Surveillance of adamantane resistance among influenza A H3 viruses isolated in Argentina between 2001 and 2007

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ABSTRACT

A dramatic rise in the frequency of resistance to adamantane drugs by influenza A H3 viruses, associated with a single amino acid replacement in the viral matrix M2 protein, has occurred in multiple countries worldwide in recent years. We investigated the frequency of adamantane-resistant influenza A H3 viruses in Argentina during the period 2001-2007. We used reverse transcription followed by polymerase chain reaction. The obtained products were sequenced for the detection of mutations of the M2 gene relevant to the resistance phenotypes. The HA1 sequences of the sensitive and resistant strains were also analyzed to clarify whether they had any relevance to the resistant mutations. Twenty out of 55 (36%) strains were identified with the resistance-conferring substitution at amino acid 31 (Serine 31 Asparagine). No resistant viruses were detected between 2001 and 2005. All strains isolated in 2006 and four out of five isolates from 2007 were resistant. None of the patients had received previous treatment with amantadine and/or rimantadine. The HA1 analysis showed that there were only two changes (Serine193 Phenylalanine and Aspartic acid 225 Asparagine) present in the strains with the M2 substitution at position 31. Our data indicate that since 2006 there has been a significant increase of adamantane-resistant influenza A H3 viruses, which raises concern over the spread of these viruses in Argentina.

Key words: Influenza, drug resistance, antivirals, M2 protein, hemagglutinin

RESUMEN

Vigilancia de la resistencia a los adamantanos entre los virus influenza A H3 aislados en Argentina entre 2001 y 2007. En los últimos años, se ha detectado un aumento de virus influenza A H3 resistentes a los adamantanos en distintos países, asociados mayoritariamente con el reemplazo de un único aminoácido de la proteína matriz M2. Se investigó la frecuencia de virus influenza A H3 resistentes a los adamantanos en Argentina entre 2001 y 2007. Se utilizó la transcripción reversa seguida de la reacción en cadena de la polimerasa y de la técnica de secuencia directa para la detección de mutaciones en el gen que codifica para la proteína M2, relevantes para los fenotipos de resistencia. También se analizó la secuencia de la porción HA1 de cepas resistentes y sensibles, para intentar establecer alguna relación con las mutaciones de M2. De un total de 55 cepas, 20 (36%) fueron resistentes debido a un cambio aminoacídico en la posición 31 (serina 31 asparagina). No se detectaron cepas resistentes entre 2001 y 2005. Las cepas aisladas en el 2006 y 4 de 5 cepas obtenidas en el 2007 fueron resistentes. Ninguno de los pacientes de los que se habían aislado esas cepas había recibido tratamiento antiviral con anterioridad. En la porción secuenciada de HA1 se encontraron dos cambios (serina 193 fenilalanina y ácido aspártico 225 asparagina), presentes sólo en las cepas que tuvieron la mutación en la posición 31 de M2. Desde el año 2006 se ha registrado en Argentina un aumento significativo de la circulación de virus influenza A H3 con genotipo resistente, lo que genera expectativa con respecto a su diseminación en nuestro país.

Palabras clave: Influenza, resistencia a antivirales, proteína M2, hemaglutinina

INTRODUCTION

Influenza A and B viruses cause significant morbidity and mortality in humans, even in otherwise healthy individuals (11). Influenza A viruses pose a especially serious threat due to their ability to produce pandemics (13). While vaccines remain the major public health strategy for prevention, antivirals, if available in sufficient quantities, could play a particularly important role in response to the early phases of a pandemic (6).

Currently, four drugs are available to treat or prevent influenza. These include the adamantanes (i.e., amanta-

dine and rimantadine) and the neuraminidase inhibitors (i.e., oseltamivir and zanamivir). Adamantane derivatives have been successfully used worldwide for the prevention and treatment of influenza A virus infections for more than 30 years (19). These drugs block the proton channel of the influenza A virion, the M2 protein, and thus inhibit the pH change necessary for the uncoating process (20). The medicines are inexpensive and chemically stable, but emergence of resistance and adverse effects are matters of concern (18).

