High Frequency of Amantadine-Resistant Influenza A (H3N2) Viruses in the 2005-2006 Season and Rapid Detection of Amantadine-Resistant Influenza A (H3N2) Viruses by MAMA-PCR

Mami Hata*, Masako Tsuzuki1, Yasuhiro Goto2, Norimichi Kumagai3, Miki Harada1, Michiko Hashimoto1, Seidai Tanaka, Kenji Sakae**, Takashi Kimura1, Hiroko Minagawa and Yutaka Miyazaki1

Department of Microbiology, 1Aichi Prefectural Institute of Public Health, Nagoya 462-8576; 2Kamiiida Daiichi General Hospital, Nagoya 462-0802; and 3Chubu International Airport Quarantine Branch Office, Ministry of Health, Labour and Welfare, Tokoname 479-0881, Japan

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SUMMARY: Using the newly designed mismatch amplification mutation assay (MAMA) PCR, we demonstrated the high frequency of amantadine-resistant influenza A (H3N2) viruses isolated during the 2005-2006 season by detecting the mutation at amino acid position 31 of the M2 protein (S31N). Further, phylogenetic analyses of the HA1 sequences of the S31N viruses revealed that they comprised a clonal lineage that would result in the common characteristic amino acid changes at positions 193 (Ser to Phe) and 225 (Asp to Asn) of the HA protein. We also demonstrated that the S31N/S193F/D225N viruses had already emerged in Aichi Prefecture by the end of the previous 2004-2005 season.

Adamantanes, e.g., amantadine and rimantadine, have been used as antiviral agents against influenza A viruses in developed countries including Japan. Adamantanes block the proton channel of the influenza A virion, M2 protein, and thus inhibit the pH change necessary for the uncoating process (1). Amantadine resistance is caused by a single point mutation at any of the amino acid positions 26, 27, 30, 31 or 34 of the M2 protein (2,3). Among these, the mutation at position 31 has been the most frequently reported in resistant virus isolates (4-6).

During the 2005-2006 influenza season, reports from the United States warned that 92.3% of the influenza A viruses were already resistant to adamantanes (7,8). This predominance of the resistant virus in the 2005-2006 season was prominent, although some increase in adamantane resistance had been noted in the previous seasons (from 0.4% in 1994-1995 to 14.5% in 2004-2005) (9). In Japan, amantadine-resistant influenza A viruses were isolated with high frequency from patients treated with this drug (10), whereas the isolation of resistant viruses in communities occurred only occasionally (5,11) since the drug was licensed for the treatment of influenza in 1998. However, in the 2005-2006 influenza season, a dramatic increase of resistant viruses in communities was also observed in Japan (12), Southeast Asia and Oceania (13) as well as in North America (7,8). Further, the resistant viruses were already detected in September 2005, just before the influenza season, in Nagasaki, Japan (14).

In this study, we investigated the frequency of the amantadine-resistant influenza A (H3N2) viruses (AH3) in Aichi Prefecture during the 2005-2006 season. To screen for the resistant viruses, we developed a rapid reverse transcription (RT)-polymerase chain reaction (PCR) protocol for the detection of mutations of the M2 gene relevant to the resistance phenotypes. The sequences of the M2 gene of these viruses were further determined. The HA1 sequences of the resistant viruses were also analyzed to clarify whether they have any relevance to the resistant mutations.

Throat swab specimens of patients suspected of having influenza were collected as part of the nationwide infectious agents surveillance, and were also collected from immigrants who reported flu-like symptoms to the Chubu International Airport Quarantine Branch Office (Tokoname, Japan). Intra-nasal specimens from pediatric patients at the outpatient ward of Kamiiida Daiichi General Hospital (Nagoya, Japan) were also used in this study. All specimens were inoculated onto Madin-Darby Canine Kidney (MDCK) cells and observed for characteristic cytopathic effects (CPEs) for up to 2 weeks. The serological types of each isolate were determined by a hemagglutination inhibition (HI) test of CPE-positive MDCK culture supernatant using the type-specific sera against influenza viruses (provided by the National Institute of Infectious Diseases, Tokyo, Japan). Viral RNA was extracted from culture supernatants using the High Pure Vial RNA Kit (Roche Applied Science, Penzberg, Germany).

