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Adamantane resistance in circulating human influenza A viruses from Alberta, Canada (1970–2007)

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Abstract

Mutation in one of five key amino acid residues (positions 26, 27, 30, 31 and 34) within the M2 protein of influenza A viruses, leads to resistance against the adamantane class of anti-influenza drugs. To investigate the emergence and prevalence of adamantane resistance in Alberta, Canada (between 1970 and 2007), 381 influenza A positive samples (original patient specimens) or isolates (virus cultured from patient specimens) were analyzed for changes in these critical amino acid residues. Our results show a significant increase in adamantane resistance in circulating H3N2 viruses in Alberta from 2005 and 2006 when compared with those from 2004 ($p < 0.001$). Adamantane resistance peaked at 74% in 2006 and then decreased (to 38%) in 2007 ($p = 0.001$). All resistant H3N2 viruses contained the substitution Ser to Asn at amino acid position 31 of the M2 protein with two viruses having an additional Ala to Val substitution at position 30. Resistance was not observed in the H1N1 viruses tested. Results presented here are concordant with, and extend, previous reports of increased resistance to adamantanes in Asia and North America in recent years. It is important to continue studies to evaluate circulating influenza A viruses for antiviral resistance markers to ensure their optimal use for prophylaxis and treatment of influenza.

Keywords: Influenza; Adamantane; Amantadine; Resistance; M2 protein; Antiviral

1. Introduction

The economic significance of influenza infections, combined with the continuing threat of an influenza pandemic, has made it important to reassess the utility of available antivirals for prophylaxis and treatment. There are two main classes of anti-influenza drugs: the adamantanes and the neuraminidase inhibitors. The adamantanes (amantadine and rimantadine) target the transmembrane domain of the M2 protein of the influenza A virus. This domain acts as a proton channel allowing the flow of ions to the inner part of the virion resulting in a decrease of pH and dissociation of the M protein from the ribonucleoprotein complex. This complex is released into the cytoplasm of the infected cell to initiate viral replication (Holsinger et al., 1994; Pinto et al., 1992). Adamantanes inhibit this process by blocking proton flow through the M2 channel (Wang et al., 1993).

Mutations at amino acid positions 26, 27, 30, 31, and 34 in the M2 protein can render influenza A viruses resistant to the action of adamantanes (Belshe et al., 1988; Hay et al., 1986). Reports of increased resistance to this class of antivirals by the Center for Disease Control [CDC, Atlanta, GA (Bright et al., 2005, 2006)] and the National Microbiology Laboratory [NML, Public Health Agency of Canada (PHAC), Winnipeg, Canada (Dr. Yan Li, personal communication)] in early 2006 prompted the Provincial Laboratory for Public Health (ProvLab, Alberta, Canada) to initiate a study to investigate the emergence and prevalence of resistance to adamantanes in Alberta, Canada.

The study described here utilized influenza A virus positive samples (original respiratory specimens) and virus isolates (obtained after culture of respiratory specimens) from Alberta, Canada, stored over a 37 year period. The data provides comprehensive information concerning levels of adamantane resistance for this geographic area and adds to the global information available on the emergence and distribution of resistant viruses. The methods described in this study will be utilized for prospective surveillance of adamantane resistance in circulating influenza A

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viruses in Alberta. This will provide information relevant to the management of influenza infections and outbreaks.

2. Methods

2.1. Study samples

A total of 381 influenza A positive samples (original specimens) or virus isolates (obtained from respiratory samples after culture), which were submitted for diagnostic investigation during the years 1970–2007 in Alberta, Canada were screened for genetic markers of adamantane resistance. Influenza A virus isolates were cultured by inoculation of respiratory samples into Rhesus monkey kidney cells (RhMK, Diagnostic Hybrids, Athens, Ohio). For 55 samples (from 2005 to 2007), total nucleic acid extracts were obtained from the original sample, without prior culture. Both upper and lower respiratory samples were utilized in this study including auger suctions, bronchoalveolar lavages, nasopharyngeal fluids/ swabs and throat swabs. A total of 317 H3 subtype and 64 H1 subtype influenza A viruses were screened for mutations in the M2 protein. The number of samples tested per year varied according to availability and are listed in Table 1.

