

## Short Communication

# Frequency of Amantadine-Resistant Influenza A Virus Isolated from 2001 -02 to 2004 -05 in Nara Prefecture

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**SUMMARY:** We investigated the frequency of amantadine-resistant influenza A viruses in Nara Prefecture during four epidemic seasons from 2001 -02 to 2004 -05. Point mutations within the *M2* gene were identified using RT-PCR and DNA sequencing analysis. Five viruses (3.4%) with point mutation were observed from 145 strains analyzed. Three viruses (2.0%) possessed a change at position 31 (AGT→AAT, Ser to Asn), one virus (0.7%) showed a change at position 26 (CTT→TTT, Leu to Phe), one virus (0.7%) showed a change at position 27 (GTT→ATT, Val to Ile), and none showed a change at position 30. All of these changes were the transition type of mutation. These results indicated that the possible circulation of drug-resistant viruses to the community was not supported by the findings obtained during the 2004 -05 season in Nara.

Amantadine, which was developed by a U. S. chemical company in 1964, is the first influenza antiviral drug for humans. In human cells, the drug inhibits virus replication during the early stage of infection by blocking the ion channel formed by the *M2* protein. Therefore, amantadine has been shown to be effective for treating and preventing human influenza A virus infections. This drug is approved for treatment of Parkinson's disease, and for prevention of influenza A virus infections in Japan. However, it has been reported that the influenza virus becomes resistant to the drug through a single amino-acid substitution at four positions within the transmembrane region of the *M2* protein (1-3). In 2002, Saito et al. (4) reported a high frequency (approximately 24%) of amantadine-resistant virus in closed settings, such as nursing homes. In this study, we examined amantadine-resistant viruses in field isolates of the influenza A (H3) virus collected in Nara Prefecture between 2001 -02 and 2004 -05.

Throat swab specimens were collected for influenza diagnosis of surveillance system in the winter seasons from 2001 -02 to 2004 -05. Supernatants of the throat swabs were inoculated into MDCK cells for influenza virus isolation. Subtypes of the viruses were determined by hemagglutination-inhibition tests with type-specific antisera. A total of 145 influenza A (H3N2) virus specimens were isolated. A screening test of the resistant viruses was performed with minor modification of Saito et al's method (4). First, a susceptibility test was done with two series of a 10-fold dilution of viruses from a cytopathic effect-positive culture, and plated in triplicate in a 96-well microplate on MDCK cells with one dilution series containing amantadine (1-adamantylamine hydrochloride; Sigma, St. Louis, Mo., USA) (1.9 µg/100 µl) in the medium. Amantadine-resistant strains were identified when a <2.0-fold difference in the log TCID<sub>50</sub>/0.2 ml titer was observed with and without the drug after 48 h inoculation.

Detection of the virus gene was carried out by the RT-PCR method reported (4), using specific primers (*M2-For3* and *M2-Rev*) for the *M2* protein region of the AH3 type virus. In addition, amino acid substitutions were confirmed by partial nucleotide sequencing (Thermo Sequenase Cy5.5 Dye Terminator Cycle Sequencing Kit; Amersham Biosciences, Corp., Piscataway, N. J., USA).

As a result of the amantadine-susceptibility test, 15 specimens were observed as possibly positive. Finally, point mutation of the *M2* gene was confirmed by nucleotide sequencing. The results of the amantadine-resistant influenza A viruses in each season are summarized in Table 1. One (3.1%) out of 32 strains was from the 2003 -04 season, three (5.3%) out of 53 strains were from the 2002 -03 season, one was confirmed as a resistant strain with amino acid substitutions in 35 strains by the 2001 -02 season, and no strains were found from the 2004 -05 season. In total five strains (3.4%) out of 145 were found during these four seasons were frequency of 3.4%: mutation at position 26 was one: CTT→TTT, leucine to phenylalanine, the position 27 was one (GTT→ATT, valine to isoleucine) and of the position 31 was three (AGT→AAT, serine to asparagine). All mutations were a transition type.