An increase in the proportion of amantadine-resistant influenza A virus began in China and Hong Kong in 2002-

2003, and an extremely high frequency of these viruses was observed in the United States in 2005-2006. Similar tendencies have been reported in Australia, New Zealand, Southeast Asia, Macau and Cambodia (1). Amantadine resistance is caused by a single point mutation at any of the aminoacid positions 26, 27, 30, 31 or 34 in the transmembrane region of the M2 protein (9), being the mutation at position 31 the most frequently reported in resistant virus isolates (2). M2 inhibitor-resistant variants are pathogenic, transmissible from person to person, and can be occasionally recovered from patients who have never been exposed to amantadine. Importantly, recent human isolates of highly virulent A/H5N1 influenza viruses are naturally resistant to these drugs (14).

As in many countries, the use of these antivirals is not common in Argentina. Adamantane virus resistance data available from South America are poor. It is necessary to establish the effectiveness of adamantane antivirals in the Argentine population for treatment or prophylaxis in the event of influenza outbreaks. Because of that, in this study we report preliminary results of a surveillance study for adamantane resistance among circulating influenza A H3 viruses collected in Argentina from 2001 to 2006 and a few ones from the 2007 season.

Hemagglutinin protein (HA) is responsible for attachment of the virus to cellular sialic acid receptor. HA mutations adjacent to a key receptor binding site (at position 226) appear to change the affinity of the HA for receptors that may impact viral fitness (16). On account of that, we chose some of the influenza A H3 strains to study the HA1 portion and to try to match all the information available in order to know the evolutionary pattern of these viruses.

MATERIALS AND METHODS

Epidemiological information and specimen collection
The National Influenza and Respiratory Viruses Laboratory Network routinely collects nasopharyngeal aspirates and nasal swabs coming from pediatric and adult outpatients and inpatients presenting acute respiratory infection. None of these patients had received previous treatment with amantadine and/or rimantadine. A total amount of 28,000 to 30,000 clinical samples are usually studied annually for respiratory viruses by the Network. Although influenza A H1 and influenza B also circulated during the studied period, influenza A H3 viruses were more predominant. These samples are examined by immunofluorescence assay (Respiratory Panel IFA-Light Diagnostics, Chemicon International, CA, USA) for diagnosis of influenza A, influenza B and other respiratory viruses. Positive samples for influenza A or B in viral transport media at 4 °C are transferred to the National Influenza Center for further virological testing together with precise patient information.

Clinical samples
Fifty five influenza A H3 positive samples collected in 9 provinces throughout the country: Misiones, Chaco, Salta, Tucumán, Santa Fe, Mendoza, Buenos Aires, Chubut and Neuquén between 2001 and 2007 were selected for this study: 6 from 2001, 2 from 2002, 9 from 2003, 9 from 2004, 8 from 2005, 16 from 2006 and 5 from 2007.

Virus isolation
Two hundred μl of supernatant of clinical samples was inoculated into Madin-Darby Canine Kidney (MDCK) cells prepared in 24-well plates. The plates were kept at 35 °C under 5% CO_2 atmosphere for up to 7 days, to observe the appearance of cytopathic effects (CPEs) (12). CPE was confirmed using commercial type-specific monoclonal antibodies against the influenza virus. Positive supernatants were then passaged twice to obtain sufficient virus titer to perform virus characterization. Isolates were frozen at –80 °C (as stock). Influenza viruses were subtyped by the hemagglutination inhibition (HI) test with antigens and antisera included in the WHO Influenza Reagent kit provided annually by the Centers for Disease Control and Prevention of Atlanta, Georgia, USA and using turkey red blood cells.

