An Off-Seasonal Amantadine-Resistant H3N2 Influenza Outbreak in Japan

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Department of Public Health, Graduate School of Medical and Dental Sciences, Niigata University, Niigata, Japan,1 Shimomura Clinic, Nagasaki, Japan,2 Department of Internal Medicine, Institute of Tropical Medicine, Nagasaki University, Nagasaki, Japan,3 National Institute of Hygiene and Epidemiology, Hanoi, Vietnam, and4 Pasteur Institute, Ho Chi Minh City, Vietnam

SAITO, R., LI, D., SHIMOMURA, C., MASAKI, H., LE, M.Q., NGUYEN, H.L.K., NGUYEN, H.T., PHAN, T.V., NGUYEN, T.T.K., SATO, M., SUZUKI, Y. and SUZUKI, H. An Off-Seasonal Amantadine-Resistant H3N2 Influenza Outbreak in Japan. Tohoku J. Exp. Med., 2006, 210 (1), 21-27 —— An off-season community influenza outbreak with high prevalence of amantadine-resistant influenza A/H3N2 occurred during September-October 2005 in Nagasaki Prefecture, Japan, prior to standard influenza circulation. A total of 48 patients with influenza-like-illness (ILI) visited a clinic during the outbreak and 27 (69.2%) of 39 ILI patients were positive for influenza A with rapid antigen testing (Quick Vue Rapid SP Infl). Nine patients were not tested because their symptoms were compatible for influenza without examination. Nasopharyngeal swabs were obtained from 4 of 27 rapid test positive patients, and influenza H3N2 strain was isolated from one out of four. The 4 nasopharyngeal samples were positive for influenza A M2 gene in polymerase chain reaction, and sequencing results all showed identical mutation at position 31, serine to asparagine (S31N) in the gene, conferring amantadine resistance. The phylogenetic tree analysis demonstrated that the hemagglutinin (HA) gene sequences of the 4 samples formed a distinct cluster (named clade N) from recent circulating H3N2 strains, characterized by dual mutations at position 193, serine to phenylalanine (S193F), and at position 225, aspartic acid to asparagine (D225N). Our findings suggested that an off-season community influenza outbreak in Nagasaki was caused by a distinct clade in H3N2 (named clade N), which possessed characteristics of amantadine resistance. ——— influenza; amantadine; antiviral resistance; outbreak

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Influenza in zones with a temperate climate is characterized by the occurrence of annual epidemic during the winter months and influenza season falls in November through April in Japan. However, several off-season community influenza outbreaks occurred in 2005 (Hirano et al. 2005;
Inoue et al. 2005; Morikawa et al. 2005; Taira et al. 2005), and we had a chance to study one of those outbreaks.

M2 inhibitors, amantadine and rimantadine, are effective for prevention and treatment of influenza A infections. The medicines are economical and chemically stable, but emergence of resistance and adverse effect are matters of concern (Suzuki et al. 2003). The genetic basis for resistance to these drugs is associated with amino acid substitutions at positions 26, 27, 30, 31 or 34 in the transmembrane region of the M2 protein (Pinto et al. 1992; Holsinger et al. 1994). It was reported that roughly 1/3 of patients develops resistance after treatment (Hayden and Hay 1992; Saito et al. 2002), but resistance in pre-treatment samples, or community prevalence, remained low in the past, as 0-3% in Japan (Suzuki et al. 2001, 2003), and roughly 1% in the United States and the United Kingdom (Ziegler et al. 1999; Tooley 2002). However, Bight and colleagues (Bright et al. 2005, 2006) recently highlighted a dramatic increase in the prevalence of amantadine resistant H3N2 influenza strains in Asian countries and USA.

Upon our epidemiological study of an off-season community influenza outbreak occurred in Nagasaki Prefecture in 2005, we found high prevalence of amantadine resistant H3N2. Here, we report this outbreak, and tried to clarify the relationship between the genetic characteristics of amantadine-resistant H3N2 influenza strains and its high prevalence.

**Materials and Methods**

**Epidemiological information and specimen collection**

During September-October 2005, an influenza outbreak was reported in Nagasaki Prefecture, Japan (Hirano et al. 2005). This study was conducted with patients consulted to Shimomura Clinic during the outbreak in Tokitsu-cho town with a total population of approximately 30,000, Nagasaki Prefecture.

