Overview of influenza virus antiviral resistance in Romania in the last four epidemic seasons – phenotyping, genotyping and molecular analysis study

Rezistența la antivirale a virusului influenza în ultimele patru sezoane epidemice în România – studiu fenotipic, genotipic şi de analiză moleculară

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Abstract

*Influenza v*irus i*s a major public health problem, having an important socio-economic impact. The primary strategy for fighting against influenza and its complications is annual vaccination. However, in the epidemic season or in the pandemic season, and especially in the period of the time until the production of a pandemic influenza vaccine, the antiviral medication is very useful both for treatment and prophylaxis. Previous studies demonstrated significant differences between the characteristics and the ratio of influenza strains resistant to antivirals, depending on the geographic area and the degree of antiviral medication used. In this study we evaluated the molecular profile of the influenza virus regarding the resistance to antivirals in four consecutive annual influenza epidemic seasons in Romania, using phenotyping, genotyping and molecular methods of analysis. The study was focused on testing the susceptibility to antiviral drugs of influenza viruses using samples from hospitals and from the sentinel surveillance system, for all subtypes of influenza A (H1N1, H3N2, pandemic H1N1) and also influenza B. This study also had the aim of developing, implementing the tools necessary for the rapid detection of known markers or suspected of affecting neuraminidase susceptibility to inhibitors, for national coverage.*

Keywords: *influenza, pandemic, antivirals, resistance*

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Rezumat

Gripa reprezintă o problemă de sănătate publică majoră, având un impact socio-economic important. Strategia primară de luptă impotriva gripei şi a complicaţiilor sale este vaccinarea anuală. În cursul unui sezon epidemic sau într-o pandemie, producerea unui vaccin, prin metodele actuale, nu poate fi realizată într-un ritm efectiv pentru a opri progresul unei noi tulpini de virus gripal, astfel încât medicaţia antivirală este foarte utilă atât pentru tratament cât şi profilaxie. Studiile anterioare au demonstrat diferenţe semnificative între caracteristicile şi proporţia tulpinilor rezistente la antivirale, în funcţie de zona geografică şi de gradul de utilizare a medicamentelor antivirale. În acest studiu s-a evaluat profilul molecular al virusurilor gripale privind rezistenţa la antivirale în patru sezoane epidemice anuale consecutive (2007-2011), în România utilizând teste fenotipice, de genotipare precum şi analiza moleculară a mutaţiilor. Pentru testarea susceptibilităţii tulpinilor circulante la medicamentele antivirale s-au folosit probe biologice recoltate de la pacienţi în spitalele de boli infecţioase sau din sistemul naţional de supraveghere a gripei. Studiul a avut totodată scopul de implementare a metodelor de detecţie rapidă a markerilor cunoscuţi sau suspectaţi de inducere a rezistenţei la medicamentele antivirale, cu acoperire naţională.

Cuvinte cheie: *influenza, pandemic, antivirale, rezistenţă*

Introduction

Influenza vaccination with inactivated vaccines, containing strains homologous to the prevalent wild-type virus can reduce influenza illness by 75%–80% of healthy adults, but in patients with reduced immunity, protection rates are not as high, with only 40% for institutionalized elderly people (1). Patients with uncomplicated human influenza, especially adolescents and young adults can be treated symptomatically and do not need specific medication. The situation is different in patients that are likely to progress to severe complications (children, elderly) or patients with underlying medical conditions (chronic diseases, other concomitant acute illness, etc). In those cases, antiviral therapy option is one that should be seriously considered. Also, infection with highly pathogenic animal influenza viruses (e.g. avian influenza H5N1) have shown that in severe forms the use of antivirals is strongly recommended, and early initiation of therapy is very important (2).

There are currently two well known classes of antiviral drugs available for influenza viruses, M2 channel inhibitors (amantadine and rimantadine), targeting only influenza A, and neuraminidase (NA) enzyme inhibitors (oseltamivir and zanamivir) targeting influenza A and B.

Amantadine and rimantadine were discovered in the '60s (3). They block ion channel formed by Matrix 2 (M2) protein which crosses the viral membrane (4) and is necessary for viral decapsidation. They are effective as treatment if used within 24 hours of disease onset. M1 and M2 proteins are coded by a single gene and single point mutations can lead to amino acid changes in the transmembrane domain of M2 providing high level of resistance to amantadine, and the mutant strains retain the virulence capacity of the wild-type virus (5, 6). The genetic basis of resistance appears to be single amino acid substitutions at positions: L26F, V27A, A30T, S31N, G34E, and V27A/S31N in the transmembrane region of the M2 ion channel (7). In the 2004-2005 epidemic season, 91% of influenza A H3N2 strains isolated from patients in the U.S. were resistant to amantadine, therefore CDC Atlanta discouraged their use in the 2005-2006 season in U.S. (6, 8).

