Surveillance of Resistance to Adamantanes among Influenza A(H3N2) and A(H1N1) Viruses Isolated Worldwide

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Our previous reports demonstrated an alarming increase in resistance to adamantanes among influenza A(H3N2) viruses isolated in 2001–2005. To continue monitoring drug resistance, we conducted a comprehensive analysis of influenza A(H3N2) and A(H1N1) viruses isolated globally in 2005–2006. The results obtained by pyrosequencing indicate that 96.4% \( (n = 761) \) of A(H3N2) viruses circulating in the United States were adamantane resistant. Drug resistance has reached 100% among isolates from some Asian countries. Analysis of correlation between the appearance of drug resistance and the evolutionary pathway of the hemagglutinin (HA) gene suggests at least 2 separate introductions of resistance into circulating populations that gave rise to identifiable subclades. It also indicates that resistant A(H3N2) viruses may have emerged in Asia in late 2001. Among A(H1N1) viruses isolated worldwide, resistance reached 15.5% in 2005–2006; in the United States alone, it was 4.0%. Phylogenetic analysis of the HA and M genes indicates that the acquisition of resistance in A(H1N1) viruses can be linked to a specific genetic group and was not a result of reassortment between A(H3N2) and A(H1N1) viruses. The results of the study highlight the necessity of close monitoring of resistance to existing antivirals as well as the need for new therapeutics.

Type A influenza viruses represent important pathogens for humans, birds, horses, pigs, and other species. It is estimated that influenza A viruses cause the death of approximately half a million individuals worldwide every year [1]. In the United States alone, 5%–20% of the population is infected with influenza virus annually [2–4], and ~36,000 people die from influenza-associated illnesses—a majority of the victims are elderly persons [5].

Vaccination is the primary measure to control influenza infections in humans. However, for individuals who cannot be or have not been vaccinated or when vaccine is not available, antiviral agents can provide an important alternative. Until recently, only adamantanes (amantadine and rimantadine) have been available for the prophylaxis and treatment of influenza A infections [6–8]. Amantadine and rimantadine are chemical derivatives of adamantane and have been shown to inhibit viral replication by blocking the proton channel formed by the M2 protein of influenza A viruses. The channel activity is essential for uncoating the viral particle during early stages of viral replication [9]. A single substitution at 1 of 5 residues of the transmembrane domain of the M2 protein (positions 26, 27, 30, 31, or 34 aa) has been shown to confer resistance to ada-
mantine [10, 11]. Amantadine- or rimantadine-resistant mutants are cross-resistant and show no evidence of fitness impairment [6]. Moreover, resistance emerges readily in the drug-treated patients, and resistant variants are transmissible [12–15].

Adamantanes have been used for many years because of their wide availability and low cost, yet the frequency of adamantane resistance among field isolates has been low (<1%) until recently [16]. During the period from 1991 to 1995, surveillance for adamantane resistance among A(H3N2) viruses [17] revealed the global frequency of resistance to be as low as 0.8%. However, a recent study that included A(H3N2) viruses collected worldwide over the course of a decade showed that the incidence had increased to 12.3% by 2004 [18]; by next year, it had reached 96%, 72%, and 14.5% in China, South Korea, and the United States, respectively. Resistance to adamantane among A(H3N2) viruses circulating in the United States had reached 92% [19], according to the results of testing of a limited number of viruses collected between October and December of 2005. With the alarming incidence of drug resistance, the US Centers for Disease Control and Prevention (CDC) issued a public health alert recommending that clinicians avoid the use of adamantanes for the remainder of the 2005–2006 influenza season [20].

In the present study, we report the results of drug-susceptibility testing for A(H3N2) and A(H1N1) viruses collected worldwide between October 2005 and September 2006 and submitted to the CDC. We also performed a more complete analysis of drug resistance for isolates collected during the preceding 2004–2005 season. Phylogenetic analysis of the hemagglutinin (HA) genes of the strains used for resistance monitoring was conducted to explore a possible role for changes in the HA as a selective advantage for the spread of drug resistance.

MATERIALS AND METHODS

Viruses. Influenza A(H3N2) (n = 3146) and A(H1N1) (n = 619) viruses were collected by various laboratories in the United States and other countries during the 2004–2005 (1 October 2004–30 September 2005) and 2005–2006 (1 October 2005–30 September 2006) influenza seasons and submitted to the World Health Organization Collaborating Center for Surveillance, Epidemiology and Control of Influenza at the CDC (Atlanta, GA). All of the viruses were screened for resistance to adamantanes.

