

Adamantane Resistance Among Influenza A Viruses Isolated Early During the 2005-2006 Influenza Season in the United States

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INFLUENZA A VIRUSES ARE A MAJOR cause of morbidity and mortality in the United States. They infect, on average, 10% to 15% of the population annually.^{1,2} It has been estimated that influenza A viruses are associated with approximately 31 000 US deaths annually, with 90% of these deaths occurring among elderly persons.³ Although annual vaccination is the primary strategy for preventing influenza infections, influenza antiviral drug therapy has been shown to be an effective means of preventing and treating influenza.

Two antiviral drugs, adamantane derivatives amantadine and rimantadine, licensed for antiviral indications in the United States in 1966 and 1993, respectively, are used for prophylaxis and treatment of influenza, especially for controlling outbreaks in settings such as nursing homes and long-term care facilities.^{4,5} Amantadine is also licensed for use in the treatment of Parkinson disease.

For influenza, these drugs bind to and block the function of the influenza A virus M2 ion channel protein, preventing virus replication within the infected cell.⁶ The effectiveness of pro-

Context The adamantanes, amantadine and rimantadine, have been used as first-choice antiviral drugs against community outbreaks of influenza A viruses for many years. Rates of viruses resistant to these drugs have been increasing globally. Rapid surveillance for the emergence and spread of resistant viruses has become critical for appropriate treatment of patients.

Objective To investigate the frequency of adamantane-resistant influenza A viruses circulating in the United States during the initial months of the 2005-2006 influenza season.

Design and Setting Influenza isolates collected from 26 states from October 1 through December 31, 2005, and submitted to the US Centers for Disease Control and Prevention were tested for drug resistance as part of ongoing surveillance. Isolates were submitted from World Health Organization collaborating laboratories and National Respiratory and Enteric Virus Surveillance System laboratories.

Main Outcome Measures Using pyrosequencing and confirmatory assays, we identified viruses containing mutations within the M2 gene that are known to confer resistance to both amantadine and rimantadine.

Results A total of 209 influenza A(H3N2) viruses isolated from patients in 26 states were screened, of which 193 (92.3%) contained a change at amino acid 31 (serine to asparagine [S31N]) in the M2 gene known to be correlated with adamantane resistance. Two of 8 influenza A(H1N1) viruses contained the same mutation. Drug-resistant viruses were distributed across the United States.

Conclusions The high proportion of influenza A viruses currently circulating in the United States demonstrating adamantane resistance highlights the clinical importance of rapid surveillance for antiviral resistance. Our results indicate that these drugs should not be used for the treatment or prophylaxis of influenza in the United States until susceptibility to adamantanes has been reestablished among circulating influenza A isolates.

JAMA. 2006;295:891-894

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phylaxis with these drugs is between 80% and 90%, and when treatment is begun within 48 hours of symptom onset, their use can reduce the duration of illness by 1.5 days.⁷⁻⁹

Resistance to adamantanes can emerge during treatment. A single point mutation in the sequence coding for the amino acid at position 26, 27, 30, 31, or 34 of the M2 protein confers resis-

tance to the adamantanes.^{10,11} Replication and transmission of adamantane-resistant viruses are not hindered by

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For editorial comment see p 934.

Table. State of Origin and Median Age of Patients From Whom Influenza A(H3N2) Isolates Were Tested for Adamantane Resistance

	No. Resistant/ Tested	Patient Age, Median (Range), y*
Alaska	2/2	16.5 (5-28)
Arizona	14/14	24 (<1-87)
California	6/7	6 (<1-28)
Colorado	6/6	25.5 (7-78)
Florida	3/3	21 (11-50)
Georgia	4/4	20 (12-35)
Hawaii	6/14	18 (3-92)
Idaho	5/5	44.5 (11-93)
Illinois	12/12	7 (5-93)
Iowa	8/8	16.5 (8-85)
Kentucky	2/2	68.5 (64-73)
Maryland	1/1	19
Massachusetts	2/4	49.5 (22-80)
Minnesota	1/1	65
Mississippi	2/2	48.5 (14-83)
Missouri	3/3	11 (<1-22)
Nevada	2/2	14
New Mexico	0/1	8
New York	5/5	57 (43-88)
Oklahoma	1/1	37
Oregon	16/17	58 (13-91)
Pennsylvania	3/3	26 (8-88)
Texas	25/25	19.5 (<1-76)
Utah	7/7	22 (<1-55)
Washington	2/2	56.5 (21-92)
Wisconsin	55/58	27 (<1-92)
Total	193/209 (92.3%)	23 (<1-93)

*Patient age was not reported for 10 isolates tested.

these mutations.^{10,12-14} A recent report describing global surveillance for adamantane-resistant influenza viruses showed a significant increase in resistance from below 2% in 1995-2002 to 12.3% in 2004.¹⁵ In the United States, the frequency of resistance increased significantly from 1.9% in 2004 to 14.5% during the first 6 months of the 2004-2005 influenza season (October 2004 through March 2005).¹⁵ The rate of resistance in the United States for the entire 2004-2005 season was 11% (R.A.B., CDC, unpublished data, 2006). The rapid emergence of adamantane-resistant viruses highlights the need to use results from antiviral susceptibility surveillance to guide therapeutic decisions for patients with laboratory-confirmed influenza infections.

