R292K Substitution and Drug Susceptibility of Influenza A(H7N9) Viruses

Katrina Sleeman,1 Zhu Guo,1 John Barnes, Michael Shaw, James Stevens, and Larisa V. Gubareva

Neuraminidase inhibitors are the only licensed antiviral medications available to treat avian influenza A(H7N9) virus infections in humans. According to a neuraminidase inhibition assay, an R292K substitution reduced antiviral efficacy of inhibitors, especially oseltamivir, and decreased viral fitness in cell culture. Monitoring emergence of R292K-carrying viruses using a pH-modified neuraminidase inhibition assay should be considered.

The recent emergence of an avian influenza A(H7N9) virus causing human infections in China (1–2) is of global concern. Most patients infected during this outbreak have experienced severe disease and required hospitalization; the mortality rate is 21% (3). Although epidemiologic investigations have revealed no evidence of sustained human-to-human transmission (4), suspected limited human-to-human transmission has been reported (3).

As with any emergent influenza virus, it is critical to assess the susceptibility of the influenza A(H7N9) outbreak virus to antiviral drugs, which are the first line of defense before an effective vaccine becomes available. Two classes of antiviral drugs are approved for management of influenza A infections, neuraminidase (NA) inhibitors (NAIs) and matrix 2 protein (M2) blockers (adamantanes). The outbreak viruses carry the established adamantane resistance marker, an S31N substitution in the M2 protein (2), leaving NAIs as the only licensed treatment option. Among the 4 NAIs, oseltamivir and zanamivir are approved in many countries; peramivir has been approved in Japan, South Korea, and China; and laninamivir is approved only in Japan. In contrast to those for adamantanes, genetic markers of resistance to NAIs are often subtype specific and drug specific (5). Therefore, monitoring drug susceptibility of the influenza A(H7N9) viruses requires testing in phenotypic assays using all available NAIs.

The Study

Our aim was to assess NAI susceptibility of 2 influenza A(H7N9) outbreak virus isolates provided by the Chinese Center for Disease Control and Prevention. The influenza A/Anhui/1/2013 isolate was recovered from an untreated patient and contained no notable NAI-resistance markers in the NA gene. When tested in the NA inhibition (NI) assay (6), the virus yielded subnanomolar IC_{50} (concentration of neuraminidase inhibitor required to reduce enzyme activity by 50%) with all 4 NAIs, similar to results for the drug-sensitive seasonal influenza A viruses used as controls (Table 1). The second isolate, influenza A/Shanghai/1/2013, was collected from a patient who had received 2 doses of oseltamivir; the isolate was reported to contain an NA substitution, R292K (2). R292K is known to alter NAi susceptibility in viruses of N2 (7) and N9 (8) subtypes. However, A/Shanghai/1/2013 virus was reported to be susceptible to both oseltamivir and zanamivir on the basis of NI assay data (2). To clarify the effect of R292K on NAi susceptibility of influenza A(H7N9) viruses, the A/Shanghai/1/2013 egg-grown isolate (E1) was received and tested at the US Centers for Disease Control and Prevention by using the NI assay (6). Our data showed full susceptibility of A/Shanghai/1/2013 virus to oseltamivir (Table 1), an observation consistent with a previous report (2). Analysis of the E1 isolate by pyrosequencing assay (9) revealed a polymorphism at NA residue 292, containing arginine (23%) and lysine (77%); Table 1). Further analysis of the E1 isolate by PacBio deep sequencing confirmed that 77% of the virus population possessed the lysine 292 variant (Table 1).

The inability to detect changes in oseltamivir IC_{50} despite the presence of R292K raised 2 questions: are conventional NI assays sufficiently sensitive to detect oseltamivir resistance caused by R292K, and is R292K truly a marker of oseltamivir resistance when it is present in these A(H7N9) outbreak viruses? We hypothesized that failure to detect the oseltamivir-resistant population by using the NI assay may stem from substantially reduced activity of the R292K variant NA. Previous studies have shown that the optimal pH for R292K enzyme activity is ~5.3 (7), whereas the conventional NI assay uses a buffer at pH 6.5. We retested A/Shanghai/1/2013 (E1) by using the NI assay under the lower pH condition. The E1 isolate exhibited a higher oseltamivir IC_{50} (643 nmol/L vs. 0.6 nmol/L; Table 2) than that determined by the conventional assay, a finding consistent with our hypothesis. IC_{50} of A/Anhui/1/2013 and reference viruses were either unchanged or found to increase slightly at the lower pH (Table 2).