Virus propagation and antigenic subtyping. Viruses were propagated in either embryonated chicken eggs or in MDCK cells and were antigenically subtyped in the hemagglutination inhibition tests using postinfection ferret serum in accordance with standard procedures [21, 22].

Reverse-transcription polymerase chain reaction (RT-PCR) and sequence analyses. Viral RNA was extracted, and a 264-nt amplicon of the M gene covering the region encoding the transmembrane domain of M2 was then targeted for RT-PCR amplification with a biotinylated primer as described in detail elsewhere [18, 19]. The 44-bp sequences were aligned using software from DNASTAR (Lasergene version 7.0) as described elsewhere. For a subset of virus isolates (n = 20), the results obtained from pyrosequencing were confirmed using the traditional Sanger sequencing method [18, 19].

The HA1 domains of HA genes from a subset of influenza A(H3N2) and A(H1N1) viruses collected worldwide from 1999 to August 2006 were also sequenced and analyzed as described elsewhere [23, 24]. These isolates were selected to reflect the genetic and geographic distribution spectrum of influenza A viruses.

Phylogenetic analysis. The HA gene sequences were assembled, aligned, and edited using DNASTAR and BioEdit (version 5.0.6; North Carolina State University) software. Phylogenetic trees were generated by use of MEGA3.1 software [25].

Biological assay. Drug sensitivity phenotype for a set of 11 A(H1N1) viruses was determined with the use of a biological assay in MDCK cells [18, 19]. Virus was considered to be drug sensitive if, in the presence of drug, the viral yield in cell culture supernatant was reduced by at least 4-fold based on the results of hemagglutination assay, as described elsewhere [26].

RESULTS

Resistance among Influenza A(H3N2) Viruses

2004–2005 influenza season. A total of 2087 influenza A(H3N2) viruses isolated worldwide and submitted to the CDC were analyzed for the presence of M2 protein mutations known to confer resistance to adamantanes; 314 (15.0%) isolates were found to be resistant. A more detailed analysis by country and/ or region of virus origin (table 1) revealed that the highest frequency of resistance was detected among isolates received from Asia (93.4%). In the United States, it had reached 10.6%. Among those 314 isolates, 306 (97.5%) had an amino acid change from Ser to Asn at residue 31 (Ser31Asn). This is the most common mutation known to confer resistance to adamantanes [18, 19, 27, 28]. In 5 isolates (1.6%), the amino acid change was detected at position 27 (Val→Ala), and 2 isolates (0.7%) had a Val→Ala substitution at position 30. A single isolate (0.3%) had a Leu→Phe amino acid change at residue 26. The results for the season 2004–2005 were partially reported elsewhere [19], when a smaller number of isolates was available for analysis.

2005–2006 influenza season. The proportion of isolates resistant to adamantanes continued to increase during the 2005–2006 season. Specifically, 90.5% of the 1169 viruses tested were resistant to adamantanes (table 1). Moreover, isolates col-
Table 1. Proportions of A(H3N2) and A(H1N1) viruses resistant to adamantanes, by geographic origin and year of isolation.

<table>
<thead>
<tr>
<th>Region</th>
<th>A(H3N2)</th>
<th>A(H1N1)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Africa</td>
<td>0/4</td>
<td>0/2</td>
</tr>
<tr>
<td>Asia</td>
<td></td>
<td></td>
</tr>
<tr>
<td>China</td>
<td>71/76 (93.4%)</td>
<td>8/8 (100%)</td>
</tr>
<tr>
<td>Hong Kong</td>
<td>38/52 (73.1%)</td>
<td>7/7 (100%)</td>
</tr>
<tr>
<td>Japan</td>
<td>3/20 (15.0%)</td>
<td>7/7 (100%)</td>
</tr>
<tr>
<td>South Korea</td>
<td>9/26 (34.6%)</td>
<td>41/41 (100%)</td>
</tr>
<tr>
<td>Taiwan</td>
<td>1/5 (20.0%)</td>
<td>8/8 (100%)</td>
</tr>
<tr>
<td>Other Asia</td>
<td>17/229 (7.4%)</td>
<td>42/73 (57.5%)</td>
</tr>
<tr>
<td>Total Asia</td>
<td>139/408 (34.1%)</td>
<td>113/144 (78.5%)</td>
</tr>
<tr>
<td>Europe</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Canada</td>
<td>1/20 (5.0%)</td>
<td>8/8 (100%)</td>
</tr>
<tr>
<td>Mexico</td>
<td>5/16 (31.2%)</td>
<td>70/72 (97.2%)</td>
</tr>
<tr>
<td>United States</td>
<td>140/1310 (10.7%)</td>
<td>761/789 (96.4%)</td>
</tr>
<tr>
<td>Total North America</td>
<td>146/1346 (10.8%)</td>
<td>839/869 (96.5%)</td>
</tr>
<tr>
<td>Oceania</td>
<td>1/8 (12.5%)</td>
<td>2/3 (66.7%)</td>
</tr>
<tr>
<td>South America</td>
<td>18/248 (7.2%)</td>
<td>73/76 (96.0%)</td>
</tr>
<tr>
<td>Overall</td>
<td>314/2087 (15.0%)</td>
<td>1059/1169 (90.6%)</td>
</tr>
</tbody>
</table>

