Research articles

ANTIVIRAL RESISTANCE OF INFLUENZA A (H3N2) STRAINS ISOLATED IN NORTHERN GREECE BETWEEN 2004 AND 2007

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The prevention and control of influenza with vaccines and antiviral drugs is of great importance. M2 inhibitors, amantadine and rimantadine have been extensively used in some countries. The next generation of antiviral drugs, neuraminidase (NA) inhibitors oseltamivir and zanamivir, are being stockpiled for a potential influenza pandemic. The emergence of resistant strains is thus an important issue. The purpose of this study was to examine the sensitivity to M2 and NA inhibitors of Greek influenza A(H3N2) strains isolated during three influenza seasons between 2004 and 2008 and to determine the phylogenetic clades of those strains. M2 and NA sequences of 34 patient isolates were checked for known resistance mutations. In addition, haemagglutinin (HA) sequences were used to determine the phylogenetic relationship between resistant and sensitive strains. All influenza A(H3N2) strains isolated during the season 2004-5 were found susceptible to adamantanes, bearing the S31N mutation, compared to 88% of the strains isolated in 2005-6 and 75% of the strains isolated in 2006-7. Molecular analysis of the HA gene showed a correlation of the mutants with specific phylogenetic clades. No known mutations in the NA or HA gene that have been implicated in resistance to NA inhibitors were found in the A(H3N2) strains isolated in the three influenza seasons. Despite the fact that amantadine is the only drug approved for prophylaxis in Greece, it has not been extensively used. So it seems that resistant strains circulating in the area after 2005 followed the global trend of replacement of susceptible strains by resistant ones. Oseltamivir and zanamivir are currently approved only for therapeutic use in Greece and has not been extensively used either.

Introduction

The prevention and control of influenza through the use of vaccines and antiviral drugs is of great importance. Adamantanes, amantadine and rimantadine, are inhibitors of influenza A virus M2 protein. They were the first antiviral drugs licensed for treatment and prophylaxis of influenza A infections and have been extensively used in some countries. Since the emergence of viruses resistant to M2 inhibitors, many countries have begun to stockpile also the next generation of anti-influenza drugs, the neuraminidase (NA) inhibitors oseltamivir and zanamivir. The emergence of resistant strains is thus an important issue.

Adamantanes inhibit viral replication during the early stage of infection by blocking the ion channel that is formed in the

envelope of influenza virus particles by the M2 protein. Five amino acid substitutions at positions 26, 27, 30, 31 or 34 within the transmembrane domain of the M2 protein have been implicated in loss of sensitivity to M2 inhibitors [1,2]. In many countries like Canada, China, Japan, or the United States (US), adamantanes have been extensively used the past years. The emergence of resistant viral strains is now a major concern, as viral resistance to adamantanes occurs rapidly *in vivo* and *in vitro*. Moreover, the resistant strains that are becoming increasingly common in communities in Asia and the US appear to be virulent, genetically stable and capable of competing with wild-type drug-sensitive strains [3].

Oseltamivir and zanamivir block the function of NA, thus inhibiting the spread of newly formed viral particles. Various mutations have been implicated in the resistance to oseltamivir and/or zanamivir, the most common being amino acid substitutions at positions 119, 222, 274, 292 and 294, and a deletion at positions 244-247 of the NA gene. Further, it is under investigation whether specific mutations at positions 198, 229 and 262 in the haemagglutinin (HA) gene could correlate with the resistance of influenza viruses to NA inhibitors [4-6].

The purpose of this study was to examine the sensitivity to M2 and NA inhibitors of Greek influenza A(H3N2) strains isolated during the influenza seasons between 2004 and 2008, and to determine the phylogenetic relationship between those strains.

Methods

This study included molecular analysis of the M2, NA and HA genes of influenza A(H3N2) strains that were isolated in northern Greece during the last three influenza seasons. Samples from patients with influenza-like illness (ILI) were collected by sentinel general practitioners and outpatient hospital clinics and sent to the National Influenza Centre for Northern Greece for the seasonal influenza surveillance of the four influenza seasons 2004-5, 2005-6, 2006-7 and 2007-8 (December to March). The sentinel network is organised by KEELPNO, the Hellenic Center for Diseases Control and Prevention (HCDCP), and covers the whole of northern Greece.