2.2. Sub-typing of influenza A positive samples and isolates

The 381 influenza A positive samples or isolates used for analysis of adamantane resistance markers were tested for their hemagglutinin (HA) subtype. Serotyping of 91 influenza A isolates was performed at the NML by hemagglutination inhibition assays using standard procedures. As a part of this study, 285 isolates/positive samples were HA typed using a real-time reverse transcriptase-polymerase chain reaction (RT-PCR) assay (procedure from Dr. Yan Li, NML) and five isolates were HA typed using a real-time RT-PCR protocol previously reported (Schweiger et al., 2000).

Table 1
Samples and virus isolates tested along with frequency of adamantane resistance by year of isolation

Year	Number of samples/isolates tested		
	Influenza A H1	Influenza A H3	Adamantane resistant (% positive)[a]
1970	0	1	0/1 (0%)
1972	0	1	0/1 (0%)
1975	0	3	0/3 (0%)
1977	0	1	0/1 (0%)
1978	0	2	0/2 (0%)
1979	2	0	0/2 (0%)
1995	7	11	0/18 (0%)
1996	2	19	0/21 (0%)
1997	0	21	1/21 (5%)
1998	0	14	0/14 (0%)
1999	0	19	0/19 (0%)
2000	3	11	1/14 (7%)
2001	18	2	0/20 (0%)
2002	10	24	0/34 (0%)
2003	8	54	0/62 (0%)
2004	0	25	1/25 (4%)
2005	0	27	8/27 (30%)
2006	12	60	53/72 (74%)
2007	2	22	9/24 (38%)

[a] All influenza A viruses with markers of adamantane resistance were of the H3 subtype.

2.3. Nucleic acid extraction

Nucleic acid extraction from influenza A isolates grown in RhMK cells or from respiratory samples was undertaken using the easy MAG extractor (BioMérieux, Inc., Durham, NC). Extraction from 200 μL of culture material/original sample was performed according to the manufacturer's instructions with an elution volume of 110 μL providing total nucleic acid.

2.4. One-step RT-PCR for amplification of M2 gene

One-step RT-PCR reactions were performed using the One-step RT-PCR Kit (Qiagen, Mississauga, Ontario) in a total volume of 50 μL. This included 10 μL 5× RT-PCR buffer, 10 μL Q solution, 2 μL of 10 mM dNTPs, 0.125 μL of 40 U/μL RNaseOUT (Invitrogen, CA), 1 μL One-step RT-PCR Enzyme Mix, 1.5 μL each of 20 μM primer and 18.9 μL of RNase free water. Primers FluA-AmanRes-For (5′ CAAATGGCTGGATCGAGTGAG 3′) and FluA-AmanRes-Rev (5′ GTTCCTTTCGATATTCTTCCCTC 3′) were designed to amplify 355 bp of the M2 gene including the region of interest for adamantane resistance. The primers were used at a final concentration of 600 nM with 5 μL of template RNA. The thermal cycling conditions consisted of a 30 min RT step at 50 °C, 15 min initial PCR activation step at 95 °C, 45 cycles of 94 °C, 50 °C and 72 °C at 45 s each, followed by a 10 min extension at 72 °C. All thermal cycling was performed using a 2700 Thermal Cycler (Applied Biosystems, Foster City, CA). The PCR products were purified using QIAquick columns (Qiagen) and eluted in 50 μL of molecular grade water.

2.5. Cycle sequencing and analysis

Amplified products were sequenced in both directions using the ABI PRISM BIGDYE Terminator v3.1 Cycle Sequencing kit in the ABI PRISM 3100-Avant Genetic Analyzer (Applied Biosystems) on a 50 cm array. Data collection was undertaken using the Applied Biosystems Software v2.0. The sequencing reaction was performed according to the manufacturer's protocol in the final volume of 20 μL with approximately 50 ng of amplified product, BIGDYE Terminator v3.1 premix and 5 pmol of the primer used for RT-PCR. Unincorporated nucleotides were purified by DyeEx exclusion columns (Qiagen). The sequences were analyzed using Seqscape v2.1.1 and Sequencing Analysis Software v5.1.1 (Applied Biosystems); alignments were performed using ClustalW v1.4 included in BioEdit v7.0.0 (http://www.mbio.ncsu.edu/BioEdit/bioedit.html).

