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Emergence of Amantadine-Resistant H3N2 Avian Influenza A Virus in South Korea▽

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We found a relatively high frequency of unique amantadine-resistant H3N2 and H9N2 avian influenza viruses (Val27Ile on M2 protein) isolated from live poultry markets in South Korea and confirmed that a Val27Ile single substitution in the M2 protein is enough to acquire the amantadine resistance phenotype by using reverse-genetically created human-avian reassortant viruses.

Influenza A viruses are important pathogens for humans, birds, pigs, and other species that possess eight segments of single-stranded negative-sense RNA. Vaccination is the primary measure to control influenza virus infections in humans. However, for individuals who have not been vaccinated, or for when a vaccine is not available, antiviral agents can provide an important alternative. Two adamantane derivatives, amantadine and rimantadine, are used for prophylaxis and treatment of influenza virus infection. These drugs bind to and block the function of the influenza A virus Matrix 2 (M2) ion channel protein, preventing virus replication within the infected cell (4, 9, 18, 22). However, single amino acid substitutions in the M2 transmembrane domain leading to amantadine resistance occur at residue 26, 27, 30, 31, 34, or 38 (2, 6, 17). Furthermore, resistant mutants emerge readily in drug-treated patients, and the mutant viruses are transmissible (7, 11, 19). Between 1991 and 1995, surveillance for adamantane-resistant (H3N2) type A influenza viruses revealed the global frequency of resistance to be as low as 0.8% (23). However, a substantially rising percentage of drug-resistant H3N2 viruses were isolated during 1995 to 2005 from the United States (14.5%) and specific Asian countries, including China, (96%), Hong Kong (72%), South Korea (36%), and Japan (65.3%) (3–5, 16).

With the relatively high frequencies of amantadine-resistant influenza A viruses being reported in human isolates in Asia (3, 5, 15), we surveyed the frequency of amantadine-resistant viruses isolated in Korea from December 2004 to April 2008 by a previously described method (12) from humans (nasopharyngeal suction) and avian species (tracheal swabs and fecal specimens) of live poultry markets (LPMs), backyard poultry farms (BPFs), and wild birds in order to investigate the presence of amantadine-resistant mutants and to discover any potential relationships between human and animal drug-resistant viruses.

DNA sequences of the M2 genes were compiled and edited using the SeqMan program (DNASTAR) and aligned using Clustal_X (1, 21). A phylogram was generated by the neighbor-joining method using the tree drawing program NJplot (14).

A total of 207 influenza A viruses isolated in South Korea were analyzed, and a subset was found to contain point mutations in regions implicated as hot spots for amantadine resistance. In 17 avian H3N2 LPM isolates and 1 H9N2 BPF isolate, we observed a single amino acid substitution in the M2 protein (Val27Ile) previously correlated with amantadine resistance. The base change was more common in the H3N2 subtype (94.4%) than in the H9N2 subtype (5.6%) (Table 1). Interestingly, an H5N2 wild bird virus (Dk/Korea/W224/07) had the same mutation. Six out of 12 H3N2 human viruses isolated in 2008 had a different amino acid substitution (Ser31Asn) in the M2 protein. No other amino acid changes associated with influenza virus resistance to amantadine were detected in other various subtypes of wild bird and poultry isolates.

Phylogeny of the M2 genes of avian and human isolates from South Korea revealed that avian influenza viruses were clearly

TABLE 1. Summary of amantadine-resistant influenza A viruses characterized in this study[a]

Source	Host/subtype	No. of samples tested	No. of samples with M2 substitution (position)
Wild bird	Dk/H1-H12	83	0
	Dk/H5N2	16	1 (Val27Ile)
LPM	DkH3N2	13	9 (Val27Ile)
	Dk/H3N6	2	0
	Dk/H9N2	2	0
	Ck/H3N2	8	8 (Val27Ile)
	Ck/H9N2	11	0
BPF	Ck/H9N2	37	0
	Dk/H9N2	11	1 (Val27Ile)
	Dk/H3N2	12	0
Human	Hu/H3N2	12	6 (Ser31Asn)
Total		207	25

[a] LPM, live poultry market; BPF, backyard poultry farm; Dk, duck; Ck, chicken; Hu, human.