NOTE. Data are no. of resistant isolates/no. of all tested (% resistance [if >0]). Countries where A(H3N2) appeared during the 2004–2005 season were Egypt and South Africa (Africa); Guam, India, Indonesia, Iraq, Kazakhstan, Kyrgyzstan, Malaysia, Mongolia, Oman, Singapore, Thailand, and Turkey (“other Asia”); Bulgaria, Finland, France Germany, Italy, Norway, Romania, Spain, Ukraine, United Kingdom, and Russia (Europe); Australia and New Zealand (Oceania); and Argentina, Bolivia, Brazil, Chile, Columbia, Costa Rica, Dominican Republic, El Salvador, French Guiana, Honduras, Martinique, Panama, Paraguay, Peru, Uruguay, and Venezuela (South America). During the 2005–2006 season, A(H3N2) appeared in Guam, India, Indonesia, Kuwait, Mongolia, Nepal, Singapore, and Thailand (“other Asia”); Egypt (Africa); France, Italy, Russia, Ukraine, and United Kingdom (Europe); and Argentina, Brazil, Chile, Colombia, Guatemala, Paraguay, and Peru (South America). Countries where A(H1N1) appeared during the 2004–2005 season were Bangladesh, India, Indonesia, Kazakhstan, Kyrgyzstan, Mongolia, Oman, Singapore, Thailand, and Turkey (“other Asia”); France, Greece, Ireland, The Netherlands, Romania, Russia, and United Kingdom (Europe); and Paraguay (South America). During the 2005–2006 season, A(H1N1) appeared in Egypt (Africa); India, Indonesia, Kuwait, Malaysia, Mongolia, Qatar, Singapore, and Thailand (“other Asia”); France, Russia, Serbia and Montenegro, and Ukraine (Europe); and Argentina, Brazil, Chile, and Peru (South America).

* These data include some isolates reported by in Bright et al. [20].

lected during this period in South Korea, Taiwan, Japan, Hong Kong, China, and Canada exhibited 100% drug resistance. In the United States, the frequency of resistance was estimated at 96.4%, which is a significant increase, compared with 10.6% in 2004–2005. Resistance to adamantanes in isolates received from Central and South America showed a similar increase, from 7.2% to 96.0% (table 1). The vast majority of the resistant viruses (n = 1059) contained the Ser31Asn change in the M2 protein. Additionally, 1 isolate had the Ala30Thr in the M2 protein; this change is known to confer resistance to adamantanes. Seven other isolates had Val27Ile substitutions; however, these changes are not associated with resistance.

Resistance in Influenza A(H1N1) Viruses

2004–2005 influenza season. A total of 195 isolates collected from 25 countries worldwide were screened for resistance to adamantanes. On the basis of pyrosequence analysis, 8 (5.8%) of 139 isolates collected in Asia were found to contain mutations conferring resistance to adamantanes (table 1). Among the resistant isolates, 7 had Ser31Asn changes and 1 had the Leu26Phe change.

2005–2006 influenza season. During the 2005–2006 influenza season, the global incidence of resistance among A(H1N1) isolates was found to be 15.5% (table 1). The analysis revealed that 65 of 66 resistant viruses contained the Ser31Asn change, and 1 isolate had the Leu26Phe change. The resistant viruses were detected in several countries in Asia, with the highest frequency of resistance found in China (71.7%). In Eastern Europe, mainly Russia and Ukraine, 44.8% of isolates were drug resistant. In the United States, resistance was estimated at 4.0%, whereas no resistance was detected among isolates received from South America.