2.6. Pyrosequencing

The template for the pyrosequencing reaction was generated by two-step RT-PCR using the forward biotin labeled primer flu-

ApyroFor (5′ GGGACTCATCCTAGCTCCAGTGC 3′) and the reverse non-biotinylated primer fluApyroRev (5′ CGATCAAGAATCCACAATATCAAGTGC 3′) to amplify a 164 bp region of the M2 gene. The reverse transcription step was performed using SUPERSCRIPT II and RNaseOUT (Invitrogen). The master mix comprised of DTT at a final concentration of 5 μM, total dNTPs at 375 nM, 600 ng of random hexamer, 20 U of RNaseOUT and 100 U of SUPERSCRIPT II in a final volume of 20 μL. PCR was carried out in a final volume of 50 μL containing 5 μL cDNA from the reverse transcription step described above or PCR product obtained from amplification with FluA-AmanRes-For and FluA-AmanRes-Rev as template (from Section 2.4), 5 μL of 10× PCR buffer, 1.5 mM $MgCl_2$, 0.1 mM of each dNTP, 0.6 μM of each primer, and 1.25 U Taq DNA polymerase (Invitrogen). After denaturation at 95 °C for 3 min, PCR amplification was performed with 35 cycles of 95 °C, 50 °C and 72 °C for 30 s each followed by a final extension at 72 °C for 7 min resulting in a biotinylated PCR product.

Pyrosequencing was performed on the PSQ™96 System (Biotage AB, Uppsala, Sweden) using the Pyro Gold kit (Biotage) according to the manufacturer's instructions. The sequencing program comprised 10 cycles with the nucleotide dispensation order ACGT to obtain the sequence for approximately 24 bases spanning residues 24–31 of the M2 protein. Briefly, 15–40 μL of biotinylated PCR product was bound to 3 μL of streptavidin-coated sepharose beads (Amersham, Little Chalfont, UK). The bound PCR product was washed with 70% (v/v) ethanol, denatured with sodium hydroxide and washed according to Manufacturer's instructions using the Vacuum Prep Tool (Biotage). The denatured product was transferred to a 96-well plate containing 2 μL of 10 μM sequencing primer fluApyroSeq (5′ GTGCAAGATYCCRATGAT 3′) for the H3 isolates and fluApyroSeqH1 (5′ GTGCACAATCCCAATTAT 3′) for the H1 isolates, mixed with 40 μL annealing buffer and incubated at 80 °C for 2 min. The reaction cartridge was then loaded with Pyro Gold reagents (dNTPs, enzyme and substrate) and placed in the instrument. Pyrosequencing data was evaluated using Peak Height Determination Software v1.1 (Biotage).

2.7. Statistical analysis

SPSS software v14.0 was used for statistical analysis of data. Significance values were calculated using Pearson Chi-squared (χ^2) analysis, p value of less than 0.05 was used to assess statistical significance.

3. Results

3.1. Prevalence of adamantane resistance in influenza A positive samples

A total of 73 (23%) of the 317 influenza A H3 subtype viruses tested contained one or two amino acid substitutions in the M2 protein known to cause resistance to adamantanes. No resistance was observed in the influenza A H1 subtype viruses tested. The prevalence of adamantane resistance over the time period of the study is shown in Table 1. No resistant viruses were detected in samples collected between 1970 and 1997. One influenza A H3 virus, with mutations conferring resistance to adamantanes, was seen in each of the following years: 1997, 2000 and 2004. The level of resistance increased significantly in 2005 and 2006 with 30% and 74% of influenza A viruses respectively containing genetic markers of resistance in critical amino acid residues of the M2 protein, compared with only 4% in 2004 (χ^2, $p < 0.001$). A decrease in influenza A virus resistance to adamantanes (to 38%) was observed, when results for samples from 2007 were compared with those from 2006 (χ^2, $p = 0.001$).

