EDITORIAL

Drug resistance among influenza A viruses isolated in Italy from 2000 to 2005: are the emergence of Adamantane-resistant viruses cause of concern?

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The main option in reducing the impact of influenza is immunoprophylaxis with an inactivated vaccine, a preventive measure particularly recommended for groups with a high risk of complications. Antiviral drugs represent another therapeutic chance to reduce deaths, complications, duration of hospitalization and could play a role in prevention of the infection by chemio-prophylactic action and by reducing the viral shedding of the infected patient. Two classes of antiviral agents have been used to treat influenza, the M2 ion channel inhibitors (MI), that include Adamantane derivates and have been used for more than 30 years [1, 2], and the Neuraminidase inhibitors (NI), Zanamivir and Oseltamivir, available since the half of ninety [3, 4]. These two classes of drugs target different viral proteins and have different mechanisms of action on the replication cycle of the virus. Amantadine inhibit virus replication during the early stage of infection by blocking the ion channel formed by the transmembrane domain of M2 protein [5, 6], and NI interrupt replication cycle by preventing virus release [7]. MI drug resistant mutants are present in about 30% of treated patients although Japanese authors have reported that the frequency of resistant viruses in children treated with Amantadine could be high as 80% [8, 9]. Recent reports on the global prevalence of adamantine-resistant viruses indicate a significant increase of drug resistance, from 1.8% during the 2001/02 influenza season to 12.3% during the 2003/04 season [10]. In USA, > 90% of the virus isolated during the first months of 2005/06 showed amino acid mutations known to be correlated with Adamantane resistance. In contrast to Amantadane resistance, NI resistance remains rare worldwide [11].

In South Europe the susceptibility profile of influenza isolates is poorly investigated and the possible clinical and epidemiological consequences are unclear. A subset of the viruses isolated during the last five influenza seasons (2000-2005) by CIRI-IV national network were characterized, including sequence analysis for antiviral susceptibility.

A total of 40 isolates were selected as representative of viruses circulating during the 1999-2005 influenza seasons: 5 were subtyped A/H1N1 and 35 were A/H3N2, using primer-specific polymerase chain reaction (PCR). No patients from whom viruses were isolated were treated with MI or NI. The sequence analysis of hemoagglutinin (HA), neuraminidase (NA) and matrix (M) genes of the viral isolates were performed: reverse transcription PCR (RT-PCR) and sequencing were performed using primers specific for the HA, NA and M gene segments, kindly supplied by Alan Hay, WHO Influenza Centre, London, UK and BigDye Terminator Cycle Sequencing Kit on an ABI PRISM 3100 DNA Analyzer (Applied Biosystems). Amino acid substitutions in position 26, 27, 30, 31, 34 in the M2 protein and 119, 152, 274, 292, 294 in the NA gene were considered related to drug resistance [12, 13]. HA sequence was performed as, although such NA-inhibitors act mainly on neuraminadase, viruses with HA substitutions, that are usually selected first, reduce the sialic acid-binding activity of the HA; the HA mutant becomes less dependent on the sialase activity of the NA, whereas subsequent NA mutations render the virus fully resistant [14].

In Table I were reported the amino acid substitutions observed in the collected viruses in the hydrophobic sequences in the trans-membrane region of the M2 protein. No strain isolated before 2004 showed amino acid substitution related to Amantadine or Rimantadine resistance, while 4 out of 24 (16.7%) viruses collected during the 2004/05 season showed S31N substitution. Interessangly, in the 4 S31N escape mutants, other mutations at codon 21, 27 and 28 (D21G, V27T and V28I) in the trans-membrane region were observed; in particular, substitution in position 27 was observed in Amantadine or Rimantadine resistant viruses isolated in Asia and America, but Valine was usually substituted with Alanine (V27A) [10, 15].

The 4 escape mutants were A/H3N2 viruses collected in the first weeks of the 2004/05 epidemic and the above mentioned mutations were not present in the A/H1N1 and A/H3N2 viruses isolated during the peak and when the incidence decreased. The patients from whom drug-resistant viruses were isolated were < 8 year children who lived in different areas of Genoa and they did not appear epidemiologically correlated. The hemagglutination inhibition (HI) test and HA-NA sequence analysis allowed to characterize the 4 viruses as belonging to a distinct cluster very close to A/H3N2/Panama/2007/99.

Tab. I. Amino acid substitutions in the hydrophobic sequences in the trans-membrane region of the M2 protein.

Period	N. Isolates	M2 amino acid								M2-Inhibitor Resistance Pattern
		21	26	27	28	30	31	32	34	
1999-2003	7	D	L	V	V	A	S	I	G	Susceptible
	3	.	.	.	.	.	.	V	.	Susceptible
2003-04	6	.	.	.	.	.	.	.	.	Susceptible
2004-05	4	G	.	T	I	.	N	.	.	Resistant
	20	.	.	.	.	.	.	.	.	Susceptible

No mutation in NA gene related to resistance to Zanamivir and Oseltamivir were observed in the 40 strains.