Conventional sequencing of complete M gene from 20 isolates showed 100% concordance with pyrosequencing data. Ad-
ditionally, 11 A(H1N1) isolates were subjected to drug-resistance testing in a virus yield reduction assay. In the presence of rimantadine, the yield of viruses with Ser31 was reduced by at least 4-fold, whereas the viruses that contained Asn31 in the M2 protein all exhibited drug resistance. Furthermore, amino acid changes Val28Ile, Ala29Thr, and/or Ala29Val were detected in 5 isolates (A[H1N1] and A[H3N2]). Drug-resistance testing in the virus yield reduction assay of these 5 isolates showed that these mutations do not confer resistance to adamantanes.

The majority of drug-resistant A(H1N1) viruses contain Asn31, similar to resistant A(H3N2); this prompted us to investigate whether the M genes of these A(H1N1) viruses were inherited by reassortment with resistant A(H3N2) viruses. A phylogenetic analysis of the complete M gene sequences from 10 A(H3N2) and 47 A(H1N1) viruses was conducted. The results showed that all A(H1N1) resistant mutants tested in the present study contained an M gene similar to that of A(H1N1) sensitive strains and different from that of A(H3N2) viruses. Our results suggest separate introduction and spread of resistance between the 2 antigenic subtypes of influenza A viruses (figure 1).

Emergence of Resistance to Adamantanes and Evolution of HA Genes of A(H3N2) and A(H1N1) Viruses

In this part of the study, we aimed to determine whether appearance of drug resistance could be linked to the acquisition of a particular change in the HA gene that could provide a selective advantage and thus could explain the spread of resistant variants worldwide. We first analyzed the genetic evolution of the HA gene in A(H3N2) isolates collected from 1999 through 2006. The earliest adamantane-resistant isolate among these viruses was A/Wuhan/396/2001, collected on 29 November 2001, followed by several viruses received from China in 2002 and 2003. Viruses circulating since 2003 fell into 2 distinct clades (figure 2). The first clade consists of mainly resistant viruses. The second and larger clade is further divided into 2 subclades. Subclade 2a is predominantly formed by sensitive viruses, whereas subclade 2b consists mainly of resistant ones. Viruses within the latter subclade contain signature amino acid changes—Ser193Phe and Asp225Asn—in their HA genes (figure 2). Analysis of the HA gene sequences of the drift A(H3N2) variants showed no evidence of an association between the emergence of drug resistance and specific amino acid change(s) in the HA.

Analysis of the HA gene sequences of 72 A(H1N1) viruses revealed 2 major clades (figure 3). Clade 1 viruses differ from the A/New Caledonia/20/99 reference strain by a conserved amino acid change, Tyr256Phe, in their HA and retain drug sensitivity. The second clade includes viruses that share 4 amino acid changes: Thr90Lys, Arg149Lys, Arg212Lys, and Thr269Asn. Viruses within this clade are further clustered into 3 different subclades. Subclade 2a is composed of resistant viruses. Viruses of subclade 2a were collected from different geographic areas, including Asia, Europe, and North America. Subclade 2b includes sensitive viruses that were collected in Asia and the United States; subclade 2c consists of both sensitive and resistant viruses. The subclades 2a, 2b, and 2c do not have signature amino acid change(s) that would allow their differentiation.

DISCUSSION

We report here a continuing worldwide increase in adamantane resistance among influenza A(H3N2) viruses that reached 100% among viruses isolated in some Asian countries during the 2005–2006 influenza season. A significant increase in resistance (up to 15.5%) was also detected among A(H1N1) viruses collected during the same period.

The findings of the present study extend those reported in 2005 [18] and early 2006 [19] and document a continuous and proportional increase in A(H3N2) isolates showing resistance to adamantanes. Globally, the incidence of resistance among A(H3N2) has dramatically increased, from 1%–2% before 2002, to 15% during the 2004–2005 influenza season, and to 90.5% during the 2005–2006 season. In the United States, the frequency of adamantane resistance has reached 96.4% for the entire 2005–2006 season. Similarly, 97.2% and 100% of the viruses received from Mexico and Canada, respectively, were drug resistant. The proportion of A(H3N2) viruses resistant to adamantanes isolated in Australia and neighboring countries has been reported to be 40.7% (43/103) [27].