A pyrosequencing method described previously permits rapid analysis for mutations within the M2 gene associated with resistance to adamantanes.¹⁵ In this study, we used this approach to screen influenza A viruses isolated early during the 2005-2006

influenza season in the United States for resistance to adamantanes.

METHODS

Isolates

All influenza isolates collected in the United States and submitted to the Influenza Branch, Centers for Disease Control and Prevention (CDC), from October 1 through December 31, 2005, were included in this study. This group of isolates came from 26 states, distributed throughout the country (TABLE). Isolates were selected and submitted by state public health laboratories participating in the World Health Organization collaborating laboratories or the National Respiratory and Enteric Virus Surveillance System. Each laboratory is requested to submit isolates representative of those circulating early in the influenza season. Laboratories that participate in the World Health Organization influenza surveillance program were provided clear guidelines for submitting early season influenza isolates that are representative of those circulating in the state. No effort was made at the state level to bias the sample selection to a specific population. Every isolate submitted to the CDC during this period was screened for drug resistance and therefore there was no intentional or systematic bias in the sample selection process. No antiviral susceptibility screening was done prior to submission of isolates to the CDC. Antiviral testing was performed using isolates submitted to the CDC, prior to further propagation or handling. All viral isolates were submitted as part of ongoing influenza surveillance. The National Center for Infectious Diseases has determined that these surveillance activities are public health practice and not research; thus, they are exempt from institutional review board regulations.

Virus Propagation and Subtype Determination

Viruses were propagated in primary rhesus monkey kidney or Madin-Darby canine kidney (MDCK) cells. Virus hemagglutinin subtypes were de-

termined by state public health or other local laboratories either by hemagglutination inhibition assay or by direct immunofluorescence assay and were confirmed at the CDC.

RNA Extraction and Sequencing

Viral RNA was extracted from 100 μ L of virus culture, using a total nucleic acid extraction kit with a MagNaPure instrument (Roche Diagnostics, Mannheim, Germany). Reverse transcription polymerase chain reaction (PCR) for complementary DNA synthesis and PCR for DNA amplification were previously described to amplify a 264-base pair product encompassing nucleotides 764 to 1027 of the influenza A matrix gene.¹⁵ Pyrosequencing was performed according to the manufacturer's (Biotage, Uppsala, Sweden) protocol as previously described to sequence a 44-base pair region that included nucleotides 784 to 827 of the matrix gene, which encodes amino acids 25 to 38 of the M2 protein.¹⁵ Confirmatory sequencing by conventional Sanger methods was performed on the same region of the M2 gene as previously described.¹⁵ Sequences were aligned using DNASTar analysis programs (DNASTar, Madison, Wis).

Biological Assay

A biological assay, previously described,¹⁵ was used to confirm antiviral resistance results on a blinded group of viruses. Briefly, monolayers of MDCK cells were pretreated with 0, 0.2, 2.0, or 20 μ g/mL of rimantadine for 30 minutes. Viruses diluted at 1:2 or 1:20 in culture medium, containing 2 μ g/mL of TPCK (L-1-tosylamide-2-phenylethyl chloromethyl ketone)-trypsin, were added to cell monolayers. Following a 1-hour adsorption period, virus medium was replaced by culture medium containing the respective concentration of rimantadine. After a 24-hour incubation period, virus replication was determined by measuring hemagglutination titers of the supernatant. Viruses sensitive to rimantadine were identified by a 4-fold or greater reduction in hemagglutina-

tion titer compared with the control cells without drug.

RESULTS

Influenza A isolates were collected from October 1 through December 31, 2005, from patients living in 26 states. A total of 193 (92.3%) of 209 influenza A(H3N2) and 2 (25%) of 8 influenza A(H1N1) viruses analyzed contained point mutations resulting in a serine-to-asparagine change at amino acid 31 (S31N) of the M2 protein that conferred adamantane resistance (Table). No other amino acid change associated with influenza resistance to adamantanes was detected in these viruses. Six H3N2 isolates contained a valine-to-isoleucine change at amino acid 27 (V27I) in conjunction with the S31N mutation; viruses with this combination have been shown to retain their drug-resistance phenotype in a biological assay (R.A.B., CDC, unpublished data, 2006).

Conventional sequencing was performed on a blinded subset of 40 viruses to confirm results obtained using the pyrosequencing method. In addition, a biological assay was used on a blinded subset of 10 isolates (4 sensitive and 6 resistant) to evaluate the drug-resistance phenotype, and these results correlated 100% with genotypic results obtained with pyrosequencing.

Adamantane-resistant H3N2 isolates were collected from 26 states, showing a wide distribution across the United States (Table). Fourteen isolates were obtained from 2 outbreaks within long-term care facilities. The median age of patients from whom H3N2 isolates were screened was 23 years, ranging from younger than 1 to 93 years (Table). Patient age was not reported for 10 isolates tested.