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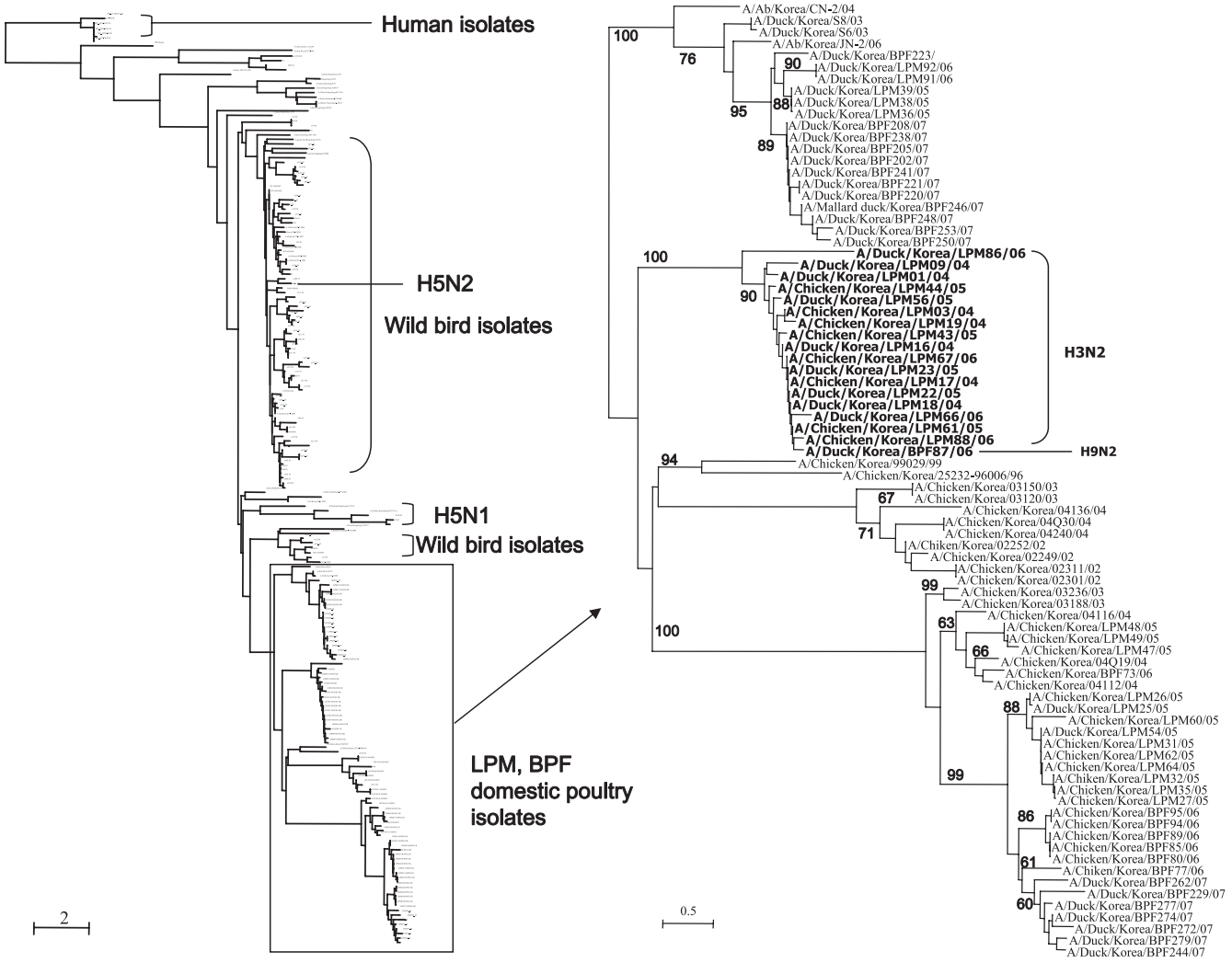