The emergence and subsequent spread of adamantane resistance among A(H3N2) viruses may be attributed in part to more widespread use of those antivirals, given that resistant viruses typically emerge readily in treated patients and are transmissible [29]. In other countries, such as the United States, adamantanes are used less because a physician’s prescription is required.

The adamantane-resistant A(H3N2) viruses were found in both major clades of viruses circulating from 2003 to 2006. The HA phylogenetic analysis indicates at least 2 independent introductions of resistant viruses into human populations.

The data also showed no apparent association between resistance and a particular change(s) in the HA. In this respect, our conclusions differ from those reported by Barr et al. [27], where drug-resistant viruses fell into a single HA clade with characteristic substitutions at residues 193 and 225. This difference could be explained by a broader geographic range and a larger number of isolates tested in our study.

The acquisition of resistance by recent A(H1N1) viruses was not a result of gene reassortment with drug-resistant A(H3N2) viruses and therefore appears to be an independent event. The HA phylogenetic analysis of A(H1N1) viruses provides evidence of a link between appearance of resistance and a specific genetic
Figure 1. Genetic evolution of the M gene of A(H3N2) and A(H1N1) viruses. Resistant isolates are labeled with a black arrowhead; sensitive isolates are unmarked. In all figures, resistance is conferred by amino acid change Ser31Asn, unless exceptions are indicated.
Figure 2. Phylogenetic analysis of the HA1 domain of the hemagglutinin (HA) gene and comparison with spread of resistance associated with the M gene among influenza A(H3N2) viruses. Black arrowheads indicate resistance to adamantane; sensitive isolates are unmarked.
Adamantane Resistance in Influenza Viruses

Figure 3. Analysis of the genetic evolution of the hemagglutinin (HA) gene (HA1 domain) from representative influenza A(H1N1) isolates and its correlation with the appearance of resistance to adamantane. Resistant viruses are marked with black arrowheads; sensitive isolates are unmarked. A, A(H1N1) isolates from both clades 1 and 2; B, clade 2 only.

group (figure 3, subclade 2a). However, there was no association of drug resistance with specific amino acid changes in the HA and/or HA antigenicity (results not shown). It remains to be seen whether strains sensitive to amantadine and rimantadine can reemerge and replace the resistant variants if the use of adamantanes were restricted.

Because of the high frequency of adamantane resistance, the use of the second class of antiinfluenza drugs, known as neuraminidase inhibitors (NAIs), is likely to increase. Two of these drugs, zanamivir and oseltamivir, have been approved by the US Food and Drug Administration for management of influenza infections since 1999 [30]. Recently, a comprehensive report on susceptibility to NAIs among 2287 influenza A and B viruses collected between 1999 and 2002 was published [31].
The authors reported that only a small number (0.33%) of viruses circulating worldwide exhibited a decrease in susceptibility to oseltamivir [31]. The low level of NAI resistance among field isolates and isolates recovered from immunocompetent drug-treated adults [32, 33] makes these drugs an important tool for influenza treatment and prophylaxis. It should be mentioned, however, that the resistance to oseltamivir was reported to be higher in treated pediatric populations, most likely because of prolonged virus shedding [34, 35].

Continuous global surveillance is essential to monitor the emergence and spread of drug resistance. Our findings indicate that adamantane-resistant viruses carrying the Ser31Asn change in the M2 protein can efficiently compete with viruses lacking the substitution. Additionally, Ser31Asn was also the predominant amino acid change conferring resistance in the recent A(H1N1) viruses, whereas the report by Saito et al. [28] indicated that Val27Ala was the most predominant amino acid change in a set of 45 A(H1N1) viruses isolated between 1999 and 2001.

Adamantane resistance was first detected in Asia at a time when severe acute respiratory syndrome and avian flu outbreaks might have resulted in an increased use of antivirals in that densely populated area. These factors cannot, however, explain the continuous spread of resistant viruses worldwide and their dominance in a low or absent selective pressure due to drug use. It is possible that the spread of resistant mutants was a result of association with unknown advantageous mutations and a lack of fitness impairment of these mutant viruses. Further studies are needed to investigate possible mechanisms underlying the selective advantage of the adamantane-resistant variants in human populations. One of the mechanisms to be explored could be an investigation of viral replication machinery and/or the role of the host response in infections with resistant viruses.

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