### Reverse-transcription polymerase chain reaction (RT-PCR) and sequence analyses
RNA was extracted from 140 μl of influenza A H3 virus cultures using the QIAmp Viral RNA Mini Kit (QIAGEN, USA), according to the manufacturer's instructions. RT-PCR was performed with the One-Step RT-PCR Kit (QIAGEN, USA) on 55 influenza A H3 RNAs using specific primers to amplify the M2 region (286 bp product encompassing nucleotides 741-1027) and a subset of 25 of them were selected to study the HA1 portion of the HA gene (929 bp encompassing nucleotides 55-984). PCR products (5 μl) were examined on 1% agarose gel to confirm amplification of an appropriately sized DNA band. PCR products were purified with the SigmaSpin™ Post-Reaction Clean-Up Columns Kit (Sigma, USA) and sequenced to examine mutations at the specific positions. The templates were labeled by cycle sequencing reactions with fluorescent dye terminators (Big Dye™ Terminator v3.1 Cycle Sequencing Kit, Applied Biosystems, USA) and the products were analysed using an ABI 3100 Avant Genetic Analyzer (Applied Biosystems, USA). Phylogenetic tree analysis was performed with the obtained HA1 sequences. Sequence data were edited and analyzed using Clustal X (version 1.8). Multiple alignments, and phylogenetic analysis were performed using PHYLIP computer software package (version 3.57) (5).

RESULTS

A total of 55 influenza A H3 field isolates collected between 2001 and 2007 were screened for specific aminoacid substitutions in the M2 region known to confer drug resistance. Of these viruses, 20 (36%) were identified with the resistance-conferring substitution at amino

Table1. Summarized results of amantadine-resistant influenza A H3 viruses detected in Argentina, period 2001-2007.

Season	N° of specimens	N° of resistant strains
2001	6	0
2002	2	0
2003	9	0
2004	9	0
2005	8	0
2006	16	16
2007	5	4
Total	55	20
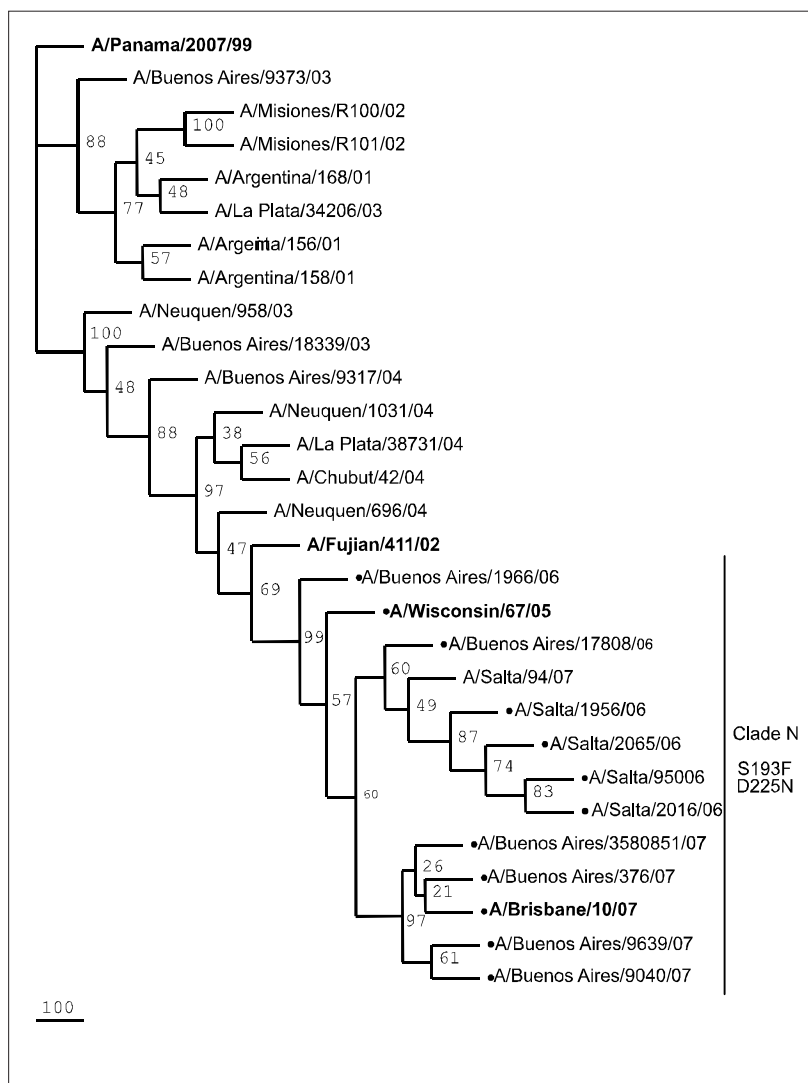
Figure 1. Phylogenetic tree analysis based on the HA1 portion nucleotide sequence (929 bp) of the HA gene from human H3 viruses collected during the period 2001-2007 (n=25) and selected strains in genetic database. Strains labeled with solid circles indicated amantadine resistant strains possessing the S31N mutation in M2 protein. Reference strains A/Wisconsin/67/05 and A/Brisbane/10/07 are also S31N amantadine-resistant viruses. Group named "clade N" presented the HA motif, S193F and D225N, in amantadine - resistant strains. Virus names in boldface denote WHO recommended Southern Hemisphere influenza vaccine strains for the A (H3N2) component during the period 2001- 2007. Phylogenetic trees were inferred from 929 nucleotide sequences by the neighbor-joining method. The horizontal spacing represents the number of nucleotide differences between branch points. Vertical lines are for spacing and labels. Numbers represent bootstrap confidence values.

