4·2%) analyzed H1N1 isolates presented the H274Y substitution (Figure 2). No other substitutions described in the literature as conferring resistance to oseltamivir were found in these isolates. Interestingly, the seven resistant viruses were detected recently, in 2008, and came from the Central American region (Honduras, Nicaragua and Venezuela).

Influenza A M2 Protein Sequence Analysis for Adamantanes Resistance

Sequence	Subtype	Nº	%	Ada
11 26 31 70 IRNEWGCRCNDSSDPLVVAASIIGIVHLILWIIDRLFSKSIYRIFKHGLKRGPSTEGVPE	A/H1N1	166	99.5	S
IRNEWGCRCNDSSDP**F**VVAASIIGIVHLILWIIDRLFSKSIYRIFKHGLKGGPSTEGVPE		1	0.5	R
11 26 31 70 IKNEWGCRCNDSSDPLVVAASIIGILHLILWILDRLFFKCVYQLFKHGLKRGPSTEGVPE	A/H3N2	13	4.5	S
IRNEWGCRCNDSSDPLVVAA**N**IIGILHLILWILDRLFFKCVYRLFKHGLKRGPSTEGVPE		285	95.5	R

Nº: Number of Samples Analyzed
%: Percentage
Ada: Adamantanes Resistance (R= Resistant, S=Sensitive)

Figure 1. Adamantanes resistance among influenza A viruses. M2 protein sequence analysis for influenza A viruses (A/H1 viruses on upper panel and A/H3 viruses on lower panel). The figure shows the consensus sequence from residues 11 to 70. Positions where a substitution can confer resistance to adamantanes (26 and 31) are boxed and when substituted their position is marked in bold. Number (*n*) of samples found for each of the strains with their percentage among the population (%), Ada is the susceptibility to adamantanes (R, resistant; S, susceptible).

Table 1. *In vitro* resistance to amantadine

Sample	Date	Country	Type/ Subtype	Substitution conferring resistance	Predicted sensitivity	Predicted IC_{50}(μg amantadine/ml)
FLU5148	23-November-2006	Ecuador	A/H1N1	Leu26Phe	R	>1000
FLU4129	01-August-2006	Peru	A/H1N1	–	S	0·25
FLU5376	31-January-2007	Peru	A/ H1N1	–	S	0·3
FLU4499	13-October-2006	Nicaragua	A/H1N1	–	S	0·3
FLU3443	18-April-2006	Peru	A/H3N2	Ser31Asn	R	>1000
FLU3601	31-March-2006	Peru	A/H3N2	Ser31Asn	R	>1000
FLU6219	20-March-2007	Venezuela	A/H3/N2	Ser31Asn	R	>1000
FLU6849	09-May-2007	Ecuador	A/H3N2	Ser31Asn	R	1000
lQE5463	14-May-2007	Peru	A/H3N2	Ser31Asn	R	>1000
FLU5674	06-March-2007	Peru	A/H3N2	–	S	0·25
FLU5854	08-March-2007	Peru	A/H3N2	–	S	0·5
FSC0799	30-July-2005	Peru	A/H3N2	–	S	0·2
FLU6151	17-April-2007	Peru	B	–	R	>1000

The table shows the concentration of amantadine giving 50% of plaque assay inhibition (IC_{50}) for different viruses types and subtypes. The table shows the date and country where the samples were collected, the type and subtype determined based on sequence analysis of RT-PCR amplicons of the corresponding hemagglutinin and neuraminidase genes, the substitution conferring resistance to amantadine, the inferred sensibility to amantadine and finally their sensibility to amantadine found *in vitro*.