Influenza-like illness (ILI) cases were defined on the basis of a sudden fever, cough and sore throat. Their nasopharyngeal swabs were examined with rapid antigen test kits for diagnosis of influenza A or B (Quick Vue Rapid SP INFLU, DS Pharma Biomedical Co., Ltd., Osaka) prior to antiviral drug treatment (oseltamivir) at the initial office visits. Upon provision of oral informed consent, precise patient information and nasopharyngeal swabs were obtained from selected influenza A positive patients for further virological testing. They were stored at 4°C in viral transport media until transferred to the Department of Public Health, Niigata University, Graduate School of Medical and Dental Sciences, Niigata City, Japan.

**Virus isolation**

One hundred μl of supernatant of nasopharyngeal swabs was inoculated into Madin-Darby canine kidney cells (MDCK), prepared in 48-well multiple well plates. The plates were kept at 34°C under a 5% CO₂ atmosphere for up to 10 days to assess cytopathic effects (CPEs). Fifty μl aliquots of supernatants of CPE positive samples were then passaged twice to obtain a sufficient virus titer to perform virus identification. Influenza isolates were typed and subtyped by hemagglutination inhibition (HAI) assay with commercially available influenza

<table>
<thead>
<tr>
<th>Patient (sample name)</th>
<th>Age (y.o.)</th>
<th>Sex</th>
<th>Date of onset (d/m/y)</th>
<th>Time to clinic visit</th>
<th>Prior medication</th>
<th>Medication at the Clinic</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 (N1)</td>
<td>5</td>
<td>F</td>
<td>16/9/2005</td>
<td>≤ 24 hours</td>
<td>No</td>
<td>Oseltamivir</td>
</tr>
<tr>
<td>2 (N2)</td>
<td>2</td>
<td>F</td>
<td>16/9/2005</td>
<td>1 day</td>
<td>No</td>
<td>Oseltamivir</td>
</tr>
<tr>
<td>3 (N3)</td>
<td>13</td>
<td>M</td>
<td>18/9/2005</td>
<td>3 days</td>
<td>No</td>
<td>Oseltamivir</td>
</tr>
<tr>
<td>4 (N4)</td>
<td>8</td>
<td>F</td>
<td>21/9/2005</td>
<td>≤ 24 hours</td>
<td>No</td>
<td>Oseltamivir</td>
</tr>
</tbody>
</table>

1 Nested PCR for M2 gene of influenza A detection.
2 Nested PCR for H3 subtype hemagglutinin (HA) gene of influenza A detection.
3 Hemagglutinin inhibition test reacted with A/New York/55/2004(H3N2).

RT-PCR, reverse transcription-polymerase chain reaction; N/A, not addressed; S31N, mutation at position 31.
vaccine strain antisera for 2005/2006 season in Japan, A/New Caledonia/20/99 (H1N1), A/New York/55/2004 (H3N2), B/Shanghai/361/2002 (Denka Seiken Co., Ltd., Tokyo), using guinea pig red blood cells. An amantadine susceptibility test was conducted with two series of 10-fold dilution of viruses from CPE-positive cultures, plated in triplicate in a 96-well microplate confluent with MDCK cells with one dilution series containing 1.0 \( \mu g/ml \) of amantadine in the media as reported previously (Masuda et al. 2000). Amantadine resistant strains were identified when a less than 1.0 fold difference in log TCID\(_{50} \) (50% tissue culture infective dose) titer was observed between series of rows with and without the drug after incubation for 48 hrs at 37°C.

**Polymerase chain reaction (PCR) and sequencing**

RNA was extracted from 100 \( \mu l \) of nasopharyngeal swabs or influenza isolates using Extragen II kit (Kainos, Tokyo), according to the manufacturer’s instructions. Reverse transcription (RT) to create complementary DNA (cDNA) was performed using an influenza A generic primer, Uni12, as reported elsewhere (Hoffmann et al. 2001). First, a nested PCR was performed using specific primers to amplify the M2 region, segment 7, of influenza A, as described previously (Masuda et al. 2000) and then the PCR products were sequenced to examine mutations at positions 26, 27, 30, 31 and 34 in the transmembrane region of the M2 protein, conferring amantadine resistance (Klimov et al. 1995). The templates were labeled by cycle sequencing reactions with fluorescent dye terminators (BigDye Terminator v 3.1 Cycle Sequencing Kit, Applied Biosystems), and the products were analyzed using an ABI 310 (Applied Biosystems) automatic sequencer following the manufacturer’s instructions. Amplification and sequencing of the hemagglutinin (HA) gene, segment 4 of influenza H3N2 was performed with sets of nested PCR primers as reported elsewhere (Besselaar et al. 2004). Phylogenetic tree analysis was made with the obtained HA sequences, together with other recent H3N2 strains registered in Influenza Virus Resource (National Center for Biotechnology Information, [http://www.ncbi.nlm.nih.gov/genomes/FLU/FLU.html](http://www.ncbi.nlm.nih.gov/genomes/FLU/FLU.html)). Multiple alignment, phylogenetic analyses were performed using MEGA ver 3.1 (Kumar et al. 2004). In order to avoid possible influence of laboratory contamination, RNA extraction, cDNA synthesis and PCR amplification from samples were repeated more than twice by different examiners. Both positive and negative controls were included along with the samples for every PCR reaction.