Amantadine is approved for treatment of Parkinson's disease, and for prevention of the influenza A virus infection. Interestingly, Saito et al. (4) and Masuda et al. (5) provided clear evidence that the amantadine-resistant influenza virus strains were circulating at a high frequency (31/141 [24.1%] and 8/26 [30.8%], respectively) in nursing homes where Parkinson's disease patients resided. Their result indicates that the high frequency of amantadine-resistant virus was induced because of the repeated use of the drug for treatment of these patients. Therefore, we were concerned about the possibility of drug-resistant viruses spreading in society at large through exposure by hospital staff, and investigated the frequency of amantadine-resistant influenza A viruses in sentinel surveillance sites during four epidemic seasons for influenza from 2001 -02 to 2004 -05.

In our study, we found a low frequency of amantadine-resistant strains in 145 strains from sentinel surveillance sites in four successive seasons, which correlated with a recent report from the Centers for Disease, Control and Prevention

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Table 1. Summarized results of amantadine-resistant influenza A viruses in Nara Prefecture (from 2001-02 to 2004-05)

Season	No. of specimens	No. of possible positives (Susceptibility test)	No. of strains with amino acid substitution (%)	No. of strains with the amino acid substitutions in the M2 gene at position (%)			
				26	27	30	31
2001-02	35	3	1 (2.8)				1 AGT ↓ AAT
2002-03	53	5	3 (5.3)	1 CTT ↓ TTT			2 AGT ↓ AAT
2003-04	32	3	1 (3.1)		1 GTT ↓ ATT		
2004-05	25	4	0 (-)				
Total	145	15	5 (3.4)	1 (0.7)	1 (0.7)	0 (-)	3 (2.0)

(6). The incidence of resistant viruses in Shizuoka and Niigata Prefectures (7) were 3.2% (2/62) and 0% (0/55), respectively, a low incidence compared with the report of Saito et al. This information may indicate that the spread of amantadine-resistant viruses is limited to environments where close contact occurs, such as nursing homes. From epidemiological data obtained worldwide, the surveillance study by Ziegler et al. (8) reported a drug-resistance frequency of 0.8% (16 of 2,017) among AH1N1 and AH3N2 viruses during a 4-year period. In a recent report, however, high incidences of resistant viruses in China and Hong Kong were observed in the 2004-05 epidemic season (73.8 and 69.6%, respectively) (6,9). Furthermore, the incidence of resistant viruses in the United States was reported to be 92.3% (193 of 209 influenza AH3N2) in the 2005-06 season (10). In China, amantadine or rimantadine are available in over-the-counter formulations and are included in various cold remedies that do not require a prescription. Because of this phenomenon, adamantanes are now broadly available as generic drugs.

Amantadine inhibits influenza A virus replication by interfering with M2 protein ion channel activity (11). Viruses become resistant to amantadine through a single amino acid substitution at positions 26, 27, 30 or 31 in the transmembrane region of the M2 protein (1-4). Mutations at position 31 (serine to asparagine) are most frequent in amantadine-resistant AH3 viruses. Approximately 30% of children treated with amantadine have been reported to shed resistant viruses. Similarly, in our data, the predominant mutation portion was a change at position 31, and its frequency was 60% (three of five mutation-positive strains). Interestingly, Shiraishi et al. (12) indicated a high frequency of mutations at position 31 (serine to asparagines) and 30 (alanine to valine) in AH3 viruses, as well as 27 (valine to alanine) in AH1 viruses. Because AH3 and AH1 viruses have phylogenetically different M2 proteins, this difference may be influenced by structural differences in M2. In the present study, furthermore, residue at position 26 was one (CTT → TTT, leucine to phenylalanine), which was critical for ion channel activity and amantadine sensitivity (7). To our knowledge, the amino acid residue at the first position of the position 27 mutation (GTT → ATT, valine to isoleucine) has not been previously reported in Japan, and is thus a rare occurrence. Though all of these mutations were transition, it may be possible to be some mechanisms for the induction of mutations, this change mechanism is not known.

In conclusion, a high spread of the amantadine-resistant viruses may be a problem in environments, such as nursing

homes, where close contact occurs, but based on our findings, it is unlikely that these will spread through society at-large.

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