For the 2008-2009 influenza season, Centers for Disease Control and Prevention (CDC) U.S. found that 100% of seasonal H3N2 and 2009 pandemic influenza samples tested have shown resistance to amantadine (9).

Neuraminidase inhibitors were introduced in the treatment of influenza in 1999 and 2000. NA is a surface protein of influenza virus with enzymatic activity, responsible for the

cleavage of sialic acid, promoting the invasion of airway epithelium and release of virions from infected cells (10). Neuraminidase inhibitors interfere with normal function of the virus by mimicking sialic acid structure, aggregating virions on the surface of host cells, limiting the spread of the infection to the mucosal secretion thus reducing viral infectivity (11).

The emergence of oseltamivir resistance in clinical isolates of influenza A viruses has been associated with substitutions at residue V116, I117, E119, Q136, K150, D151, D199, I223, H274, and N295 in the NA active site and for Influenza B mainly two substitutions, residues R152 and D198 (12, 13).

The 2007–2008 influenza season was marked by an emergence of oseltamivir-resistant seasonal influenza A/H1N1 viruses with the H274Y mutation in NA gene, reported to the World Health Organization (WHO) by Norway in January 2008. Before this season, oseltamivir resistance was <1% worldwide (14, 15). Norway reported 67% oseltamivir-resistant influenza (H1N1) whereas selective pressure exerted by oseltamivir treatment was not confirmed due to the low use of oseltamivir, suggesting an effective transmission of an oseltamivir resistant strain. Similarly, other resistant viruses were detected in Europe at the same time, implying oseltamivir use related resistance and transmission from other parts of the world (16).

In order to accommodate the side chain of oseltamivir, in the active site, the neuraminidase molecule must undergo rearrangement to create a pocket, while several mutation that limit the necessary molecular rearrengement may diminish the binding of oseltamivir. The most prevalent mutations H274Y and R292K, N294S inhibit the rotation of E276 which binds with R224 thus preventing the formation of the pocket, resulting in resistance to oseltamivir (17). Unlike amantadine-resistant viruses, all of the NA mutants have severely compromised enzyme activity, stability, or infectivity *in vitro* and in animal models (18). Viruses bearing the H274Y substitution, selected *in vivo* have reduced sensitivity to oseltamivir carboxylate with 3 log reduction of replication in cell culture and pathogenicity significantly reduced in ferret model (19). Mutation I222V induces only a 1/2 reduction to oseltamivir carboxylate whereas sensitivity of the double mutant (with H274Y) was reduced more than 1000-fold (19).

The emergence of the 2009 H1N1 influenza pandemic raised concerns over the possible occurrence of a transmitting oseltamivir resistant strain. Initially, the World Health Organization (WHO) reports indicated that pH1N1 2009 viruses are sensitive to both oseltamivir and zanamivir and that their susceptibility to neuraminidase inhibition is very similar to that of drug-sensitive seasonal viruses (2). In the summer of 2009 oseltamivir-resistant isolates started to emerge with 100 strains reported worldwide together with a few outbreaks where transmission of resistant (H274Y) viruses may have occurred (20) but the majority of these were mainly detected following antiviral drug treatment (13). In August 2009 WHO issued pharmaceutical recommendations stating that patients in higher risk groups should be treat with oseltamivir or zanamivir as soon as possible or with zanamivir if there is suspicion of oseltamivir resistance. In case of severe or progressive clinical presentation, the previous statement applies, as well as for immunosuppressed patients for which higher doses and longer duration of treatment should be considered (21).

As part of the WHO Global Influenza Surveillance Network, the Romanian National Influenza Center (NIC) from Cantacuzino Institute submitted influenza viruses to the WHO Influenza Collaborating Centre in the United Kingdom for extensive antigenic and genetic analyses. We were also able to use the results from the genotyping analyses to assess the NA susceptibility to inhibitors and to verify our own genotyping and phenotyping results.

The goals of this surveillance study was to determine the baseline oseltamivir and M2 channel

inhibitors susceptibility of influenza viruses transmitted in Romania, to detect drug-resistant viruses, and to track the emergence of drug-resistant viruses over four annual epidemic seasons: 2007-2008, 2008-2009, 2009-2010 and 2010-2011. Because of the significant differences between the percentages and characteristics of influenza strains resistant to antiviral depending on geographic area and the use of antivirals it is critically important to evaluate the resistance profile of any influenza virus subtype. Also it was very important to implement and optimize the tools necessary for the rapid detection of known or suspected markers of low susceptibility to antivirals, at national level.

