# Distribution of Amantadine-Resistant H5N1 Avian Influenza Variants in Asia

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**We examined the distribution of genetic mutations associated with resistance to the M2 ion channel–blocking adamantane derivatives, amantadine and rimantadine, among H5N1 viruses isolated in Vietnam, Thailand, Cambodia, Indonesia, Hong Kong, and China. More than 95% of the viruses isolated in Vietnam and Thailand contained resistance mutations, but resistant mutants were less commonly isolated in Indonesia (6.3% of isolates) and China (8.9% of isolates), where human infection was recently reported. The dual mutation motif Leu26Ile–Ser31Asn (leucine→isoleucine at aa 26 and serine→asparagine at aa 31) was found almost exclusively in all resistant isolates from Vietnam, Thailand, and Cambodia, suggesting the biological selection of these mutations.**

Avian influenza H5N1 viruses have caused repeated disease outbreaks in poultry in Asia since 2003. Outbreaks have occurred most recently in central Asia and parts of Europe [1]. Long-term endemicity of H5N1 viruses in Asia has led to the evolution of multiple genetic and antigenic sublineages, and regular control measures may be ineffective in the eradication of H5N1 viruses from poultry [2, 3]. Human infection has

been reported in Vietnam, Thailand, and Cambodia, and more recently in Indonesia, China, Turkey, and Iraq [4, 5]. The H5N1 virus, or a derivative of it, could become the next pandemic influenza virus [6].

The World Health Organization has recommended preparedness for a potential H5N1 influenza pandemic caused by currently circulating H5N1 viruses. There is concern that an effective vaccine might not be available in quantities sufficient to counter this virus if it mutates to cause human-to-human transmission. In the absence of an effective, widely available vaccine, protection in the face of a pandemic would rely largely on the prophylactic and therapeutic properties of antiviral treatment. This reliance on treatment places emphasis on the stockpiling of antiviral drugs, particularly of those for which resistance is not regularly detected [7].

Currently available anti-influenza drugs include the adamantanes (amantadine and rimantadine, which block the ion channel formed by the M2 protein and inhibit early stages of virus replication) and the neuraminidase inhibitors oseltamivir and zanamivir. Some clinical isolates of human influenza viruses and the majority of H5N1 viruses isolated from humans and poultry in Vietnam and Thailand show resistance to the adamantanes [3, 8–10]. Given the uncertainty about its therapeutic value, amantadine is not stockpiled as frequently as other antivirals are, in preparation for a pandemic. However, it is not known whether resistance to amantadine is prevalent in H5N1 viruses outside of Vietnam and Thailand. The increasing genetic diversity of H5N1 viruses in affected areas prompted us to analyze the geographic distribution of amantadine-resistance mutations.

*Materials and methods.* The genetic sequences of the M2 ion channel protein of the matrix gene of 638 H5N1 viruses of human and avian origin (191 virus sequences were obtained from the Influenza Sequence Database [11], and 447 were generated in our laboratory) were analyzed as described elsewhere [10]. The M2 sequences of an additional 220 H9N2 viruses from China (158) and Hong Kong SAR (62) were randomly selected from our database (186) and the Influenza Sequence Database (34) and were similarly analyzed. Substitution of residues Leu26, Val27, Ala30, and Ser31 of the M2 ion channel protein were used to screen for predicted amantadine-resistant mutants [12]. Amantadine resistance was assessed in an MDCK cell-based virus reduction assay. Briefly, triplicate MDCK monolayer cultures in 96-well format were infected with 100 $TCID_{50}$ of virus, then cultured in the presence of amantadine hydrochloride (Sigma) at concentrations of 10 nmol/L to 500 $\mu$mol/L

**Table 1. Distribution of amantadine-resistant mutants among H5N1 viruses isolated in affected Asian countries, 1996–2005.**

| Source | Leu26Ile | Val27Gly/Val27Ala/Val27Ile | Ala30Ser | Ser31Asn | Leu26Ile and Ser31Asn |
|---|---|---|---|---|---|
| Hong Kong SAR ($n = 231$) | 1 | 16[a] | 9 | 33 | 1 |
| China ($n = 123$) | 0 | 5[b] | 0 | 11 | 0 |
| Vietnam ($n = 175$) | 160 | 1 | 1 | 160 | 160 |
| Thailand ($n = 58$) | 58 | 0 | 0 | 58 | 58 |
| Indonesia ($n = 32$) | 0 | 0 | 0 | 2 | 0 |
| Cambodia ($n = 9$) | 9 | 0 | 0 | 9 | 9 |
| Malaysia ($n = 2$) | 2 | 1 | 0 | 2 | 2 |
| Japan and Korea ($n = 8$) | 0 | 0 | 0 | 0 | 0 |

**NOTE.** Data are no. of isolates with given mutation(s). A total of 638 virus isolates were analyzed; of these, 39 were from human, and 599 were from birds.