FIG. 1. Phylogenetic analysis of the Matrix 2 (M2) genes of influenza virus isolates from South Korea. Phylogenetic neighbor-joining trees comparing the nucleotide sequences of the M genes of the 207 avian and human isolates in South Korea in this study with nucleotide sequences from other selected human and avian influenza virus strains. The scale represents the number of substitutions per nucleotide. Viruses in boldface type indicate Korean LPM and BPF isolates bearing the amantadine resistance phenotype. Branch labels record the stability of the branches over 1,000 bootstrap replicates. Only bootstrap values of ≥60% are shown in the tree due to limited space and the reliability of groupings above 60%. Standard nomenclature is used for human isolates. Dk, duck; Ck, chicken.

distinct from human isolates, separating into at least seven different sublineages (Fig. 1). It is noteworthy that the M2 genes of all amantadine-resistant avian H3N2 and H9N2 viruses were clustered together except for that of one H5N2 virus that also has the M2 mutation conferring drug resistance. Nevertheless, this result suggests that all amantadine-resistant H3N2 avian influenza viruses analyzed have the same M gene lineage.

To evaluate whether the observed valine-to-isoleucine substitution would consequently result in an amantadine resistance phenotype, a previously reported phenotypic assay for amantadine susceptibility (13) was performed on a blind subset of 60 avian H3N2 isolates (41 sensitive and 19 resistant isolates). The results showed the presence of 19 drug-resistant isolates (with a ≥2.5-fold higher $\log_{10}$ 50% tissue culture infective dose/0.2 ml virus titer) conforming to isolates also possessing the amino acid substitution at residue 27. Therefore, amantadine resistance correlated 100% with the presence of the mutation obtained by sequencing. These results are in agreement with published reports of the Val27Ile mutation conferring amantadine resistance (10).

To determine whether human influenza virus would acquire the phenotype if the characteristic mutation is present, we generated two recombinant viruses by plasmid-based reverse genetics in 293T cells, as described previously (8). The A/PR/8/34/L03M reassortant virus bore the M gene from the A/duck/Korea/L03/05 (H3N2) virus, encoding the amantadine resistance-conferring amino acid substitution (Val27Ile), and the other genes from the A/PR/8/34 (H1N1) strain (A/PR/8/34 plasmids kindly provided by Robert G. Webster). The A/PR8/34/ML03M reassortant virus contained the mutant M gene from the A/duck/Korea/L03/05 isolate, encoding the single amino acid change (Ile27Val) by PCR mutagenesis. To test for susceptibility, the titers of the viruses were determined in the

presence and absence of drugs. As expected, there were no differences in growth between the viruses in the media without the antiviral drug; however, the A/PR/8/34/L03M virus had a 2.5-fold-higher $\log_{10}$ 50% tissue culture infective dose/0.2 ml titer than did the A/PR/8/34/ML03M virus in the media containing the 1 μg/ml of amantadine. These results clearly demonstrate that the Val27Ile single substitution in the M2 protein is enough to acquire the amantadine resistance phenotype.

Historically, most adamantane-resistant influenza virus isolates from humans (70 to 80%) contain mutations at position 31 of the M2 protein, whereas the Val27Ile mutation is extremely rare (1.6%) in frequency (3–5, 10). In this study, we found a total of 19 out of 195 (9.7%) avian influenza viruses in LPMs, BPFs, and wild birds in South Korea encoding the Val27Ile mutation in the M2 protein. In line with the fact that the Val27Ile mutation was sufficient to acquire the amantadine resistance phenotype, our data strongly support a previous report (13) that it could also be a potential marker for drug resistance and, thus, should be considered during chemoprophylaxis. It is noteworthy that the majority of the amantadine-resistant viruses in this study were of the H3N2 subtype, commonly isolated from LPMs in South Korea (20). Their genetic evolution toward amantadine resistance indicates that the presence of an undetermined selective pressure is involved; however, more detailed studies are needed to identify the possible means of selectivity in LPM settings compared to that in wild bird habitats.