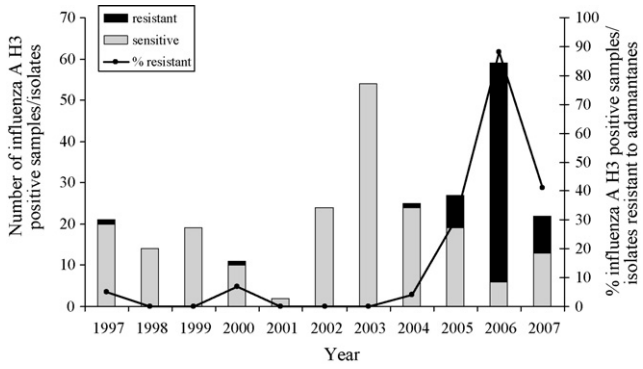

Fig. 1. Prevalence of adamantane resistance in influenza A H3 positive samples/isolates tested from 1997 to 2007. The total number of adamantane resistant and sensitive samples are shown for each year; the percentage of resistant samples is also given.

Analysis of the results for influenza A H3 viruses, by year, is given in Fig. 1 and the monthly distribution of influenza A H3 viruses tested and levels of resistance observed from 2004 to 2007 are given in Fig. 2. Resistance was first detected consistently in H3 viruses from early 2005 with a sharp increase in November 2005. As shown in Fig. 2, the level of resistance between December 2005 and March 2006 was 93%; this was followed by a decline in resistance for isolates tested after September 2006. The highest level of resistance was seen in February and March 2006 at 100%.

A total of 60 specimens were tested between December 2005 and March 2006; 17 of these specimens were from outbreaks at long term and assisted care centers, three specimens were from

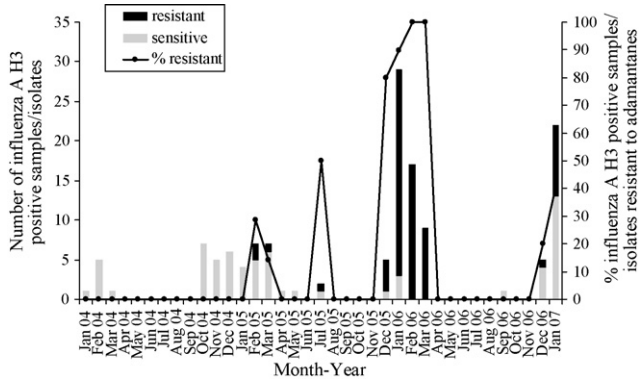

Fig. 2. Monthly distribution of adamantane resistance in influenza A H3 positive samples/isolates tested from January 2004 to January 2007. The total number of sensitive and resistant samples detected during the different months and the percentage of resistant samples is shown.

Table 2
Changes in critical amino acid residues conferring resistance to adamantanes

M2 mutations associated with resistance to adamantanes	Number of samples with change in amino acid (% positive) of total resistant
26 Leu to Phe	0/73 (0%)
27[a] Val to Ala	0/73 (0%)
30 Ala to Val	2/73 (3%)[b]
31 Ser to Asn	73/73 (100%) (two had mixed sensitive/resistant sequences)[b]
34 Gly to Glu	0/73 (0%)

[a] Thirteen influenza A H3N2 viruses had a Val to Ile change at amino acid position 27. Viruses with only this change have been designated as sensitive to adamantanes in our analysis. Further work will be undertaken to investigate whether this mutation confers adamantane resistance.

[b] The two viruses with Ala to Val change at position 30 had mixed sensitive/resistant sequences at position 31.

outbreaks at schools and 40 specimens were not related to outbreaks. The level of resistance in the outbreak and non-outbreak specimens was 100% and 90%, respectively. Computation of the p value showed no significant difference (χ^2, $p = 0.193$) in the level of resistance when comparing cases that were not exposed to amantadine (non-outbreak related) with cases from long term and assisted care centers (outbreak related) that may have been exposed to amantadine.

3.2. Amino acid changes observed leading to resistance

Table 2 shows the critical residues, which are known to confer resistance to adamantanes and the changes detected at these positions in the samples/isolates tested. All resistant viruses identified in this study (73 resistant of 381 tested) contained the Ser to Asn substitution at amino acid 31. Two of these viruses had mixed sensitive/resistant sequences at position 31; in addition, they also showed a change from Ala to Val at amino acid 30. The latter change has been reported to render the virus resistant to the action of adamantanes independent of the Ser 31 Asn change. No changes from the sensitive (wild type) sequence were detected for amino acid positions 26 and 34. Thirteen influenza A H3 viruses had a Val to Ile change at amino acid position 27. This particular amino acid variation has not been reported independently in the literature and we intend to investigate if it leads to adamantane resistance without other amino acid changes. This previously unreported change has been classified as sensitive in our analysis.