[a] Of these 16, 2 had Val27Gly, and 14 had Val27Ala.

[b] Of these 5, 4 had Val27Ile, 1 had Val27Ala.

for 48 h; hemagglutination titers of individual well supernatants were then tested in duplicate.

*Results.* As reported previously [3, 10], viruses containing the M2 Ser31Asn mutation predominated among H5N1 isolates from humans and poultry in Vietnam, Thailand, and Cambodia. Of the 175 available isolates from Vietnam, 160 contained the Ser31Asn mutation, whereas Val27Ala and Ala30Ser mutations were rarely observed (1 of each was found) (table 1). All isolates from Thailand (58/58) and Cambodia (9/9) contained the Ser31Asn mutation. A Leu26Ile mutation was observed in all isolates from these 3 countries that contained the Ser31Asn mutation. To our knowledge, this is the first report of a Leu26Ile mutation in the M2 gene of an H5N1 virus. The strong association between this mutation and Ser31Asn is also novel; the first time it was detected was in Vietnamese isolates from 2003. Interestingly, all Vietnamese H5N1 viruses isolated in 2004 contained the dual Leu26Ile–Ser31Asn amantadine-resistance mu-

tations, and the amantadine-sensitive viruses isolated in 2005 were genetically associated with a sublineage believed to have been introduced during 2005 (figure 1) [2].

The M2 sequences of H5N1 viruses from Japan, Korea, China, Hong Kong, and Indonesia were also examined. No known resistance-linked mutation was identified in isolates from poultry outbreaks in Japan and Korea in late 2003. Of the 231 viruses isolated in Hong Kong between 1997 and 2005 (221 from poultry and 10 from infected humans), 33 (including 2 human isolates) carried the Ser31Asn mutation, but only 1, A/Chicken/Hong Kong/YU250/03, carried the dual Ser31Asn–Leu26Ile mutations (table 1). Preliminary in vitro tests indicate that this virus is resistant to amantadine; viral titers in culture supernatants were not reduced by the highest drug concentration tested (500 $\mu$mol/L), whereas replication of viruses lacking resistance mutations was inhibited by the presence of 1–10 $\mu$mol/L amantadine. The first time amantadine-resistance mutations were observed in
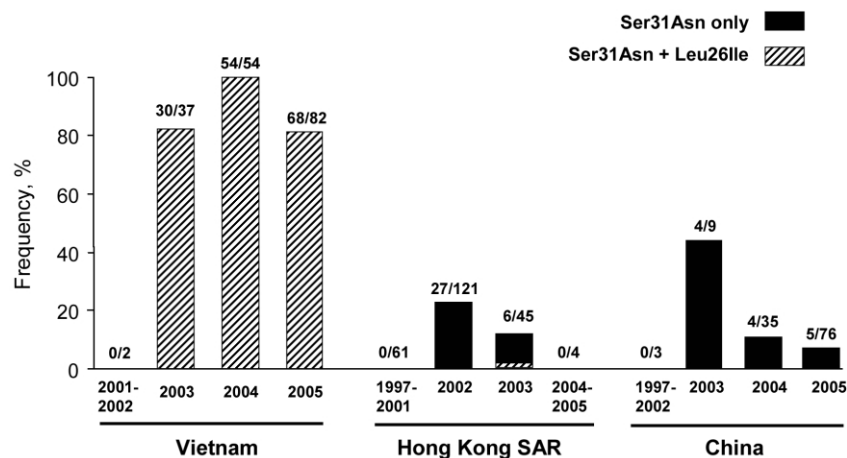


**Figure 1.** Prevalence of amantadine-resistant mutants among H5N1 viruses isolated in Vietnam, Hong Kong, and China during different years. All Thai viruses studied were isolated during a single year (2004), which precluded their inclusion in this analysis.

Hong Kong avian H5N1 viruses was in 2002; none of 61 isolates collected between 1997 and 2001 contained any of the mutations for which we screened (figure 1 and data not shown).