The alarming increase in incidence of Amantadine-resistant and Rimantadine-resistant influenza A viruses over the past decade described in Asia and America was not entirely observed in this Italian surveillance: the drug-resistant virus proportion of 16.7% observed during the 2004/05 season was similar than that registered in USA (15%) during the same season and very much lower than that reported in China and Hong Kong during the 2003/04 season (70 and 74%, respectively) and in the U.S.A. during the 2005/06 season (92%). The 4 drug resistant viruses present very peculiar epidemiological and virological figures: the early circulation during the 2004/05 epidemic, the young age of the infected patients and the belongings to a antigenic cluster that circulated very rarely during the 2004/05 season. A/H3N2/California/7/04-like viruses, that predominated during the epidemic did not showed mutation related to MI-resistance [16].

The effect of Adamantane use on epidemiological data is very difficult to assess as data on Adamantine treatment or exposure are available only in the U.S.A., where of the 92 patients from whom drug-resistant viruses were isolated since 2004, only two (2%) were known to have receive MI treatment before virus collection [10]. Furthermore, MI are available in over-the-counter formulations and do not need prescription in China, Russia and some other countries [17, 18]. In Italy the use of MI is very limited and no patients from whom viruses were isolated were treated. MI-resistant mutations could be occurred without the drug pressure as reported by Bright [10]: he described A/H1N1 viruses, isolated in 1930's (A/PR/8/34 and A/WSN/33), before these drugs were developed, presenting amino acid substitution associated with resistance.

More studies are needed to define the circulation of MI-resistant viruses, the frequency with which they are transmitted and their impact on effort to control influenza. This data are of fundamental importance in relation to strategies for MI or combined MI-NI use in case of epidemic or pandemic. On the other hand, no virus showed amino acid mutations related to NI-resistance: the lack of emergent resistance make them the drug of choice for prophylactic or therapeutic purpose.

References

[1] Belshe RB, Smith MH, Hall CB, Betts R, Hay AJ. *Genetic Basis of resistance to rimantadine emerging during treatment of influenza virus infection.* J Virol 1988;62:1508-12.

[2] Dolin R, Reichman RC, Madore HP, Linton PN, Webber-Jones J. *A controlled trial of amantadine and rimantadine in the prophylaxis of influenza A infection.* N Engl J Med 1982;307:580-84.

[3] Gubareva LV, Kaiser L, Hayden FG. *Influenza neuraminidase inhibitors.* Lancet 2000;355:827-35.

[4] Hayden FG, Osterhaus AD, Treanor JJ, Fleming DM, Aoki FY, Nicholson KG, et al. *Efficacy and safety of the neuraminidase inhibitors zanamivir in the treatment influenzavirus infection.* N Engl J Med 1997;337:874-80.

[5] Hay A. *A molecular basis of resistance of influenza A viruses to amatadine.* J Antimicrob Chemoter 1986 8:19-29.

[6] Pinto LH, Holsinger LJ, Lamb RA. *Influenza virus M2 protein has ion channel activity.* Cell 1992;69:517-28.

[7] Madren LK, Shipman C, Hayden FG. *In vitro inhibitory effects of combinations of anti-influenza agents.* Antivir Chem Chemoter 1995;6:109-13.

[8] Hayden FG. *Perspective of antiviral use during pandemic influenza.* Phil Trans R Soc London 2001;356:1877-84.

[9] Shiraishi K, Mitamura K, Sakai-Tagawa Y, Goto H, Sugaya N, Kawaoka Y. *High frequency of resistant viruses harboring different mutations in amantadine-treated children with influenza.* J Infect Dis 2003;188:57-61.

[10] Bright RA, Medina MJ, Xu X, Perez-Oronoz G, Wallis TR, Davis XM, Povinelli L, Cox N, Klimov AI. *Incidence of adamantine resistance among A(H3N2) viruses isolated worldwide from 1994 to 2005: a cause of concern.* Lancet 2005;366:1175-9.

[11] Moscona A. *Neuraminidase inhibitors for influenza.* N Engl J Med 2005;353:1363-73.

[12] Gubareva LV, Kaiser L, Matrosovich MN, Soo-Hoo Y, Hayden FG. *Selection of influenza virus mutants in experimentally infected volunteers treated with oseltamivir.* J Infect Dis 2001;183:523-31.

[13] Zambon M, Hayden FG and the Global Neuraminidase Inhibitor Susceptibility Network. *Position statement: global neuraminidase inhibitor susceptibility network.* Antiviral Research 2001;49:147-56.

[14] Ilyushina NA, Bovin NV, Webster RG, Govorkova EA. *Combi-*

nation therapy, a potential strategy for reducing emergence of druf-resistent influenza A variants. Antiviral Res 2006 in press

[15] Suzuki H, Saito R, Masuda H, Oshitani H, Sato M, Sato I. *Emergence of amantadine-resistant influenza A viruses: epidemiological study.* J Infect Chemother 2003;9:195-200.

[16] Ansaldi F, Icardi G, Gasparini R, Campello C, Puzzelli S, Bella A, et al. *New A/H3N2 influenza variant: a small genetic evolution but a heavy burden on the Italian population during the 2004-2005 season.* J Clin Microbiol 2005;43:3027-9.

[17] China Nonprescription Medicine Association. The active ingredients of the OCT product as of September, 2002. http://en.cnma.org.cn.

[18] Ministry of Health Russian Federation. Order N287, list of medication allowed to be sold without prescriptions, 1999. http://www.webapteka.ru.

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