The close proximity of humans and poultry products in LPMs could provide a convenient ground for interspecies virus transmission. Amantadine-resistant avian viruses that find their way into human hosts would present an additional problem when administering antiviral drugs for infection control. Overall, the results of this study highlight the need to closely monitor drug resistance in avian influenza viruses to aid in the early detection of potentially pandemic strains, and they also underscore the need for new therapeutics.

Gene sequences determined in this study have been deposited in the GenBank database (EU819089 to EU819140).

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REFERENCES

1. **Aiyar, A.** 2000. The use of CLUSTAL W and CLUSTAL X for multiple sequence alignment. Methods Mol. Biol. **132:**221–241.
2. **Belshe, R. B., M. H. Smith, C. B. Hall, R. Betts, and A. J. Hay.** 1988. Genetic basis of resistance to rimantadine emerging during treatment of influenza virus infection. J. Virol. **62:**1508–1512.
3. **Bright, R. A., M. J. Medina, X. Xu, G. Perez-Oronoz, T. R. Wallis, X. M. Davis, L. Povinelli, N. J. Cox, and A. I. Klimov.** 2005. Incidence of adamantane resistance among influenza A (H3N2) viruses isolated worldwide from 1994 to 2005: a cause for concern. Lancet **366:**1175–1181.
4. **Bright, R. A., D. K. Shay, B. Shu, N. J. Cox, and A. I. Klimov.** 2006. Adamantane resistance among influenza A viruses isolated early during the 2005–2006 influenza season in the United States. JAMA **295:**891–894.
5. **Deyde, V. M., X. Xu, R. A. Bright, M. Shaw, C. B. Smith, Y. Zhang, Y. Shu, L. V. Gubareva, N. J. Cox, and A. I. Klimov.** 2007. Surveillance of resistance to adamantanes among influenza A(H3N2) and A(H1N1) viruses isolated worldwide. J. Infect. Dis. **196:**249–257.
6. **Hay, A. J., M. C. Zambon, A. J. Wolstenholme, J. J. Skehel, and M. H. Smith.** 1986. Molecular basis of resistance of influenza A viruses to amantadine. J. Antimicrob. Chemother. **18**(Suppl. B)**:**19–29.
7. **Hayden, F. G., R. B. Belshe, R. D. Clover, A. J. Hay, M. G. Oakes, and W. Soo.** 1989. Emergence and apparent transmission of rimantadine-resistant influenza A virus in families. N. Engl. J. Med. **321:**1696–1702.
8. **Hoffmann, E., S. Krauss, D. Perez, R. Webby, and R. G. Webster.** 2002. Eight-plasmid system for rapid generation of influenza virus vaccines. Vaccine **20:**3165–3170.
9. **Iwahashi, J., K. Tsuji, T. Ishibashi, J. Kajiwara, Y. Imamura, R. Mori, K. Hara, T. Kashiwagi, Y. Ohtsu, N. Hamada, H. Maeda, M. Toyoda, and T. Toyoda.** 2001. Isolation of amantadine-resistant influenza A viruses (H3N2) from patients following administration of amantadine in Japan. J. Clin. Microbiol. **39:**1652–1653.
10. **Kitahori, Y., M. Nakano, and Y. Inoue.** 2006. Frequency of amantadine-resistant influenza A virus isolated from 2001–02 to 2004–05 in Nara Prefecture. Jpn. J. Infect. Dis. **59:**197–199.