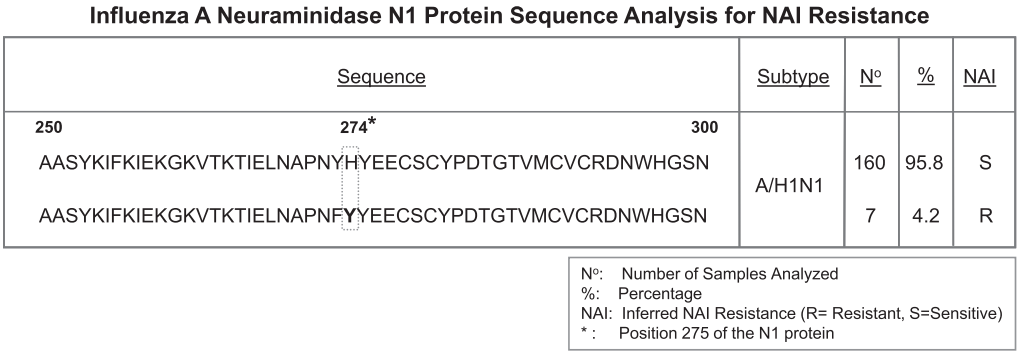

Figure 2. Resistance to NAIs among Influenza A viruses. Neuramidase protein sequence analysis for influenza A/H1N1 viruses. The figure shows the concensus sequence of the neuraminidase N1 gene from residues 250 to 300. Positions where a substitution can confer resistance to neuraminidase inhibitors (NAIs) are boxed and when substituted residues are in bold. Number (*n*) of isolates found for each of the strains with their percentage among the population (%), Ada is the susceptibility to adamantanes (R, resistant; S, susceptible).

In influenza A/H3N2 viruses, the substitution of many different residues of the NA 2 gene have been reported to confer resistance to oseltamivir. In this study, we have analyzed by sequencing the presence of substitutions on residues R118K, E119Q, D151E, R152K, R224K, E227D, E276D, and R292K and found that none of the 298 A/H3N2 isolates analyzed presented substitutions at any of this positions.

In the same way, we analyzed 216 influenza B viruses for substitution at positions 149, 152, 198, and 203 which can confer resistance to NAIs in influenza B viruses, and found that none of the isolates presented substitutions at these residues.

Our study shows that amantadine resistant strains of influenza have been in circulation in Central and South America during the last 3 years. As most of these resistance strains have the same substitution (Ser31Asn for the M2 gene of A/H3N2 viruses) and antiviral agents are not commonly used in this region, a common origin for these viruses is a strong possibility.[23] In agreement to previously published data,[24] these findings suggest that these resistant variants could have arisen in the absence

of any antiviral selective pressure, or if they have a common origin in places where antiviral agents are used, they must have an advantage for their maintenance even in the absence of these agents that contributed to their migration, global distribution and maintenance over a 3 year period.

The situation is slightly different in respect to neuraminidase resistance as oseltamivir resistant viruses were not detected in the first 2 years of influenza surveillance. The detection of resistant variants began only in the period 2007–2008 in Europe [25,26] and has remained at a relatively low rate, which is in agreement with the levels of oseltamivir resistance found by others until 2007.[18,27] The fact that the seven resistant viruses came from the Central American region strongly suggests that these viruses are arriving from the northern hemisphere. It is important to note that there have been other mutations described in the literature as conferring resistance and is possible that some lesser known mutants may have been missed in this study and, there are likely to be NA mutations that have not yet been described to have an impact on NAI sensitivity. Careful surveillance of influenza viruses in the next months will be needed to further characterize the spreading of this type of viruses among South American countries.

This finding somehow discourage the strategy of stockpiling NAIs, such as is under way in many industrialized countries as part of national influenza pandemic preparedness.[28] Moreover, this NAI is extremely expensive for developing countries as one treatment costs around US$15.[19] In Peru for example, there is an absence of a national sanitary strategy related to the treatment of viral respiratory agents, such as influenza, but even if there was, the high cost of oseltamivir would make it an unsustainable alternative for this low-income country to use it as common treatment.[14] Furthermore, in Peru, as in many countries in South America, amantadine is the only antiviral agent approved by the government.[20] Therefore, the use of vaccinations in persons at-risk should be highly encouraged.

Acknowledgements

We are very grateful to Ms María Ester Gamero for cell culture support in *in vitro* adamantanes resistance experiments and to Mrs Gloria Chauca for laboratory and administrative support.

Funding Statement

This study was funded by the United States Department of Defense Global Emerging Infections Systems Research Program, work unit number: 847705·82000·25GB.B0016.

Conflict of interest

We declare that we have no conflict of interest.

Disclaimers

The views expressed in this article are those of the authors and do not necessarily reflect the official policy or position of the Department of the Navy, Department of Defense, nor the US Government.

The study protocol was approved by the Naval Medical Research Center Institutional Review Board (Protocols NMRCD.2002·0019) in compliance with all applicable Federal regulations governing the protection of human subjects.

Disclosure

None of the authors has a financial or personal conflict of interest related to this study. The corresponding author had full access to all data in the study and final responsibility for the decision to submit this publication.

Copyright Statement

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