3.3. Assessment of sensitivity of RT-PCR and sequencing on clinical samples

We undertook RT-PCR amplification and sequencing on 55 nucleic acid extracts from respiratory samples without prior culture. Good quality sequence data could be obtained from the specimens without culture and interpretation of adamantane resistance based on these sequences was possible. In analyzing the 55 sequences obtained directly from specimens positive for the presence of influenza A virus RNA, 25 were classified as adamantane-sensitive and 30 were classified as resistant. Of the 55 samples tested, 17 were from 2005, 18 from 2006 and 20 from 2007. A higher percentage of resistance was seen in samples from 2006 (16 resistant of 18 tested), compared with those from 2005 (5 resistant of 17 tested) and 2007 (9 resistant of 20 tested).

3.4. Correlation between dideoxysequencing and pyrosequencing

A subset of 224 virus isolates tested by conventional (dideoxy) sequencing were analyzed by pyrosequencing the region of interest. Five of these samples were classified as resistant by conventional sequencing, two exhibited a mixed sequence with resistant and sensitive residues (codons AGT and AAT) present at amino acid position 31 in the electropherogram and 217 samples were classified as sensitive. The resistant and sensitive isolates were successfully identified by pyrosequencing. However, the mixed sequences were typed as sensitive by pyrosequencing (only AAT was detected). It is thus feasible to use pyrosequencing as a high throughput method for the detection of adamantane resistance but further methodological optimization may be necessary for identification of mixed resistant/sensitive sequences.

4. Discussion

Here we report the results of a comprehensive study monitoring adamantane resistance in circulating influenza A viruses in Alberta, Canada. Our results show a significant increase in adamantane resistance levels in circulating influenza A H3 viruses from December 2005 to March 2006. Samples obtained from September 2006 to January 2007 showed a decline in the level of resistance for H3 subtype influenza A viruses; however, the level of resistance still remains high as compared to 2004. Our data is more extensive but concordant with those documented for other provinces in Canada (http://www.phac-aspc.gc.ca/fluwatch/archive_e.html) over this time period.

The high levels of resistance reported here are comparable with other similar studies monitoring resistance world-wide (Bright et al., 2005, 2006; Barr et al., 2007; Deyde et al., 2007; Saito et al., 2007a,b). According to the CDC, 91% of the H3 subtype influenza A positive samples analyzed during the 2005–2006 season were resistant to amantadine with a Ser 31 Asn mutation (CDC, 2006). Similar high levels of adamantane resistance have been reported by the NML for H3 subtype influenza A viruses tested between September 2005 and January 2006 (PHAC, 2006). A reduction in adamantane resistance levels was reported in Canada for H3N2 virus isolates submitted in 2006/2007 compared to the previous season (http://www.phac-aspc.gc.ca/fluwatch/06-07/).

No resistant influenza A viruses were identified before 1997 in our study; however, the number of samples analyzed between 1970 to 1979 is limited and may not reflect all circulating influenza viruses at this time. These results are concordant with the reported literature in Canada (Prud'homme et al., 1997). Other studies have reported similar low levels of resistance before 2005 in North America (Bright et al., 2005; Belshe et al., 1989; Ziegler et al., 1999; PHAC, 2006). No resistance was observed in the H1N1 isolates analyzed in this study. Similarly,

a lack or low level of resistance has been noted world-wide (Bright et al., 2005), in the United States (Bright et al., 2006) and in Canada (PHAC, 2006) for influenza A (H1N1) viruses. High levels of adamantane resistance were reported for influenza A (HIN1) viruses circulating in Asia in 2005 and 2006 (Deyde et al., 2007)

The first event leading to the appearance of adamantane resistance in H3N2 viruses could have resulted from either drug selection or a spontaneous mutation, followed by the efficient spread of this resistant strain of influenza A globally (Bright et al., 2005). The mutation leading to adamantane resistance may not compromise viral fitness, replication or transmissibility and has been further suggested to confer a transmission advantage (Bright et al., 2005; Hayden, 2006). Published data suggest that adamantane resistance in H3N2 viruses first arose in Asia with no reports of resistance in other regions of the world, including North America, until much more recently (Bright et al., 2005; Deyde et al., 2007).