Materials and methods

Study design

Patients were selected based on influenza like illness (ILI) case definition (sudden onset, fever > 38° C, headache, myalgia, cough, coryza, and pharyngeal angina). Enrollment in the study was made after the informed consent (parents /legal guardians for children) and after written consent (from parents /legal guardians in children cases). The study was approved by the Bioethics Comity of the Cantacuzino Institute. Due to the wide-spread concern of amantadine/rimantadine resistant strains, all patients were treated with NA inhibitors (oseltamivir or zanamivir). Biological samples collected from NAI treated patients were used to isolate the virus on Madin-Darby Canine Kidney (MDCK) cell culture and screened for oseltamivir resistance by measuring neuraminidase activity with a chemiluminescence based phenotyping method. The strains manifesting phenotype resistance to the NAI were sequenced (partial NA gene). Screening of amantadine/rimantadine resistance (partial M2 gene sequencing) was also performed on the antiviral treated samples.

All patients were clinically evaluated using standard methodology in infectious disease hospitals. Any serious adverse event that occurred during therapy was followed by cessation of any antiviral drug treatment.

Inhibitors

Oseltamivir carboxylate for phenotyping was kindly supplied by Hoffmann, La Roche Ltd. (Basel, Switzerland).

Neuraminidase activity inhibition (NAI) screening

For NAI testing the NA-STAR kit was used (Applied Biosystems Foster City, California), which is a recent and sensitive chemiluminiscence based test for influenza A (including human, avian, swine, equine strains) as well as influenza B. The kit contains all the required reagents (NA-Star buffer, 26 mM morpholineethanesulfonic acid, 4 mM CaCl2 [pH 6.0], NA-Star 1, 2-dioxetane chemiluminescent substrate, NA-Star accelerator, and 96-well solid white plates).

All samples were assayed in duplicate together with positive and negative controls (strains susceptible and resistant to oseltamivir).

The kit user 's guide suggested a culture supernatant dilution of 1:5 to be used, which was proven to provide consistent results, reducing the concentration of phenol red, usually used as pH indicator in cell culture medium, interfering with the readings. Twenty five µl of 10 half-log dilutions of oseltamivir (1000, 317, 100, 32, 10, 3.2, 1.2, 0.32, 0.1, 0.03 nM) in NA-Star Assay Buffer were added to each well of a white 96-well microplate, 25 µl of culture supernatant was added and plates were pre-incubated at 37 °C for 20 min. Ten microliters of 1:1000 NA-Star Substrate was added to each well, the plate was shaken then incubated for 10 min at 37 °C. Finally, 50 µl of Accelerator was added to each well, plates were shaken and the luminescence was read (with 1s integration time). We used a multi-functional microplate detector LKB 3100, (Zenith Anthem - Laboratory. of Biochemistry, Victor Babes Institute, Bucharest) for the samples isolated in 2007-2008 epidemic season; in 2008-2009, 2009-2010 and 2010-2011 seasons we used the TriStar LB 941 multimode microplate reader (Berthold Technologies).

Determination of IC_{50}

Prior of determining IC_{50}, we subtracted from every reading, the mean of background or noise readout (signal from combination of reagents normally used, without virus present). Inhibitory concentration (IC_{50}) values were determined by non-linear regression analysis with sigmoid dose-response (variable slope) fitting using GraphPad Prism™ (version 5.0.4) software.

Statistical analysis

Samples with an IC_{50} value greater than 1.65SD above the mean for the season were considered minor outliers and retested Isolates with mean IC_{50} value above the mean for the season +3SD are considered major outliers and subjected to genotyping. Extreme outliers were isolates with IC_{50} in the scale of 10-fold mean IC_{50} for each respective subtype.

Virus isolation

Biological samples (nasopharyngeal swabs) were inoculated on MDCK monolayer, in 24 well plates, on Dulbecco DMEM medium supplemented with gentamicin (50 µg/ml), amphotericin (2.5µg/ml) and trypsin (1 µg/ml). The plates were incubated for 48 hours at 35 ºC with 5% CO2 atmosphere. Haemagglutination analysis was performed at 24, 48 and 72 hours post-infection by incubating samples in equal volumes of supernatant with 0.65% guinea pig erythrocyte suspension or 0.5% suspension of turkey red blood cells.

Nucleic acids extraction

Total RNA from biological samples or cell supernatant were extracted with the commercially available kit QIAamp viral RNA mini-kit (Qiagen), starting from 140 µl of sample, and finally re-suspended in a volume of 60 µl.