As part of further investigation of the role of R292K in altering NAi susceptibility, recombinant NA proteins

---

1These authors contributed equally to this article.
defines inhibition as normal [<10], reduced [10–100] or ([36x268]). IC
laninamivir (Tables 1, 2), consistent with previous findings
plied (>1,000), and comparative differences in IC
-100) occur in the absence of an NAI. However, propagation of
viruses (>). Therefore, fitness of the A/Shanghai/1/2013
reversion to wild-type (23% Arg in E1 to 100% in E1/S3),
(>1,000) isolate in eggs and in MDCK-SIAT1 cells resulted in
virus with the R292K substitution
Shanghai/1/2013 isolate, there is evidence of additional
influenza A(H7N9) isolates with the R292K substitution
in addition to oseltamivir, and reduced inhibition by zana-
mivir and laninamivir (Tables 1,2).

Conclusions
R292 is a highly conserved amino acid across all NA subtypes, and together with 2 other highly conserved residues (R118 and R371), it forms an arginine triad in the enzyme active site (5). R292K is a rare substitution and to date has only been reported in viruses collected from patients treated with oseltamivir (2,5). In addition to A/Shanghai/1/2013 isolate, there is evidence of additional influenza A(H7N9) isolates with the R292K substitution (1/). In this study, propagation of A/Shanghai/1/2013 (E1) isolate in eggs and in MDCK-SIAT1 cells resulted in reversion to wild-type (23% Arg in E1 to 100% in E1/S3), confirming results of previous studies with N2 subtype viruses (12). Therefore, fitness of the A/Shanghai/1/2013 R292K virus is probably compromised when replication occurs in the absence of an NAI. However, propagation of the E1 isolate in the presence of oseltamivir (100 nmol/L) resulted in enrichment of the R292K population (from 77% to 100%), demonstrating a growth advantage over the wild-type.

Table 1. Susceptibility of influenza viruses to neuraminidase inhibitors, according to NI assay*

<table>
<thead>
<tr>
<th>Sample type</th>
<th>Subtype</th>
<th>Virus name (passage)</th>
<th>AA at 292†</th>
<th>% K292</th>
<th>IC50 nmol/L (-fold)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Virus isolate</td>
<td>H7N9</td>
<td>A/Anhui/1/2013 (E2/S1)</td>
<td>R</td>
<td>0.15 (1)</td>
<td>0.59 (3)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>A/Shanghai/1/2013 (E1)</td>
<td>R</td>
<td>0.25 (1)</td>
<td>0.52 (2)</td>
</tr>
<tr>
<td>Recombinant</td>
<td>H7N9</td>
<td>A/Anhui/1/2013 (E1)</td>
<td>R and K</td>
<td>5153 (&gt;1,000)</td>
<td>127.60</td>
</tr>
<tr>
<td>NA</td>
<td>H7N9</td>
<td>A/Shanghai/1/2013</td>
<td>R</td>
<td>0.25 (1)</td>
<td>0.46 (1)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>A/Shanghai/1/2013</td>
<td>K</td>
<td>4987 (&gt;1,000)</td>
<td>101.89</td>
</tr>
<tr>
<td>Reference</td>
<td>H3N2</td>
<td>Oseltamivir-sensitive</td>
<td>–</td>
<td>0.07 (1)</td>
<td>0.23 (1)</td>
</tr>
<tr>
<td>virus</td>
<td></td>
<td>Oseltamivir-resistant</td>
<td>–</td>
<td>3974 (&gt;1,000)</td>
<td>16.27 (203)</td>
</tr>
<tr>
<td>H1N1‡</td>
<td></td>
<td>Oseltamivir-sensitive</td>
<td>–</td>
<td>0.19 (1)</td>
<td>0.16 (1)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Oseltamivir-resistant</td>
<td>–</td>
<td>157.25 (828)</td>
<td>15.81 (264)</td>
</tr>
</tbody>
</table>