For references of sequencing of the HA gene and its phylogenetic tree analysis, 6 amantadine resistant H3N2 isolates carrying S31N mutation collected in Hanoi and Ho Chi Minh City, Vietnam in 2005 (Hanoi/HN 30602/05, Hanoi/HN30607/05, Hanoi/ISBM63/05, Hanoi/HN30720/05, Hochiminh/14/05, Hochiminh/16/05), and 4 sensitive (Hanoi/ISBM53/05, Hanoi/ISBM69/05, Hanoi/TB285/05, Hochiminh/2/05) were included. In addition, two sensitive isolates collected in Nagasaki, Japan in January in 2005 (2004-05 season) were added to the analysis as representatives of standard circulation (Nagasaki/ND2/05 and Nagasaki/ND8/05).

**Nucleotide sequence accession numbers**

The DNA Data Bank of Japan (DDBJ) accession numbers of the nucleotide sequences for 4 strains, A/Nagasaki/N1/05(H3N2) to A/Nagasaki/N4/05(H3N2), are AB262301 to AB262304.

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<table>
<thead>
<tr>
<th>Outcome</th>
<th>Influenza rapid test</th>
<th>Influenza isolation</th>
<th>RT-PCR (M2)(^1)</th>
<th>RT-PCR (H3-HA)(^2)</th>
<th>M2 mutation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Recovered</td>
<td>A Positive(^3)</td>
<td>Positive</td>
<td>Positive</td>
<td>Positive</td>
<td>S31N</td>
</tr>
<tr>
<td>Recovered</td>
<td>A Negative</td>
<td>Positive</td>
<td>Positive</td>
<td>Positive</td>
<td>S31N</td>
</tr>
<tr>
<td>Recovered</td>
<td>A Negative</td>
<td>Positive</td>
<td>Positive</td>
<td>Positive</td>
<td>S31N</td>
</tr>
<tr>
<td>Recovered</td>
<td>A Negative</td>
<td>Positive</td>
<td>Positive</td>
<td>Positive</td>
<td>S31N</td>
</tr>
</tbody>
</table>

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3 serine to asparagine, in M2 transmembrane domain in influenza A.
RESULTS

A total of 48 patients visited Shimomura Clinic as ILI with onset dates ranging from 9 Sep to 15 Oct (Fig. 1). The average patient age was 18.9 ± 16.8 years (0.5 to 82 years old), and a half of patients were children under age of 12. Rapid test was positive with influenza A in 27 (69.2%) of 39 patients. Nine patients were not tested because their symptoms were compatible for influenza without examination. Three quarter of ILI patients received oseltamivir treatment.

Four patients underwent nasopharyngeal swab collection, and they had no history of recent overseas travel or prior amantadine administration (Fig. 1 and Table 1). They received oseltamivir treatment and recovered from the illness without complications.

Influenza virus was recovered from one patient (Nagasaki/N1/05), and reacted with A/New York/55 /2004(H3N2) on HAI characterization (Table 1). It was conferred as amantadine resistant by the TCID$_{50}$/0.2 ml susceptibility test, with a virus titer of 0.5 log$_{10}$ in amantadine added medium, and 1.3 log$_{10}$ in amantadine free medium.

All four nasopharyngeal swabs showed positive by RT-PCR with M2 protein primers, and carried amino acid substitution at position 31, serine to asparagine (S31N) in transmembrane domain in M2 protein (Table 1). HA gene PCR for A/H3 was positive with all, and sequencing results showed a motif with dual mutations at position 193, serine to phenylalanine (S193F), and at position 225, asparatic acid to asparagine (D225N), except for A/Nagasaki/N4/05 which possessed only S193F change (Fig. 2). Furthermore, six amantadine resistant strains in Vietnam also had a same motif with dual mutations. Those resistant strains formed a distinct cluster (named clade N) from other strains. However, 4 amantadine sensitive strains in Vietnam and 2 from Nagasaki did not have the motif and not clustered in the clade N.