RT-PCR

Five micro liters of nucleic acid from specimens were added to a 45-µL reaction mixture containing the following primers: N1Seq13 (5′- ggt cca gat gat gga gca g -3′); and H1NAAS5 (5′- cca tcc att tgg atc cca aat cat ctc -3′) for the N1 gene, N2Seq14 (5′- cgg aat gcg ttt gta tca atg g -3′), and N2NARt2 (5′- aga ttg atg tcc gcc cca tc-3′) for the N2 gene, that were described in Zanamivir RELENZA™ Laboratory Manual (January 2000, Updated 2006), amplify a 525-bp product of N1 gene and 695-bp product for N2 gene. For M2 gene, we used the primers: M2F1 (5'- cag cta agg cta tgg agc aa-3') and M2R1 (5'-act gtc gtc agc atc cac ag-3'). The primer pair amplifies a 402-bp product of M2 gene and was described by Hata M. et al. (22). For pandemic H1N1 we used the primer pair: (5′- tgt aaa acg acg gcc agt aat ggr car gcc tcr tac aa-3′), and (5′- cag gaa aca gct atg acc gct gct ycc rct agt cca gat-3′), amplifying a 620-bp product, primers were described by the WHO Collaborating Centre for influenza at CDC Atlanta, United States of America (23).The primer pairs used amplify a 1201 bp product of Influenza B NA gene are: F9-(5'-GGC CAA AAA TGA ACA ATG CTA CC-3'), R725(5'ATG CAA TTG CAG GCA CTT TCT TGT-3') and F691-(5'-CAC AAG AAA GTG CCT GCA ATT GC-3'), R1210-(5'-CAG AAA CAA TTA AGT CCA GTA AGG-3'). In all reactions, primers were used at a final concentration of 0.6 µM.

We used 5× QIAGEN OneStep RT-PCR buffer, dNTP mixture and enzyme mixture as recommended by manufacturer (QIAGEN OneStep RT PCR Kit; QIAGEN, Hilden, Germany). Reactions were run on BIO-RAD DNA Engine Dyad (Hercules, California, U.S.) thermal cycler with the following program: reverse transcription 60 min at 50°C, initial activation 15 min at 95°C, followed by 40 cycles 94°C for 30 sec, 55°C for 30 sec, and 72°C for 60 sec, final extension 72°C for 10 min.

Sequencing

Gel purification was performed with Wizard® SV Gel and PCR Clean-Up System Promega (Fitchburg, Wisconsin). Sequencing was performed with ABI PRISM® BigDye Terminator v3.1 Ready Reaction Cycle Sequencing kit (Applied Biosystems Foster City, California) on a 4 capillary ABI PRISM® 3100-Avant Genetic Analyzer.

Sequence analysis

Staden Package, described by James Bonfield, Rodger Staden, et al. (24) which is comprised by 4 programs: pregap4, trev, gap4 and Spin Pre-

gap4, was used for quality evaluation of ABI traces, conversion of data formats and end clipping. Gap4 was employed for sequence assembly and contig editing. The NA and M sequences were aligned with reference sequences from databases in the public domain using BioEdit software (version 7.0.5; North Carolina State University).

Molecular modeling

The homology model of pandemic 2009 NA structure was made available by the Bioinformatics Institute, A*STAR Singapore (25). MODELLER (26) and CHARMM programs were used for *in-silico* mutagenesis (of H274Y and N386Y substitution). PyMol version (0.99) was used for molecular representations. After addition of hydrogen atoms, the model was refined by energy minimization (EM) with the protocols in CHARMM program, using a CHARMM22 force field. We used the TIP3P water model for explicit solvation of the neuraminidase monomeric structure with oseltamivir in the catalytic site. The ionized systems were minimized for 10 ps and subjected to 50 ps of molecular dynamics with NAMD (27) with 1 fs time steps. Constant temperature (T = 308 K) was enforced using Langevin dynamics. Constant pressure (p = 1 atm) was applied using the Nosé-Hoover Langevin piston method. Van der Waals interactions cutoff distances were set at 12 Å and long-range electrostatic forces were computed using the particle-mesh Ewald (PME) summation.

Docking

AutoDock (version 4.2) was used for docking oseltamivir to the neuraminidase structure of pandemic H1N1 virus that was previously modeled. The docking experiments were performed with two neuraminidase models: wild-type (A/California/07/2009) model and a model with H274Y mutation and a third model with N386K mutation. AutoDock is automated docking software developed by the Scripps Institute (California, U.S.) that is designed to predict how ligands will bind to a macromolecule at its known receptor binding domain. Ligands were prepared by adding hydrogens, computing Gasteiger charges, uniting non-polar hydrogens charges and to assign rotable bonds (seven torsions). The macromolecules (NA) were prepared by first removing all water molecules, adding polar hydrogens only, and assigning Kollman charges.The NA macromolecules were kept rigid. AutoGrid software calculates the grid that describes the receptor binding domain of the macromolecule. All grid boxes were set at the following coordinates: 24 x 24 x 24 Å. AutoDock performs the docking of the ligand to the pre-calculated grids on the macromolecule (electrostatic, dielectric). AutoDock outputs estimated free energy of binding which includes the intermolecular energy and torsional free energy. It also outputs the docking energy which includes both the intramolecular and intermolecular energies. AutoDockTools is a GUI for AutoGrid and AutoDock, which was used to prepare the ligand (oseltamivir) and in the receptor molecule (NA) and to analyze the docking run results. The docking simulations were performed using a Lamarckian Genetic Algorithm (LGA) for 100 independent runs with 25.000.000 energy evaluations. The 100 LGA runs were processed using the built-in clustering analysis of AutoDockTools with a 2.0 Å cutoff.