Clinical samples, which were nasopharyngeal swabs in 2SP (sucrose-phosphate) medium were first cultured in Madin Darby Canine Kidney (MDCK) cells and embryonated chicken eggs. Virus detection was done by haemagglutination test. RNA extraction,

TABLE 1

Primer sequences, used for RT-PCR amplification of regions of the M2, NA and HA genes

Primer	Gene	Binding site (nucleotide position)	Sequence
MF8 forward	M2	8-30	5'- GCAGGTAGATATTGAAAGATGAG - 3'
MR1024 reverse	M2	1,024-1,002	5'- AGAAACAAGGTAGTTTTTACTC - 3'
N2F-1 forward	NA	1-20	5' - AGCAAAAGCAGGAGTGAAGA - 3'
N2R-1407 reverse	NA	1,408-1,389	5' - ATATAGGCATGAGATTGATG - 3'
H3HAF6 forward	HA	6-29	5'- AAGCAGGGGATAATTCTATTAACC - 3'
H3HAR1056 reverse	HA	1,056-1,037	5'- GTTCTCTGGTACATCCGC - 3'

HA: haemagglutinin; NA: neuraminidase.

TABLE 2

Influenza virus detection and typing, northern Greece, influenza seasons between 2004 and 2008

Influenza season	2004-5	2005-6	2006-7	2007-8	Total
Examined	170	140	128	93	531
Influenza A	63(37%)	11(7.9%)	45(35%)	31(33%)	150(28%)
Influenza B	3(1.8%)	42(30%)	6(4.7%)	15(16%)	66(12%)
Negative	104(61%)	87(62%)	77(60%)	47(51%)	315(59%)

Percentage is calculated from the total number of examined samples.

TABLE 3

Influenza A viruses subtyping, northern Greece, influenza seasons between 2004 and 2008

Influenza Season	2004-5	2005-6	2006-7	2007-8	Total
Examined	63	11	45	31	150
H1	36(57%)	0	0	31(100%)	67(45%)
H3	27(43%)	11(100%)	45(100%)	0	83(55%)

Percentage is calculated from the total number of influenza A viruses.

TABLE 4

Analysis of 34 influenza A(H3N2) strains isolated in northern Greece during influenza seasons between 2004 and 2008

Influenza A (H3N2) strains, northern Greece				
Influenza period	Sensitivity/resistance to M2 inhibitors	Known mutations in M2 gene	Phylogenetic groups based on HA analysis	Known mutations in NA gene
2004-5	100% sensitive	Not found	A/Lisbon/3/04-like	Not found
2005-6	13% sensitive	Not found	A/Berlin/2/06-like	Not found
	88% resistant	S31N	A/HongKong/4443/05-like	
2006-7	25% sensitive	Not found	A/Nepal/921/06-like	Not found
	75% resistant	S31N	A/Trieste/25/07-like	

HA: haemagglutinin; NA: neuraminidase.

reverse transcription PCR (RT-PCR) and sequencing were performed. RNA was extracted from HA-positive cell culture supernatants, or allantoic and/or amniotic fluids using the Viral RNA Mini Kit (Qiagen, Germany). Reverse transcription and amplification of the M2, NA and HA genes was done with the Superscript III One-step RT-PCR kit (Invitrogen, UK), using the oligonucleotide primers listed in Table 1. Purified PCR products were sequenced (Lark Technologies, Cogenics Ltd., Essex, UK) using the forward primers for each amplified product (MF8, N2F-1 and H3HAF6). NA and HA sequences from representative strains were added to GenBank under accession numbers EU744874, EU744875, EU744876, EU744877, EU744878, EU744879, EU744880, EU744881, EU744882, EU744883.

From a total of 83 PCR positive A(H3N2) viruses, 34 were cultured successfully and further studied (41% of the isolates). They covered 14 isolates from the 2004-5 season, eight isolates from the 2005-6 season and 12 isolates from the 2006-7 season were studied. Viral M2 and NA sequences were examined for resistance mutations. Viral HA sequences were also examined and compared with reference strains in order to determine the phylogenetic relationship between the circulating resistant and sensitive strains.

Results

A total number of 83 A(H3N2) viruses were isolated during three out of the four influenza seasons between 2004 and 2008

FIGURE 1

HA sequences of influenza A(H3N2) circulating in northern Greece and Europe in 2004-5, WHO reference and vaccine strains

Phylogenetic tree of HA amino acid sequences, constructed with the Neighbor Joining method and MEGA4.1 software.
HA: haemagglutinin; WHO: World Health Organization

in northern Greece. This region represents 38.5% of the Greek population. No A(H3N2) strains were isolated during the last influenza season 2007-8. An overview of the surveillance and typing results is given in Tables 2 and 3.

The results of the sequence analysis are summarised in Table 4 and show that 100% of the A(H3N2) strains that were isolated during the influenza season 2004-5 were sensitive to adamantanes, whereas 88% and 75% of the strains isolated during the 2005-6 and 2006-7 influenza seasons, respectively, contained an amino acid substitution at position 31 (serine to asparagine) known to confer resistance to adamantanes.