*NI: neuraminidase inhibition; AA: amino acid; IC50: concentration of neuraminidase inhibitor required to reduce enzyme activity by 50%; E: passage in eggs; S: passage in MDCK-SIAT1 cells (| separates passage before and after arrival to CDC); NA: neuraminidase; NT: not tested; RT-PCR, reverse transcription PCR.
†AA position: R292K (N2 numbering); R294K (straight full-length N9 numbering). Single-nucleotide polymorphism analysis was performed by using the pyrosequencing assay (RT-PCR: primers: N9-F731-Bio, 5'-CT GGA CCT GCA GAC ACA AGA ATA-3' and confirmed by deep sequencing. Pac bio RS sequencing library was constructed by using a 701-bp RT-PCR amplicon generated by RT-PCR (N9NA-PCR, reverse transcriptase-PCR) (N9NA-F204.5'-CAAACATCCAAAGTGGAGAGAA-3'; N9NA-R203 5'-TGTGCTATTGCTACTGGTCTATC-3'). A single v3 SMRT cell was used for each library, and data were collected on 2 × 55 min movies. Only circular consensus sequencing reads were used in the analysis. Subpopulation detection was analyzed by using CLC Genomics Workbench version 6.01 (CLC Bio, Aarhus, Denmark). Isolates were tested in the NI assay by using the NA-Fluor kit (6). Fold change in IC50 values represent the average taken from at least 4 replicates, with the exception of A/Shanghai/1/2013 (E1), because of insufficient sample volume. Oseltamivir-susceptible and oseltamivir-resistant reference viruses were used as controls in NI assays. Oseltamivir refers to oseltamivir carboxylate. Reference virus A/Texas/23/2012 contains H275Y oseltamivir resistance conferring neuraminidase substitution.‡Pandemic influenza A(H1N1) 2009 virus.

(rNAs) of A/Shanghai/1/2013 isolate and A/Anhui/1/2013 isolate were expressed in insect cells by using a transient expression system. The rNAs were tested with 4 NAIs in conventional and pH-modified NI assays (Tables 1, 2). Irrespective of the assay and N9 backbone used, oseltamivir showed an inhibitory effect on the R292K rNAs activity only at concentrations >1,000 nmol/L. The R292K rNAs also showed increased IC50s for peramivir, zanamivir, and laninamivir (Tables 1,2), consistent with previous findings (5). IC50s of the NAIs for the rNAs lacking R292K were comparable with those for the A/Anhui/1/2013 virus.

The NA activity of the rNAs was tested at multiple pH points in MES buffer supplemented with 4 mmol/L CaCl2. Activity of the R292K rNA peaked at pH 5.1 and increased by 5-fold compared with that measured under conventional assay conditions (pH 6.5). Conversely, the NA activity of rNA lacking this change was almost unchanged across the pH range tested (pH 4.9–6.9). These findings indicate that the R292K virus population could be concealed because of its reduced enzymatic activity under conventional assay conditions. NI assays with rNA proteins can clarify the extent of NAI sensitivity for each virus mutant and should be considered when analyzing heterogeneous virus populations with suspected NAI resistance.

To interpret NI assay results, criteria from the World Health Organization Antiviral Working Group were applied (10), and comparative differences in IC50s (which defines inhibition as normal [<10], reduced [10–100] or highly reduced [>100]) were determined by using a subtype-specific reference. The A/Shanghai/1/2013 (E1) isolate exhibited highly reduced inhibition by oseltamivir at pH 5.1. On the basis of data obtained by using rNAs, the R292K conferred highly reduced inhibition by peramivir, in addition to oseltamivir, and reduced inhibition by zanamivir and laninamivir (Tables 1,2).
Replication of the E1 isolate in the presence of any NA1 in cell culture might lead to enrichment with R292K, because even a small growth advantage would reduce the proportion of the wild type. The efficacy of NAIs in clinical management of influenza (H7N9) infection remains unknown and may be compromised to a certain extent when R292K is present. Animal model studies are needed to aid in the understanding of clinical relevance of R292K. Reduction of NA activity caused by R292K may detrimentally affect transmission of the virus, as indicated by an R292K influenza A(H3N2) virus that showed reduced infectivity in mice (13–14) and ferrets (12–13,15) and was not transmitted among ferrets (12,15). The data reported here demonstrate the continued importance of monitoring drug susceptibility in emergent influenza viruses and highlight the challenges involved in laboratory assessment of NA1 drug susceptibility testing.