The resistant strains circulating in Canada may have originated in countries where amantadine is routinely used, with the high frequency of global travel leading to repeated introductions of resistant strains into the population. In March 2006, an advisory was issued recommending that amantadine not be used for the prophylaxis of influenza A virus infections in Canada for the remainder of the 2005–2006 season with oseltamivir to be used as an antiviral medication for prevention of influenza (PHAC, 2006). Amantadine is thus not used currently for the management of influenza A cases and outbreaks in Canada. However, this antiviral was used for chemoprophylaxis (but not treatment) in influenza A virus outbreaks occurring at long term and assisted care centers before March 2006 in Alberta. The high level of adamantane resistance seen in non-outbreak related influenza A H3 viruses suggests that continued use of amantadine is not required as a selective pressure to maintain the resistant genotype. Other studies have also reported the increase of resistant isolates in patient populations not exposed to amantadine since 2005 (Bright et al., 2005, 2006; Saito et al., 2007b).

In our study described here, influenza A H3N2 viruses analyzed from September 2006 showed a reduction in the level of adamantane resistance when compared with previous months/years; however, the level of resistance still remained high as compared to 2004. The reason for decline in resistance levels could be the introduction and spread of drug sensitive influenza A viruses during the 2006–2007 respiratory season. Alternatively, the Ser 31 Asn mutation may have been lost as a result of suspension of amantadine use in Canada. It has been reported that the decrease in resistance during the 2006–2007 season coincided with the emergence of new HA antigenic variants (Deyde et al., 2007).

Pyrosequencing is a faster alternative for high throughput screening and surveillance of adamantane resistance than direct sequencing of PCR products. We were able to confirm previous studies (Bright et al., 2005, 2006; Deyde et al., 2007) that this procedure can be utilized with results concordant to those obtained by formal dideoxy capillary-based sequencing of amplified products. Use of such an approach would ensure the availability of timely results for adamantane resistance.

This is the first study to report the emergence and prevalence of adamantane resistant influenza A isolates in a Canadian province over a period of 37 years. The resistance profile of influenza A viruses cannot be easily predicted in advance of an outbreak or pandemic. Currently, studies in different countries have reported the presence of both adamantane-resistant and -sensitive H5N1 viruses (Cheung et al., 2006; Puthavathana et al., 2005; Hurt et al., 2007). The taxonomy of H5N1 viruses has recently been reviewed and refined into 10 unique clades (http://www.who.int/csr/disease/avian_influenza/guidelines/nomenclature/en/index.html). Linking adamantane resistance profile to consistent clade designation will help in monitoring and tracking such viruses. It is important that we continue prospective monitoring, not only for M2 protein inhibitors but also neuraminidase inhibitors, to help in the prudent use of available antivirals for the management of all seasonal influenza infections and outbreaks. Such work will also allow laboratories to have methods available for pandemic preparedness.

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References

Barr, I.G., Hurt, A.C., Iannello, P., Tomasov, C., Deed, N., Komadina, N., 2007. Increased adamantane resistance in influenza A (H3) viruses in Australia and neighbouring countries in 2005. Antiviral Res. 73, 112–117.

Belshe, R.B., Burk, B., Newman, F., Cerruti, R.L., Sim, I.S., 1989. Resistance of influenza A virus to amantadine and rimantadine: results of one decade of surveillance. J. Infect. Dis. 159, 430–435.

Belshe, R.B., Smith, M.H., Hall, C.B., Betts, R., Hay, A.J., 1988. Genetic basis of resistance to rimantadine emerging during treatment of influenza virus infection. J. Virol. 62, 1508–1512.

Bright, R.A., Medina, M.J., Xu, X., Perez-Oronoz, G., Wallis, T.R., Davis, X.M., Povinelli, L., Cox, N.J., Klimov, A.I., 2005. Incidence of adamantane resistance among influenza A (H3N2) viruses isolated worldwide from 1994 to 2005: a cause for concern. Lancet 366, 1175–1181.

Bright, R.A., Shay, D.K., Shu, B., Cox, N.J., Klimov, A.I., 2006. Adamantane resistance among influenza A viruses isolated early during the 2005–2006 influenza season in the United States. JAMA 295, 891–894.

