

Short Communication

High Incidence of Amantadine-Resistant Influenza AH3 Viruses Isolated during the 2005 - 2006 Winter Season in Nara, Japan

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SUMMARY: We examined the incidence of amantadine-resistant influenza AH3 viruses isolated in Nara Prefecture during the 2005-06 winter season. The genetic analyses of the M2 ion channel protein were conducted using reverse transcriptase PCR and direct sequencing. Thirteen out of 18 (72.2%) strains were identified as amantadine-resistant, and this incidence was remarkably higher than those previously recorded in Nara Prefecture. Genetic analyses of the viruses revealed that all the anti-drug strains contained a change at position 31 (AGT→AAT, Ser31Asn) in the M2 gene. One of the 13 amantadine-resistant strains also contained a change at position 27 (GTT→GCT, Val27Ala). Our data indicate that there has been a significant increase of drug-resistant influenza AH3 viruses in Nara Prefecture, and raise concern about the spread of resistant influenza AH3 viruses in Japan.

Amantadine has been widely used as a treatment for influenza A virus infections for many years. Amantadine-resistance was first described soon after the drug was discovered in early 1960, and subsequent work has established that point mutations in critical residues of the M2 protein confer high-level resistance to amantadine and rimantadine. Based on worldwide epidemiological surveillance data, Ziegler et al. (1) reported a drug-resistance frequency of 0.8% (16/2,017) among AH1 and AH3 viruses during a 4-year period. Similarly, we reported a low frequency (5/145, 3.4%) of amantadine-resistant strains from sentinel surveillance sites during four epidemic seasons from 2001-02 to 2004-05 in Nara Prefecture (2). In a recent report, however, Bright and colleagues' genotypic study (3) showed a major increase in the prevalence of drug-resistant AH3N2 strains in Southeastern Asian countries such as Hong Kong and China, which contributed to an increase of more than 30-fold in the frequency of drug-resistance between 1994-95 and 2003-04 worldwide. Furthermore, a high incidence of resistant viruses of 92.3% was reported during the 2005-06 influenza season in the United States (4). In this study, we report the results of genetic analyses of drug-resistant influenza AH3 viruses isolated between December 1, 2005 and March 31, 2006 in Nara Prefecture.

Throat swab specimens were collected for influenza diagnosis of surveillance system in 2005-06 season. Supernatants of throat swabs were inoculated into MDCK cells, prepared in 48-well multiplates, for influenza virus isolation. Influenza isolates were typed and subtyped by hemagglutination inhibition assay using antisera against A/New Caledonia/20/99 (H1N1) and A/New York/55/2004 (H3N2), which were provided by the National Institute of Infectious Diseases in Japan. In this way, 18 influenza AH3 strains were obtained. Detection of amantadine-resistant influenza virus strains was performed by reverse transcriptase (RT)-PCR and DNA

sequencing of M2 transmembrane protein genes as described previously (2,5).

The results of the genetic analysis of amantadine-resistant influenza AH3 viruses, including mutation sites and types, are summarized in Table 1. Thirteen out of 18 (72.2%) strains held a point mutation at amino acid position 31 (AGT→AAT, Ser31Asn) in the M2 protein gene. One (5.6%) of the 13 strains contained another point mutation at position 27 (GTT→GCT, Val27Ala). Both mutations were of transition-type (G-to-A or T-to-C). All strains with point mutations were confirmed as the drug-resistant virus by a serological susceptibility test based on the method of Saito et al. with minor modification (data not shown) (5). All 5 patients in December 2005, 3 out of 7 in January 2006, and 5 out of 6 in February 2006 were infected with amantadine-resistant viruses. In addition, geographical analysis of patients with drug-resistant viruses was performed. Four patients were from the northern region, 2 were from the middle region, and 7 were from the southern region of Nara.