Results and discussions

Worldwide, 2007-2008 influenza season represented a novelty in terms of resistance to neuraminidase inhibitors, increase of resistance to inhibitors, with numerous influenza A/H1N1 strains resistant to oseltamivir, with H274Y substitution, having unclear causality. These strains showed no differences compared to sensitive strains, in terms of clinical severity or infectivity. The percentage of resistant strains in Europe ranged from 1% in Italy to 67.8% in Norway.

During the 2007-2008 epidemic season, we received 51 biological samples collected from patients in infectious disease hospitals from Bucharest. Viruses were isolated from 40 patients (23 A/H1N1 and 17 influenza B) the remaining

Table 1. Reference strains used as controls in NAI testing IC_{50} (nM), with their respective mutations

Influenza A virus	Subtype	NA mutation	IC_{50} (nM)	Resistant/Sensible
A/Mississippi/3/01	H1N1	274H	0.7±0.2	Sensible
A/Mississippi/3/01	H1N1	274Y	148.9±47.0	Resistant
A/Fukui/20/04	H3N2	119E	0.4±0.1	Sensible
A/Fukui/45/04	H3N2	119V	10.3±1.2	Resistant
A/Sibiu/184/2008	H1N1	-	0.9889	Sensible
A/Dolj/393/2008	H1N1	D354G + H275Y	191.7	Resistant
A/Bucuresti/226/2008	H1N1	D354G + H275Y	183.7	Resistant

samples (11) were negative. Another 51 cases were isolated form samples received from the sentinel surveillance system and were sent for sequencing and antigenic characterization at WHO Collaborative Centre for Influenza Research and Reference in the National Institute of Medical Research (MRC), Mill Hill, UK. Of these, 4 (7.3%) were resistant to oseltamivir (having D354G in addition to H274Y substitution).

The resistant isolates A/Bucuresti/193/08; A/Bucuresti/226/08; A/Bucuresti/229/08 and A/Dolj/393/08 had NA gene sequences closely related to other European resistant strains circulating at that time, within A/Brisbane/59/07 (CY064976.1) subclade.

Of the total A/H1N1 isolates, 20 were assayed for resistance to oseltamivir together with positive and negative controls (*Table 1*), and two H1N1 oseltamivir resistant isolates (A/Bucuresti/226/08 and A/Dolj/393/08) were antigenically characterized and genotyped at MRC. Our results revealed high IC50 in both genotyped strains, A/Bucuresti/226/08 (IC50 = 183.7 nM), A/Dolj/393/08 (IC50 = 191.7 nM), comparable to the positive control (samples resistant to oseltamivir, IC50 = 183.7 nM), confirming the phenotyping and genotyping analyses performed at MRC. The assay mean IC50, excluding the IC50 values of positive control and those of the known resistant isolates was 0.49 ±0.14 nM, with negative control having IC50 = 0.498 nM. According to the MRC report, the sequences of A/Dolj/393/08 and A/Bucuresti/193/08 were identical, which does not explain the higher IC50 of A/Bucuresti/193/08, whereas our analyses indicated a higher IC50 value for A/Dolj/393/08 - 191.7 nM vs. A/Bucuresti/193/08 -183.7 nM.

One of the isolated A/Bucuresti/45.1.A/2008 presented a IC50 of 3.77 nM, value considerably higher than mean IC50 per assay (0.49 nM) + 3SD (0.14), approximately 7 - fold mean IC50. The isolate was considered a major outlier and we further analyzed it by sequencing the 210-360 amino acid coding region of NA gene. Both the known oseltamivir resistant isolates fell within the A/Brisbane/57/07 subclade, as well as the apparently resistant isolate A/Bucuresti/45.1.A/08 (CY078387) with identical sequences, with L234M substitution and without the H274Y substitution. The other isolates were sequenced and did not presented any change, with the exception of A/Bucuresti/21.1.A/08 (CY078381), which had a IC50 0.53 nM that indicated sensibility to inhibitor. This isolate did not present the H274Y substitution, instead it had the I287T change, not known to induce resistance to NAI.