Centre for Disease Control Prevention (CDC), 2006. High levels of adamantane resistance among influenza A (H3N2) viruses and interim guidelines for use of antiviral agents—United States, 2005–2006 influenza season. MMWR Morb. Mortal. Wkly. Rep. 55, 44–46.

Cheung, C.L., Rayner, J.M., Smith, G.J., Wang, P., Naipospos, T.S., Zhang, J., Yuen, K.Y., Webster, R.G., Peiris, J.S., Guan, Y., Chen, H., 2006. Distribution of amantadine-resistant H5N1 avian influenza variants in Asia. J. Infect. Dis. 193, 1626–1629.

Deyde, V.M., Xu, X., Bright, R.A., Shaw, M., Smith, C.B., Zhang, Y., Shu, Y., Gubareva, L.V., Cox, N.J., Klimov, A.I., 2007. Surveillance of resistance to adamantanes among influenza A (H3N2) and A (H1N1) viruses isolated worldwide. J. Infect. Dis. 196, 249–257.

Hay, A.J., Zambon, M.C., Wolstenholme, A.J., Skehel, J.J., Smith, M.H., 1986. Molecular basis of resistance of influenza A viruses to amantadine. J. Antimicrob. Chemother. 18, 19–29, Suppl B.

Hayden, F.G., 2006. Antiviral resistance in influenza viruses–implications for management and pandemic response. N. Engl. J. Med. 354, 785–788.

Holsinger, L.J., Nichani, D., Pinto, L.H., Lamb, R.A., 1994. Influenza A virus M2 ion channel protein: a structure–function analysis. J. Virol. 68, 1551–1563.

Hurt, A.C., Selleck, P., Komadina, N., Shaw, R., Brown, L., Barr, I.G., 2007. Susceptibility of highly pathogenic A (H5N1) avian influenza viruses to the neuraminidase inhibitors and adamantanes. Antiviral Res. 73, 228–231.

Pinto, L.H., Holsinger, L.J., Lamb, R.A., 1992. Influenza virus M2 protein has ion channel activity. Cell 69, 517–528.

Prud'homme, I.T., Zoueva, O., Weber, J.M., 1997. Amantadine susceptibility in influenza A virus isolates: determination methods and lack of resistance in a Canadian sample 1991–94. Clin. Diagn. Virol. 8, 41–51.

Public Health Agency of Canada (PHAC), 2006. National and international surveillance for antiviral resistance. Canada Communicable Dis. Rep. 32, 2–3.

Puthavathana, P., Auewarakul, P., Charoenying, P.C., Sangsiriwut, K., Pooruk, P., Boonnak, K., Khanyok, R., Thawachsupa, P., Kijphati, R., Sawanpanyalert, P., 2005. Molecular characterization of the complete genome of human influenza H5N1 virus isolates from Thailand. J. Gen. Virol. 86, 423–433.

Saito, R., Li, D., Suzuki, H., 2007a. Amantadine-resistant influenza A (H3N2) virus in Japan, 2005–2006. N. Engl. J. Med. 356, 312–313.

Saito, R., Li, D., Suzuki, Y., Sato, I., Masaki, H., Nishimura, H., Kawashima, T., Shirahige, Y., Shimomura, C., Asoh, N., Degawa, S., Ishikawa, H., Sato, M., Shobugawa, Y., Suzuki, H., 2007b. High prevalence of amantadine-resistance influenza A (H3N2) in six prefectures, Japan, in the 2005–2006 season. J. Med. Virol. 79, 1569–1576.

Schweiger, B., Zadow, I., Heckler, R., Timm, H., Pauli, G., 2000. Application of a fluorogenic PCR assay for typing and subtyping of influenza viruses in respiratory samples. J. Clin. Microbiol. 38, 1552–1558.

Wang, C., Takeuchi, K., Pinto, L.H., Lamb, R.A., 1993. Ion channel activity of influenza A virus M2 protein: characterization of the amantadine block. J. Virol. 67, 5585–5594.

Ziegler, T., Hemphill, M.L., Ziegler, M.L., Perez-Oronoz, G., Klimov, A.I., Hampson, A.W., Regnery, H.L., Cox, N.J., 1999. Low incidence of rimantadine resistance in field isolates of influenza A viruses. J. Infect. Dis. 180, 935–939.