acid 31 (AGT→AAT, Ser31Asn). No other amino acid change associated with influenza resistance to adamantanes was detected in these viruses. The results of the adamantane-resistant influenza A H3 viruses in each season are summarized in Table 1.

Analysis of the HA1 portion sequencing showed the presence in the resistant strains of a motif with dual mutations at position 193, serine to phenylalanine (S193F) and at position 225, aspartic acid to asparagines (D225N). Analysis of the HA1 sequences showed that all resistant

strains fell into a single HA1 cluster named clade N. All the sensitive strains did not have the motif and did not cluster in clade N, with the exception of isolate A/Salta/94/07 which clustered with the N-lineage, but did not carry the M2 S31N mutation (Figure 1).

DISCUSSION

Influenza antiviral drugs play an important role in a comprehensive approach to controlling influenza illness

and transmission (3). The frequency of resistance to amantadine and rimantadine among circulating influenza A viruses has increased dramatically over the past few years (2). Of the 55 A H3 viruses screened, we have found an alarming increase in the incidence of adamantine resistance in Argentina since 2006, whose circulation was also detected in 2007. These viruses were isolated from patients residing in 9 provinces, representing most of the regions of Argentina.

It is generally accepted that amantadine-resistant viruses emerge under the drug pressure, but are less virulent or transmissible than susceptible viruses (8). In the present study, none of the patients had previously received amantadine and/or rimantadine. Thus, we support the assumption that the resistant viruses may not have solely arisen as a result of drug exposure, but that naturally occurring resistant-associated mutations could have emerged (7).

All resistant strains examined in this study carried the S31N mutation in the transmembrane domain in M2 protein. This same mutation was indeed the most common change observed in drug-resistant viruses in other published studies (2, 21).

Alternatively, the global spread of S31N may be related to its interaction with advantageous mutations located elsewhere in the viral genome, such as those in the HA that facilitate immune escape (4, 17). The HA1 phylogenetic analysis showed, as it was previously described by other authors, the existence of a distinct lineage (N-lineage) containing dual mutations at positions 193 and 225, which fall into the receptor-binding region (15). All amantadine-resistant viruses isolated during 2006 and 2007 years are clustered inside the N-lineage. HA protein plays an important role in virus infection, and the dual mutations in the HA1 gene may have caused the significant increase in the frequency of the drug-resistant virus in the 2006-2007 seasons. However, the level of contribution to infectivity of dual mutations remains unknown (10). The N-lineage also contains an isolate that does not possess the S31N mutation, which could suggest a reassortment event occurring between co-circulating amantadine-resistant and sensitive viruses. Taking into account that the N-lineage is characterized by 17 amino acid replacements across the complete genome (including two in the HA1 protein), we will need to work not only on the HA1 segment but also on the complete genome, to have a better understanding of the fitness and evolution characteristics of the resistant and sensitive strains circulating in the country.

Our results indicate that these drugs should not be used for the treatment or prophylaxis of influenza in Argentina until adamantane susceptibility has been reestablished among circulating influenza A strains. The high proportion of adamantane-resistant influenza A viruses currently circulating in our country highlights the clinical importance of rapid surveillance for antiviral resistance as well as the need for new tools.

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