The isolates were also tested for resistance to amantadine or rimantadine by sequencing the M2 gene. None of the isolated strains presented any of the characteristic M2 mutations, thus indicating their sensibility to amantadines. This finding correlates with the reports for 2007-2008 season on amantadine resistance in Europe (2-9%), similar to Australia and New Zeeland, attributed to the spread of A/Brisbane/59/2007-like viruses, whereas Southern Asia had high

rates of resistance (33-100%) due to the spread of A/Hong Kong/2652/2006-like viruses (28). The accession numbers for all H1N1 M2 gene sequences are listed in *Table 4*.

During the 2008-2009 epidemic season, we received samples from 148 patients. Fifty seven samples were positive for A/H3N2 and 28 samples for Influenza B by RT-PCR. Of those, we isolated 31 A/H3N2 and 8 influenza B strains. Of the total number of confirmed cases, 26 received antiviral treatment (16 with oseltamivir and 10 with zanamivir). Clinical evolution was favorable in all cases, patients became afebrile in up to 6 days of hospitalization.

Screening of phenotypic susceptibility to oseltamivir was performed for 22 Influenza A (H3N2) and 17 Influenza B isolates from patients in infectious disease hospitals. Some of the patients received oseltamivir treatment after ILI diagnostic. We used reference strains as negative and positive controls for the assay: isolates known as sensitive (A/Fukui/20/04) and resistant (A/Fukui/45/04), provided by the Neuraminidase Inhibitor Susceptibility Network (NISN) reference panel (*Table 1*). Unfortunately, for NA-Star testing of influenza B we could not validate the assay due to lack of reference strains for influenza B.

For the H3N2 NAI assay, we obtained a mean IC50 value of 0.26 ± 0.05 nM, excluding the values for positive control (6.8 nM). Only one isolate, A/Bucharest/79.1.A/09, had an IC50 value (10.45 nM), considerably higher than the H3N2 subtype mean. The strain was evaluated as an extreme outlier and retesting the isolate resulted in a similarly high IC50 of 9.349 nM.

Despite the low reduced enzymatic activity in the presence of oseltamivir, the isolate A/Bucharest/79.1.A/09 (CY078406) did not present any mutation in the 240-450 coding region, compared to the reference strain A/Wisconsin/03/2009. Most probably, the outlier contained the 119V mutation, judging by the high inhibition concentrations (40.2 and 35.95 fold mean assay IC50) which seem to correlate with this mutation. Other studies reported similar findings: 50 to 500 fold IC50s values for the 119V substitution (29)(30)(31). Strains that contain mutations in the sequenced coding region (H274N, G248R, K249E, S345G, L338S, V313A, D251G) generally are minor outliers. The accession numbers all for all N2 sequences are listed in *Table 4*.

Testing the influenza B neuraminidase susceptibility to oseltamivir (N=17) we obtained a higher mean IC50 per assay (6.87 ± 2.53, median = 6.23) which is in normal range for influenza B (32). The mean IC50 values before and after oseltamivir treatment are significantly higher in patients with influenza B than in patients with influenza A/H3N2 or A/H1N1 (33).

We also sequenced the M2 gene from 23 isolates, obtained from patients who did not receive any amantadine or rimantadine treatment. Because of the high prevalence of M2 resistance mutations in previous seasons, it was not very surprising that all of the sequences tested contained the S31N substitution. This situation concurs with the WHO report on influenza A virus resistance to oseltamivir and other antiviral medicines, published in 2009, revealing that 1149/1150 H3N2 strains tested worldwide were resistant to amantadines (34). The accession numbers for all H3N2 M2 sequences are listed in *Table 4*.

During the 2009-2010 epidemic season, 14 pandemic H1N1 isolates were tested for inhibition susceptibility to oseltamivir. All isolates were phenotypically sensitive to oseltamivir, IC50 ranged between 0.33-0.75 nM (*Table 2*).

In the 2010-2011 season we continued to monitor antiviral resistance by testing the viral isolates from the national influenza surveillance system. The pandemic H1N1 isolates registered an IC50 mean (0.45 nM) comparable to the previous year, but with a slightly elevated SD, raising the upper limit to 1.1 nM. We detected and isolated only three H3N2 cases, which had a mean IC50 similar to the H3N2 subtype isolates

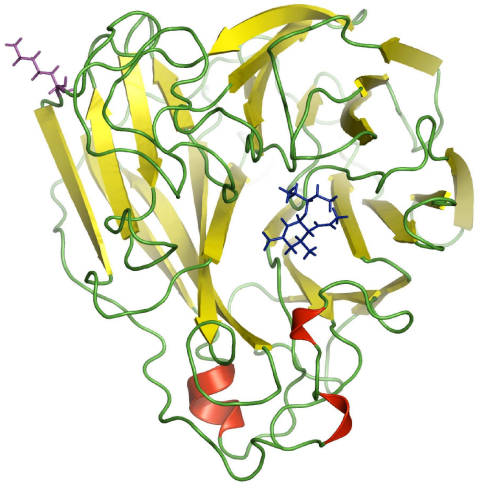

Figure 1. Representation of pandemic 2009 virus neuraminidase showing in green - loops, yellow – sheets, red – helixes, blue – oseltamivir, and magenta LYS 386