The frequency of amantadine-resistant human H3N2 influenza viruses isolated in Asia was recently reported to have increased sharply in 2003, particularly in China [13]. To determine whether a similar phenomenon had occurred in H5N1 viruses, we screened isolates from China. Resistance-related mutations were less common in isolates from China than in those from Thailand, Vietnam, and Cambodia; 4.1% (5/123) of the Chinese isolates carried Val27Ala, and 8.9% (11/123) carried Ser31Asn. None contained the dual Leu26Ile–Ser31Asn mutations. As a comparison, the M2 sequences of 220 H9N2 viruses isolated in China and Hong Kong between 1976 and 2004 were examined for amantadine-resistance mutations; 10 carried the Ser31Asn mutation, and none carried the Leu26Ile mutation (data not shown). H5N1 infection in humans has now been reported in Indonesia [4], but only 2 of the 32 isolates screened had an amantadine-resistance mutation (a single Ser31Asn substitution). The novel and distinct Leu26Ile–Ser31Asn mutation pattern therefore appears to be geographically restricted to the 3 contiguous countries Vietnam, Thailand, and Cambodia, with the exception of a single isolate identified in Hong Kong in 2003.

***Discussion.*** Mutated viruses reportedly may either lose the ability to bind M2 ion channel blockers, as with the Ser31Asn substitution, or bind the drug but retain M2 function, as with Val27Ala [14]. Because of the unavailability of viruses with only the Leu26Ile mutation, the ability of the Leu26Ile mutation to confer amantadine resistance has not yet been verified, and its contribution to amantadine resistance or to other phenotypic characteristics of isolates from Vietnam, Thailand, and Cambodia remains to be elucidated. However, the presence of the confirmed resistance mutation Ser31Asn suggests that isolates with both the Leu26Ile and Ser31Asn mutations are likely to be amantadine resistant; preliminary in vitro tests on a Hong Kong isolate—A/Chicken/Hong Kong/YU250/03, which contains dual mutations—indicate that this is indeed the case.

Although amantadine-resistant H5N1 viruses are present in Asia, their distribution appears to be largely limited to Thailand, Vietnam, and Cambodia. That most H5N1 isolates from China and Indonesia are sensitive to amantadine is striking and perhaps surprising, in view of the reportedly widespread administration of amantadine to farmed poultry in some countries. The apparent geographical disparity in the susceptibility of H5N1 isolates to amantadine is unexplained, although the conserved pattern of mutations suggests that the Thai, Vietnamese, and Cambodian viruses originated from a single virus introduced into, or generated within, the region. Given that the Ser31Asn–Leu26Ile motif was first identified in Vietnam in 2003, it appears that Vietnam might have been the location of introduction or generation. Phylogenetic evidence suggests that

H5N1 viruses in Vietnam, Thailand, and Malaysia likely arose as a result of the introduction of viruses from other affected areas [2, 10]. This conclusion is further supported by the distribution of the dual mutation, which is present in almost all viruses from the Vietnam/Thailand/Malaysia sublineage [2] and which has otherwise been recorded only in 1 isolate from elsewhere (A/Chicken/Hong Kong/YU250/03). Although it is not known how the dual mutants observed in the present study were generated, amantadine appears to retain the potential to be useful in an H5N1 pandemic in the absence of a vaccine, as a prophylactic agent [15] and as a component of combination antiviral therapy.

Most adamantane-resistant influenza viruses (70%–80%) bear mutations at aa 31 of the M2 protein, whereas only 1%–2% have mutations at aa 26 [9, 13]. Dual Leu26Ile and Ser31Asn mutations are extremely rare; we found this dual motif in only 1 (A/Swine/Scotland/410440/94 [16]) of the 1307 publicly available influenza A sequences (including isolates from all species) that we examined. The high incidence of Leu26Ile and its exclusive association with Ser31Asn only in H5N1 isolates from Vietnam, Thailand, and Cambodia suggest that viruses carrying the dual mutations were stably selected, because no single Leu26Ile or Ser31Asn mutants were found among resistant isolates from these countries. Resistance mutations at other amino acid positions (Val27Ala and Ala30Ser) were also rare in these 3 countries. Further, it is noteworthy that all available M2 sequences of H5N1 viruses isolated from humans in Vietnam carry the dual Leu26Ile–Ser31Asn mutations. Additional investigation is needed to elucidate the biological effect that the dual M2 mutations Leu26Ile and Ser31Asn have on the life cycle of the H5N1 virus.

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