Amantadine inhibits influenza A virus replication by interfering with M2 protein ion channel activity (6). While the drug is chemically stable and available inexpensively, the emergence and spread of resistant strains are a matter of concern (7). The viruses become resistant to amantadine through a single amino acid substitution at positions 26, 27, 30, 31 or 34 in the M2 protein. Generally, point mutations at position 31 (Ser31Asn) are most frequent in amantadine-resistant AH3 viruses (7,8). In 2005, surprisingly, major increases in the prevalence of resistant AH3 viruses with a specific mutation (Ser31Asn) were observed in China (57.5 and 73.8%) and Hong Kong (18.3 and 69.6%), during two epidemic seasons from 2003-04 to 2004-05, respectively (3). In China, amantadine or rimantadine are contained in various cold remedies and they are now broadly available in over-the-counter formulations without a prescription as generic drugs. In the United States, during the 2005-06 season, a total of 209 AH3 viruses isolated from patients in 26 states were screened, of which 193 (92.3%) contained a change at amino acid 31 (Ser31Asn), and 2 of 8 influenza AH1 viruses contained the same mutation. Finally, it was suggested that these drugs should not be used for the treat-

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Table 1. Frequency of amantadine-resistant influenza AH3 viruses in 2005 - 2006 season, Nara Prefecture, Japan

Month	No. of specimens isolated	No. of strains with amino acid substitution (%)	No. of strains with the amino acid substitutions in the M2 gene ¹⁾ at position (%)			
			26	27 ²⁾	30	31 ³⁾
December	5	5 (100)				5
January	7	3 (42.9)		1 ⁴⁾		3
February	6	5 (83.3)				5
Total	18	13 (72.2)		1 (5.6)		13 (72.2)

¹⁾: Substitution position of the amino acid in the M2 protein verified by sequencing.

²⁾: GTT→GCT (Val→Ala).

³⁾: AGT→AAT (Ser→Asn).

⁴⁾: A virus strain occurred the dual mutation at position 27 and 31.

ment or prophylaxis of influenza in the United States until susceptibility to this drug has been reestablished among circulating influenza A isolates (4). Furthermore, Barr et al. (9) reported that adamantane-resistant influenza AH3 viruses increased in Australia and neighboring countries in 2005. In Japan, Saito et al. (10) reported that an off-season community influenza outbreak with drug-resistant AH3 viruses occurred from September to October in 2005 in Tokitsu-cho, Nagasaki Prefecture. Therefore, we investigated the frequency of amantadine-resistant influenza AH3 viruses in sentinel surveillance sites during the 2005 - 06 epidemic season in Nara Prefecture. As a result, we observed a high frequency (72.2%) of amantadine-resistant AH3 viruses with point mutation at position 31 (Ser31Asn). In addition, our investigation revealed two interesting findings. First, a total of 13 drug-resistant viruses were observed over the epidemic season (from December 1, 2005 to March 31, 2006). Secondly, amantadine-resistant strains were isolated in all areas (northern, middle and southern) of Nara Prefecture. Although our sample size was small, our results suggested that amantadine-resistant viruses did not arise locally or sporadically but circulated to the general population in all parts of Nara Prefecture.

In a recent study, highly pathogenic avian influenza A viruses of the H5N1 subtype were found to be circulating in Eastern Asia with unprecedented epizootic and epidemic effects. Cheung et al. (11) reported the distribution of genetic mutations associated with resistance to the M2 ion channel-blocking adamantane derivatives, among H5N1 viruses isolated in Vietnam, Thailand, Cambodia, Indonesia, Hong Kong and China. More than 95% of the viruses isolated in Vietnam (162/175) and Thailand (58/58) contained resistance-causing mutations. Interestingly, the dual mutation motif at position 26 (Leu26Ile) and 31 (Ser31Asn) occurred almost exclusively in resistant viruses.

In conclusion, our data suggest the necessity of investigating the resistant influenza viruses throughout Japan without delay, and draw attention to the importance of tracking the

emergence and worldwide spread of drug-resistant viruses.

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