Acknowledgments

We thank the Chinese Center for Disease Control and Prevention for sharing the influenza A/Anhui/1/2013 (H7N9) virus and members of the US Centers for Disease Control and Prevention Influenza Division for their contributions.

Dr Sleeman is an associate service fellow on the Molecular Epidemiology Team of the Influenza Division at the Centers for Disease Control and Prevention in Atlanta, Georgia. Her research interests are negative-stranded RNA viruses and antiviral drugs, with a particular emphasis on influenza viruses and antiviral drug resistance.

References


<table>
<thead>
<tr>
<th>Sample type</th>
<th>Subtype</th>
<th>Virus name (passage)</th>
<th>AA at 292†</th>
<th>% K292</th>
<th>IC&lt;sub&gt;50&lt;/sub&gt; nmol/L (fold)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Virus Isolate</td>
<td>H7N9</td>
<td>A/Anhui/1/2013 (E2/S1) A/Shanghai/1/2013 (E1)</td>
<td>R and K</td>
<td>77</td>
<td>0.90 (1) 1.00 (1) 0.12 (1) 1.29 (1)</td>
</tr>
<tr>
<td>Recombinant NA</td>
<td>H7N9</td>
<td>A/Anhui/1/2013</td>
<td>R</td>
<td>100</td>
<td>0.53 (1) 0.85 (1) 0.92 (1)</td>
</tr>
<tr>
<td></td>
<td>H1N1‡</td>
<td>Oseltamivir-sensitive A/California/12/2012 Oseltamivir-resistant A/Texas/23/2012</td>
<td>–</td>
<td>364.74 (493) 0.86 (1) 29.72 (23)</td>
<td></td>
</tr>
</tbody>
</table>

*NI, neuraminidase inhibition; AA, amino acid; IC<sub>50</sub>, concentration of neuraminidase inhibitor required to reduce enzyme activity by 50%; E, passage in eggs; S, passage in MDCK-SIA1 cells (separates passage before and after arrival to CDC); NA, neuraminidase; NT, not tested; RT-PCR, reverse transcription PCR.
†AA position: R292K (N2 numbering); R294K (straight full-length N9 numbering). Single-nucleotide polymorphism analysis was performed by using the pyrosequencing assay (RT-PCR primers: N9-292/294-R908-seq, 5’-TAT TTG AGC CCT GCC-3’) and confirmed by deep sequencing. Pac bio RS sequencing library was constructed by using a 701bp RT-PCR amplicon generated by RT-PCR (N9NA-292, 5’-CAACATCTCCATTTGGAGTGAAGGAC-3’; N9NA-R903 5’-TGGTGCTATTGCTACTGGTAC-3’). A single v3 SMRT cell was used for each library and data was collected on 2 × 55 min movies. Only circular consensus sequencing reads were used in the analysis. Subpopulation detection was analyzed by using CLC Genomics Workbench version 6.01 (CLC Bio, Aarhus, Denmark). Isolates were tested in the NI assay by using the NA-Fluor kit (6). Fold change in IC<sub>50</sub> compared with drug-sensitive subtype-specific control. IC<sub>50</sub> values represent the average taken from at least 4 replicates, with the exception of A/Shanghai/1/2013 (E1), due to insufficient sample volume. Oseltamivir-susceptible and oseltamivir-resistant reference viruses were used as controls in NI assays. Oseltamivir refers to oseltamivir carboxylate. Reference virus A/Texas/23/2012 contains H275Y oseltamivir resistance conferring neuraminidase substitution.
‡Pandemic influenza A(H1N1) 2009 virus.