Since the Ser to Asn (AGT to AAT) mutation at codon 31 of the M2 protein gene has been reported to be the most commonly encountered mutation leading to amantadine resistance, we developed a mismatch amplification mutation assay (MAMA) protocol (15) for the specific detection of this mutation. First, cDNA was obtained from viral RNA with Superscript III (Invitrogen), and was used as the template for PCR. Conserved primers of M2F1 (5'-cagctaaggctatggagcaacc-3') and M2R1 (5'-aactgtcgtcagcatccacag-3') were designed for the control PCR amplification of a 402-bp product containing the M2 gene. A mismatch reverse primer MAMA31S
(5'-gtcaagatcccaatgattc-3') was designed for the detection of the wild-type codon for Ser-31 (AGT). The ability of the mismatch primer to generate a PCR product with the wild-type allele (AGT) and not the mutated allele (AAT) is based on the fact that the 3' end of the mismatch primer has only one mismatch with the wild-type allele, but two mismatches with the mutated allele. MAMA31S was also added to each PCR reaction to generate a 246-bp PCR product with the wild-type M2 gene but not with the mutated gene. Twenty-five microliters of PCR reaction contained 2.5 µl of cDNA, 1 U of ExTaq DNA polymerase (Takara, Ohtsu, Japan), and 5 pmol of the forward primer, 2.5 pmol of the reverse primer, and 12.5 pmol of the mismatch primer. Following the denaturation step at 94°C for 3 min, PCR cycling conditions were as follows: 30 cycles of 94°C for 30 s, 58°C for 30 s and 72°C for 30 s. The sequence of the M2 gene was determined by direct sequencing of the 402-bp control amplicon using a Model-4200 automated DNA sequencer (Li-Cor, Lincoln, Nebr., USA).

In addition, a region containing the complete HA1 gene was amplified from viral RNA using the One-step RT-PCR Kit (Invitrogen). The primer pair used was 5'-caaaagcaggggattattct-3' and 5'-ttgagatctgctgcttgtcc-3', which yields a 1,178-bp product. The 987-bp HA1 sequence was determined and used for the phylogenetic analysis with GENETYX (Ver. 7; GENETYX Corp., Tokyo, Japan).

We analyzed 23 AH3 isolates in the 2005-2006 season and 8 late in the 2004-2005 season. The mutation of the M2 gene was screened by the MAMA-PCR method. The representative result of MAMA-PCR for the mutation detection in M2 protein is shown in Fig. 1. The 246-bp product shown in lanes 1, 3, 5, 8, and 10 denoted the presence of the sequence for the wild-type Ser (AGT), and the absence of an amantadine-resistant mutation at this site. The nucleic acid sequences of the isolates negative for the 246-bp product were examined by sequencing the control 402-bp band. Twenty isolates (20/23, 87%) in the 2005-2006 season and 3 (3/8, 37.5%) isolates in the 2004-2005 season had the S31N mutation in the M2 gene.

The phylogenetic tree analyses of HA1 gene revealed that all S31N viruses belonged to a single distinct lineage, as shown in Fig. 2. All isolates in this clade had the common mutations...
in the HA1 gene, i.e., S193F and D225N. These HA1 mutations have also been reported in the latest studies (12-14). One isolate, Aichi/108/2006, had the mutation of S31D in the M2 gene, and its HA1 gene was included in the same lineage as the S31N viruses. It is possible that the parent of this isolate was a S31N virus and that an additional point mutation from AAT (N) to GAT (D) occurred in the allele.

Among the 3 strains of S31N viruses isolated at the end of the 2004-2005 season, Aichi/165/2005 was isolated from a Japanese resident who had returned from China after a short trip. The earliest isolate in Aichi with these mutations was from the specimen collected in May (Aichi/163/2005), which means that the variant virus had already emerged by the end of the 2004-2005 season, several months earlier than the report from Nagasaki (14).

In this study, a rapid PCR method was developed for the detection of base substitutions resulting in the Ser to Asn (and possibly further to Asp) mutations at amino acid position 31 in the M2 gene. Isolates with this S31N or S31D mutation did not harbor an amplified product with the mismatch reverse primer MAMA31S together with the forward primer, whereas the isolates with the wild-type gene generated a 246-bp PCR product. The MAMA-PCR protocol described here would be a useful tool to screen the strains of the influenza A virus isolate.

The nucleotide sequences of the genes used in this study can be found in GenBank under the following accession numbers: M2 gene: AB259742-51, AB259767-76, AB289679-89, HA1 gene: AB243867-72, AB259101-12, AB259739-41, AB289670-78, DQ508865 (A/Panama/2007/1999), DQ865947 (A/Wisconsin/67/2005), DQ865973 (A/California/7/2004).

REFERENCES