11. **Klimov, A. I., E. Rocha, F. G. Hayden, P. A. Shult, L. F. Roumillat, and N. J. Cox.** 1995. Prolonged shedding of amantadine-resistant influenzae A viruses by immunodeficient patients: detection by polymerase chain reaction-restriction analysis. J. Infect. Dis. **172:**1352–1355.
12. **Lee, Y. J., J. Y. Shin, M. S. Song, Y. M. Lee, J. G. Choi, E. K. Lee, O. M. Jeong, H. W. Sung, J. H. Kim, Y. K. Kwon, J. H. Kwon, C. J. Kim, R. J. Webby, R. G. Webster, and Y. K. Choi.** 2007. Continuing evolution of H9 influenza viruses in Korean poultry. Virology **359:**313–323.
13. **Masuda, H., H. Suzuki, H. Oshitani, R. Saito, S. Kawasaki, M. Nishikawa, and H. Satoh.** 2000. Incidence of amantadine-resistant influenza A viruses in sentinel surveillance sites and nursing homes in Niigata, Japan. Microbiol. Immunol. **44:**833–839.
14. **Perriere, G., and M. Gouy.** 1996. WWW-query: an on-line retrieval system for biological sequence banks. Biochimie **78:**364–369.
15. **Saito, R., D. Li, C. Shimomura, H. Masaki, M. Q. Le, H. L. Nguyen, H. T. Nguyen, T. V. Phan, T. T. Nguyen, M. Sato, Y. Suzuki, and H. Suzuki.** 2006. An off-seasonal amantadine-resistant H3N2 influenza outbreak in Japan. Tohoku J. Exp. Med. **210:**21–27.
16. **Saito, R., D. Li, Y. Suzuki, I. Sato, H. Masaki, H. Nishimura, T. Kawashima, Y. Shirahige, C. Shimomura, N. Asoh, S. Degawa, H. Ishikawa, M. Sato, Y. Shobugawa, and H. Suzuki.** 2007. High prevalence of amantadine-resistance influenza a (H3N2) in six prefectures, Japan, in the 2005–2006 season. J. Med. Virol. **79:**1569–1576.
17. **Schnell, J. R., and J. J. Chou.** 2008. Structure and mechanism of the M2 proton channel of influenza A virus. Nature **451:**591–595.
18. **Shih, S. R., C. N. Lee, H. R. Tsai, G. W. Chen, and K. C. Tsao.** 2001. Amantadine-resistant influenza A virus in Taiwan. J. Formos. Med. Assoc. **100:**608–612.
19. **Shiraishi, K., K. Mitamura, Y. Sakai-Tagawa, H. Goto, N. Sugaya, and Y. Kawaoka.** 2003. High frequency of resistant viruses harboring different mutations in amantadine-treated children with influenza. J. Infect. Dis. **188:**57–61.
20. **Song, M. S., T. K. Oh, H. J. Moon, D. W. Yoo, E. H. Lee, J. S. Lee, C. J. Kim, G. J. Yoo, H. Kim, and Y. K. Choi.** 2008. Ecology of H3 avian influenza viruses in Korea and assessment of their pathogenic potentials. J. Gen. Virol. **89:**949–957.
21. **Thompson, J. D., T. J. Gibson, F. Plewniak, F. Jeanmougin, and D. G. Higgins.** 1997. The CLUSTAL_X windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. Nucleic Acids Res. **25:**4876–4882.
22. **Wang, C., K. Takeuchi, L. H. Pinto, and R. A. Lamb.** 1993. Ion channel activity of influenza A virus M2 protein: characterization of the amantadine block. J. Virol. **67:**5585–5594.
23. **Ziegler, T., M. L. Hemphill, M. L. Ziegler, G. Perez-Oronoz, A. I. Klimov, A. W. Hampson, H. L. Regnery, and N. J. Cox.** 1999. Low incidence of rimantadine resistance in field isolates of influenza A viruses. J. Infect. Dis. **180:**935–939.