Address for correspondence: Larisa V. Gubareva, Centers for Disease Control and Prevention, 1600 Clifton Rd NE, Mailstop G16, Atlanta, GA 30333, USA; email: lgubareva@cdc.gov

EMERGING INFECTIOUS DISEASES
A Peer-Reviewed Journal Tracking and Analyzing Disease Trends

Instructions for Emerging Infectious Diseases Authors

Types of Articles

Perspectives. Articles should be under 3,500 words and should include references, not to exceed 40. Use of subheadings in the main body of the text is recommended. Photographs and illustrations are encouraged. Provide a short abstract (150 words) and a brief biographical sketch of first author. Articles in this section should provide insightful analysis and commentary about new and emerging infectious diseases and related issues. Perspectives may also address factors known to influence the emergence of diseases, including microbial adaptation and change, human demographics and behavior, technology and industry, economic development and land use, international travel and commerce, and the breakdown of public health measures. If detailed methods are included, a separate section on experimental procedures should immediately follow the body of the text.

Synopses. Articles should be under 3,500 words and should include references, not to exceed 40. Use of subheadings in the main body of the text is recommended. Photographs and illustrations are encouraged. Provide a short abstract (150 words) and a brief biographical sketch of first author—both authors if only two. This section comprises concise reviews of infectious diseases or closely related topics. Preference is given to reviews of new and emerging diseases; however, timely updates of other diseases or topics are also welcome. If detailed methods are included, a separate section on experimental procedures should immediately follow the body of the text.

Research Studies. Articles should be under 3,500 words and should include references, not to exceed 40. Use of subheadings in the main body of the text is recommended. Photographs and illustrations are encouraged. Provide a short abstract (150 words) and a brief biographical sketch of first author—both authors if only two. Report laboratory and epidemiologic results within a public health perspective. Although these reports may be written in the style of traditional research articles, they should explain the value of the research in public health terms and place the findings in a larger perspective (i.e., “Here is what we found, and here is what the findings mean”).

Policy and Historical Reviews. Articles should be under 3,500 words and should include references, not to exceed 40. Use of subheadings in the main body of the text is recommended. Photographs and illustrations are encouraged. Provide a short abstract (150 words) and a brief biographical sketch.

Articles in this section include public health policy or historical reports that are based on research and analysis of emerging disease issues.

Dispatches. Articles should be 1,000–1,500 words and need not be divided into sections. If subheadings are used, they should be general, e.g., “The Study” and “Conclusions.” Provide a brief abstract (50 words); references (not to exceed 15); figures or illustrations (not to exceed two); and a brief biographical sketch of first author—both authors if only two. Dispatches are updates on infectious disease trends and research. The articles include descriptions of new methods for detecting, characterizing, or subtyping new or reemerging pathogens. Developments in antimicrobial drugs, vaccines, or infectious disease prevention or elimination programs are appropriate. Case reports are also welcome.

Commentary. Thoughtful discussions (500–1,000 words) of current topics. Commentaries may contain references but should not include figures or tables.

Another Dimension. Thoughtful essays, short stories, or poems on philosophical issues related to science, medical practice, and human health. Topics may include science and the human condition, the anticipated side of epidemic investigations, or how people perceive and cope with infection and illness. This section is intended to evoke compassion for human suffering and to expand the science reader's literary scope. Manuscripts are selected for publication as much for their content (the experiences they describe) as for their literary merit.

Letters. This section includes letters that present preliminary data or comment on published articles. Letters (500–1,000 words) should not be divided into sections, nor should they contain figures or tables. References (not more than 10) may be included.

Book Reviews. Short reviews (250–500 words) of recently published books on emerging disease issues are welcome. The name of the book, publisher, and number of pages should be included.

News and Notes. We welcome brief announcements (50–150 words) of timely events of interest to our readers. (Anouncements may be posted on the journal Web page only, depending on the event date.) In this section, we also include summaries (500–1,000 words) of emerging infectious disease conferences. Summaries may provide a full report of conference activities and should focus on the meeting's content.

See our website for more information: http://wwwnc.cdc.gov/eid/pages/author-resource-center.htm

1524 Emerging Infectious Diseases • www.cdc.gov/eid • Vol. 19, No. 9, September 2013