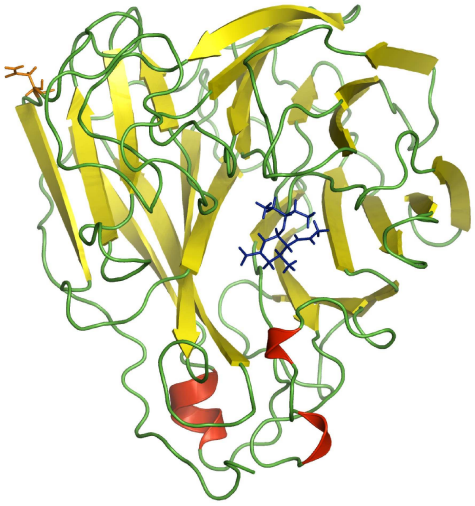

Figure 2. Neuraminidase of pandemic 2009 virus; in green - loop, yellow – sheet, red – helix, blue – oseltamivir, and in orange ASN 386.

from 2008-2009 season. One pandemic H1N1 isolate from a fatal SARI case (A/Iasi/47959/2010) presented an elevated oseltamivir IC50 value of 1.071 nM, close to the upper limit. The NA sequence (CY090790) did not revealed the presence of NAI resistance mutations.

In the case of influenza B isolates, the IC50 mean value was considerably higher (10.12 nM) in comparison to the 2008-2009 season. The influenza B isolates with elevated IC50 values were sequenced. Alignment of the sequences with the influenza reference strain (B/Brisbane/60/2008) NA sequence did not reveal any NAI resistance mutations (*Table 4*)

During the 2009-2010 influenza epidemic season, we registered 122 fatal cases. A great majority of them were clinical cases with associated chronic diseases. Oseltamivir treatment was administered to some of these severe cases. Because we wanted to test whether these patients were infected with a transmitted antiviral resistant pandemic H1N1 strain, we sequenced a 620-bp product of NA gene from original clinical sample. The sequencing results

Table 2. Influenza A and B IC_{50} seasonal mean values and upper limits

Season	**2007-2008**	**2008-2009**		**2009-2010**	**2010-2011**		
Subtype	**H1N1**	**H3N2**	**Influenza B**	**pH1N1**	**pH1N1**	**Influenza B**	**H3N2**
N	21	22	17	15	17	13	3
Mean IC_{50} (nM)	0.49	0.26	6.87	0.48	0.45	10.12	0.28
S.D. (nM)	0.14	0.05	2.53	0.11	0.21	4.13	0.17
Upper limit (nM)	0.93	0.41	14.49	0.81	1.1	22.53	0.8

N (number of isolates), mean values (nM), S.D. (standard deviation), IC50 Upper limit (= mean IC50 + 3 S.D) are indicated for oseltamivir carboxylate.