No known mutations of the NA gene that confer resistance to NA inhibitors were detected in the A(H3N2) strains that were isolated during the three influenza periods 2004-5, 2005-6 and 2006-7. Nor did we find known mutations in the HA gene that have been implicated in resistance to NA inhibitors. Phenotypic assays are needed to identify resistance to those antiviral drugs due to novel mutations and thus genotypic methods are only used for monitoring of established resistance markers.

Phylogenetic analysis of the HA gene of the isolated strains showed a correlation of phylogenetic clades with the S31N

mutation in the M2 gene that confers resistance to adamantanes. Specifically, during the 2004-5 season, all adamantane-resistant strains belonged to the phylogenetic group represented by the A/Lisbon/3/04 strain, and all were sensitive to adamantanes (Figure 1).

In the season 2005-6, the isolates that carried the S31N resistance mutation were all A/HongKong/4443/05-like, whereas the remaining 12.5% of adamantane-sensitive isolates were A/Berlin/2/06-like and thus belonged to a different phylogenetic group (Figure 2).

In the season 2006-7, 75% of the strains belonged to the phylogenetic group that was represented by A/Trieste/25/07 and were resistant to M2 inhibitors (Figure 3). The remaining strains (25%) belonged to the phylogenetic group represented by A/Nepal/921/06 and were sensitive to adamantanes [7]. In contrast, none of the phylogenetic groups carried mutations in the NA gene that are implicated to confer resistance to NA inhibitors, and thus no phylogenetic relationships could be determined.

Discussion

Our results are consistent with the results that have been published in other countries of the European Union, Australia, Asia

FIGURE 2

HA sequences of influenza A(H3N2) circulating in northern Greece and Europe in 2005-6, WHO reference and vaccine strains

Phylogenetic tree of HA amino acid sequences, constructed with the Neighbor Joining method and MEGA4.1 software.

HA: haemagglutinin; WHO: World Health Organization

FIGURE 3

HA sequences of influenza A(H3N2) circulating in northern Greece and Europe in 2006-7, WHO reference and vaccine strains

Phylogenetic tree of HA amino acid sequences, constructed with the Neighbor Joining method and MEGA4.1 software.

HA: haemagglutinin; WHO: World Health Organization.

and the US. [2,7-12] Resistance to M2 inhibitors first appeared following extensive drug use in Asia and the US after the SARS epidemic in 2004. The worldwide spread of these resistant strains occurred through replacement of sensitive with resistant viruses, probably because of other selective advantages of the resistant strains connected to other genes than M2.

Resistance of Greek influenza strains has developed despite the absence of selective drug pressure, as these drugs have not been extensively used in Greece [3,8-10,13]. It seems that viruses of different phylogenetic clades are imported to Greece from different parts of the world, not least due to the country's borders with Asia. The fact that our 2007 A/Nepal/921/2006-like isolates possessed M genes that were closely related to A/California/7/2004-like viruses, would seem to support the hypothesis that the sensitivity of viruses isolated more recently could be due to a reassortment event that caused reacquisition of an older, sensitive, M gene, rather than reversion of the resistance mutation. Unfortunately, the available information from southern Europe is limited.

According to the latest available information, the Ministry of Health and Social Solidarity of Greece has stockpiled oseltamivir to cover 5% of the population in case of an influenza pandemic. This corresponds to 500,000 therapeutic courses. A stock of 400,000 courses of amantadine has been created as well [14-16]. However, amantadine is the only drug that is approved for prophylaxis by the National Organisation for Medicines in Greece and can only be used for individuals over the age of five years, while rimantadine is not available at all. Oseltamivir and zanamivir are only approved for therapeutic use, for children over the age of one and 12 years, respectively, and their use can start only after an official announcement by the Ministry of Health that there is an ongoing influenza epidemic or pandemic [14,15,17].

Greece is a known crossroads among three continents (Europe, Asia, Africa) through which resistant strains can spread. Our results emphasise the need for constant monitoring of emerging resistance to the available antiviral drugs, especially after the recent appearance of A(H1N1) strains that are resistant to oseltamivir [18]. Analysis of 65 Greek influenza A(H1N1) strains by WHO found seven of 65 (10.8%) isolates resistant to oseltamivir [17]. In communities in which oseltamivir-resistant viruses are circulating widely, treatment with zanamivir or a combined treatment with adamantanes should be considered [19]. Decision makers in Greece are urged to take into account the results of such studies when they consider alternative stockpiling and usage policies of these drugs both for annual epidemics and for a possible pandemic situation [16].

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