DISCUSSION

In 2005, several off-seasonal H3N2 influenza outbreaks occurred in Japan (Hirano et al. 2005; Inoue et al. 2005; Morikawa et al. 2005; Taira et al. 2005), but main causes remain unknown. We had a chance to study one of those outbreaks, in Nagasaki (Hirano et al. 2005). We could analyze only 4 samples, but all of them were resistant H3N2 viruses. Even with limited number of cases in a rather small geographic area, our results suggested high prevalence of resistant strains in the outbreak area. It is generally accepted that amantadine resistant viruses emerges under the pressure of the drug, but are less virulent or transmissible than susceptible viruses (Harper et al. 2005).
Fig. 2. Phylogenetic tree analysis among hemagglutinin (HA) genes from human H3N2 viruses. Isolates and nasopharyngeal swabs collected in Nagasaki in Japan, Hanoi and Ho Chi Minh City in Vietnam in 2005, and selected strains in genetic database were included in the analysis. Strains labeled with closed circles indicated amantadine resistant strains possessing S31N mutation in M2 protein, and open circles sensitive. Presence of HA motif in amantadine resistant strains, mutations at positions 193, serine to phenylalanine (S193F), and 225, asparatic acid to asparagine (D225N), were shown by arrows, and this group was named “clade N”. Virus names in italic boldface denote Japanese and WHO recommended influenza vaccine strains for H3N2 in Northern Hemisphere in 2005/06. Phylogenetic trees were inferred from 467 nucleotide sequences by the neighbor-joining method. Bootstrap values > 70% are shown.
In Japan, the prevalence of resistance remained 0-3% in pre-treatment samples until the 2004-05 season in our survey (Suzuki et al. 2001, 2003), and less than 1% in USA and other countries (Ziegler et al. 1999; Tooley 2002). However, Bight and colleague’s (Bright et al. 2005; Guan and Chen 2005) study recently highlighted a dramatic increase in the prevalence of resistant H3N2 strains in Asian countries, suggesting association with inappropriate drug administration. In the present study, all patients had neither prior amantadine administration nor history of recent overseas travel, under the situation amantadine is prescribed only by licensed clinicians in Japan. Our study also indicate the presence of resistant H3N2 strains in Vietnam, where amantadine is not available as either a prescriptive or a commercial drug. Recent report indicated that even in USA, resistant H3N2 was quite high (Bright et al. 2006). Thus, we support the assumption that the resistant viruses may not have arisen solely as a result of exposure to drugs, but naturally occurring resistant-associated mutations could have emerged (Guan and Chen 2005).

All resistant strains isolated from this outbreak and 6 isolates from Vietnam carried S31N mutation in transmembrane domain in M2 protein. The same mutation point was observed in recent resistant H3N2 in Asia and USA (Bright et al. 2005, 2006). Our resistant strains formed a distinct cluster (clade N) with dual mutations at positions 193 and 225, except for one strain had only S193F change, while amantadine sensitive strains in Vietnam and Nagasaki clustered in other clades. Although the origin of the clade N is unexplained, our findings suggest that clade N is circulated simultaneously in Japan and Vietnam. We need further study of the relationship between our strains and other resistant strains in China, Hong Kong, Taiwan, South Korea, and USA. The high incidence of resistant H3N2 viruses was found in almost the same geographic regions as H5N1 viruses. Resistant H5N1 viruses appeared to be largely limited to be in Thailand, Vietnam, and Cambodia, and are present in almost all viruses from the Vietnam/Thailand/Malaysia sublineage, while most H5N1 isolates from China and Indonesia are sensitive to amantadine and are present in other sublineage (Guan and Chen 2005; Cheung et al. 2006). The relationship between amantadine resistant H3N2 and H5N1 viruses and its HA lineage is quite noteworthy and may be strongly associated each other, but warrant examination.

Our findings suggest that an off-season community influenza outbreak with high prevalence of amantadine resistant H3N2 (clade N) occurred in Nagasaki, Japan. Additional investigation is needed to elucidate the origin and geographical spread of H3N2 strains of clade N, and its biological effect on the life cycle of the H3N2 viruses. The increase of resistant strains is a big problem on a global scale, and we need to stress international coordination for proper use of M2 inhibitors and increase antiviral surveillance to keep this cheap drug as one of strategic options for influenza control.

Acknowledgments

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References

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