A small number of isolates collected from other countries in North America and submitted to the CDC also were screened for resistance. Ten of 10 H3N2 isolates collected from patients in Mexico and 3 of 3 isolates tested from Canada also contained the S31N mutation conferring drug resistance.

COMMENT

Influenza antiviral drugs play an important role in a comprehensive approach to controlling influenza illness and transmission. The frequency of resistance to amantadine and rimantadine among circulating influenza A viruses has increased dramatically over the past few years.¹⁵ As part of increased surveillance for the emergence of drug-resistant viruses at the CDC, all isolates submitted through December 31, 2005, were screened for resistance to adamantanes by using a pyrosequencing method. Of the 209 A(H3N2) viruses screened, we found an alarmingly high adamantane resistance rate of 92%. These viruses were isolated from patients residing in 26 states, representing all regions of the United States. This rate was much higher than the rate found among viruses collected within the United States during previous influenza seasons.¹⁵ We found identical resistance among all H3N2 viruses submitted for screening from other North American countries. Canada recently reported that among H3N2 isolates tested for the 2005-2006 influenza season, 43 of 47 (91%) contained this same mutation, showing that adamantane-resistant influenza viruses are circulating in other regions of North America.¹⁶ In addition, preliminary and limited data indicate that the rate of resistance for the 2004-2005 influenza season continued to increase over the previous report for some countries in Asia (China, 96%; Hong Kong, 72%; South Korea, 36%; Singapore, 42%) (R.A.B., CDC, unpublished data, 2006). Data have not been analyzed for the 2005-2006 influenza season in these countries.

It is known that influenza viruses resistant to amantadine and rimantadine can emerge quickly in nursing homes and long-term care facilities when these drugs are being used to control influenza outbreaks.^{13,17-22} Among isolates described in this report, the median patient age was 23 years (range, <1-93 years) for isolates for which the age of a patient was indi-

cated and most were not residents of long-term care facilities. In fact, only a small proportion of the isolates (n=14) were from such facilities. Therefore, use of these drugs for outbreak control cannot explain the high rate of resistance. It is most likely that patients were infected by H3N2 viruses circulating in the community already containing the S31N drug-resistant mutation in the M2 protein. Indeed, this same mutation was the most common change observed in drug-resistant viruses in previously published studies that examined prior years' influenza seasons.^{15,23} In these studies, in which additional patient information was obtained, adamantane treatment was rarely documented among persons outside institutional settings. A limitation of the current study conducted during the initial months of the 2005-2006 influenza season is that additional clinical and epidemiological data are not yet available.

Amantadine and rimantadine have been considered first-line drugs for the treatment of community-acquired influenza virus infections. The high frequency of adamantane-resistant viruses reported here shows that surveillance and rapid identification of resistant viruses have become critical in planning for the appropriate treatment and control of influenza infections in the community and in closed settings. Based on our results of an earlier analysis of 109 of the isolates in this report, the CDC has recommended that neither amantadine nor rimantadine be used for treatment or prophylaxis of influenza for the remainder of the 2005-2006 influenza season.²⁴ A second class of antiviral drugs for influenza is the neuraminidase inhibitors, including oseltamivir and zanamivir. These drugs, licensed in the United States in 1999, work by binding to the active site of the influenza A and B virus neuraminidase proteins and preventing viral release from infected cells. Recent reports of resistance to this class of drugs have shown very few resistant influenza A or B viruses.²⁵⁻²⁷

We have shown that pyrosequencing represents a rapid and accurate high-throughput screening assay for mutations known to confer antiviral resistance among influenza viruses. Thus, rapid assessments of drug resistance within a state or large community are possible if this technology can be introduced into regional reference laboratories. This information can be relayed quickly to health care personnel making critical decisions about the prevention and control of influenza. Our results highlight the importance of continued surveillance

for the emergence and transmission of influenza viruses resistant to antiviral drugs. They serve as a warning to the medical community of the speed at which resistant influenza viruses can become predominant circulating strains and spread throughout a continent.

Published Online: February 2, 2006 (doi:10.1001/jama.295.8.joc60020).

Author Contributions: Dr Bright had full access to all of the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

Study concept and design: Bright, Shay, Cox.

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Study supervision: Bright, Cox, Klimov.

Financial Disclosures: None reported.

Disclaimer: The findings and conclusions in this report are those of the authors and do not necessarily represent the views of the Centers for Disease Control and Prevention.

Acknowledgment: We thank the National Influenza Centers, state public health laboratories, and participating laboratories in the WHO global surveillance program for their submission of isolates. We also acknowledge Jenna Achenbach, MS, Amanda Balish, BS, Angela Foust, MA, Rebecca Garten, PhD, Henrietta Hall, BS, Jan Mabry, BSFR, Gilda Perez-Oronoz, MSPH, and Teresa Wallis, MS, from the CDC Influenza Branch for their contributions to this project.

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