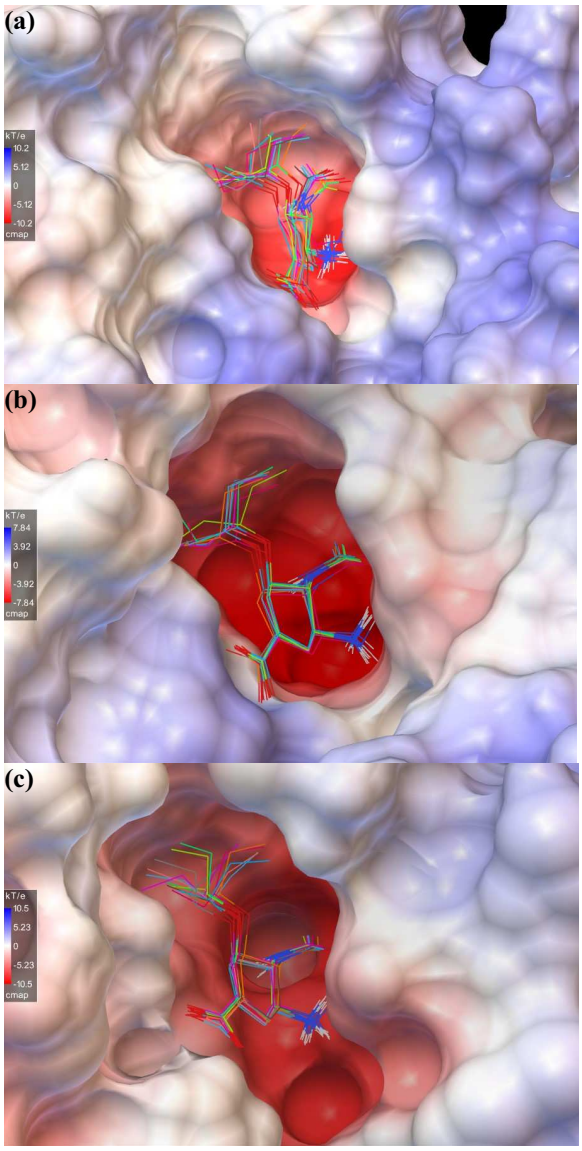

Figure 3. Alignment of oseltamivir 10 docked conformations with the lowest binding energy to the neuraminidase active site for the wild-type structure (a) N386K mutated structure (b) and for the H274Y model (c).

revealed that none of the fatal cases treated with oseltamivir had the H274Y mutation or any other well-known mutations associated with molecular resistance. One sequence had the N386K, which was reported by MRC London as forming a distinct sub-clade of recent NA genes. Also a co-variance between NA gene mutation and haemagglutinin (HA) is not overt (35). One interesting aspect is that sample with the N386K substitution in the NA sequence also presented the D222G substitution in the HA gene. This association remains to be further analyzed. Amino acid 386 is located on the globular head of the neuraminidase subunit (*Figure 1* and *Figure 2*). Because of the considerable distance between the substitution site and the location of the active site of the sialidase subunit, most probably this mutation does not interfere with the normal process of oseltamivir side-chain blocking of neuraminidase active site. The accession numbers of all pandemic NA 2009-2010 sequences are listed in *Table 4.*

The experienced difficulty with MDCK isolation of pandemic H1N1 strains from necroptic tissue, prevented us from determining oseltamivir IC50 values for the fatal cases. Thus, in order to investigate if the substitution N386K could have any possible contribution in the induction of resistance to oseltamivir, we employed several molecular analysis methods (molecular modeling, dynamics and docking). The structure of the pandemic NA glycoprotein, obtained by homology modeling was in-silico mutated in the well known amino-acidic site 274 and in 386. Molecular dynamics (MD) simulations were carried out on the mutants, followed by molecular docking with the structure of oseltamivir in NA active site. The binding energies registered across 100 binding poses of oseltamivir in the NA active sites of the wild-type and H274Y models were compared together with the binding energies of the N386K model.

The docking analysis revealed that the ki (inhibition constant) for wild-type model was 61.54 nM (-9.84 kcal/mol) and the N386K model had 32.70 (-10.21 Kcal/mol) whereas the model with H274Y mutation had 227.29 nM (-9.06 kcal/mol).The wild-type and the N386K mutant display similar ki values, within one order of magnitude diference. The model with the well-known mutation H274Y has clearly a

Table 3. Docking results of oseltamivir structure to the three NA molecular models of pH1N1 (275Y mutant, wild-type and N386K) obtained with AutoDock

Model	H275Y	H275	N386K
Number of conformation in cluster	66	49	100
RMSD (Å)	2.836	1.016	1.766
Lowest Free Binding Energy (Kcal/mol)	-9.06	-9.84	-10.21
ki (nM)	227.29 nM	61.54 nM	32.70 nM

higher binding energy (reduced sensitivity) across 100 docking conformations compared to the wild-type and N386K models in the interaction with oseltamivir (*Table 3)*.

Because of the conformational change induced by the H274Y mutation, opening of the active site is not as wide as the wild-type structure and the N386K mutant structure. For this reason the inhibitor molecule, in the H274Y actives site, suffers reorientation by 2.83 Å RMSD (Root Mean Square Deviation), compared to the initial structure, in the docked conformation with the lowest binding energy. This transition is almost triple and double compared to the wild-type and N386K mutated structure. The electrostatic interaction energy of the H274Y mutated structure with oseltamivir is on average weaker by 1 Kcal/mol compared with wild-type and N386K structure. The electrostatic mapping of H274Y active site reveals the lower electronegative (red) potential (*Figure 3*).

The docking results, although not correlated with the experimental determined IC50 values, are indicative of N386K substitution having a minimal influence in oseltamivir binding to the active site, thus suggesting normal susceptibility.

Conclusions

Because of the reduced number of samples analyzed in this study for NAI susceptibility, the results are not representative for the oseltamivir treated ILI patients. Instead this data is presented as part of the national influenza surveillance program. The dockings of oseltamivir into the NA wild-type and mutated structures (H274Y and N386K) are presented as a theoretical method of investigating the effect of an unknown mutation on inhibitor binding to the active site, when experimentally determined IC50 is not possible.

The isolates tested for NAI sensitivity, with a few exceptions, were susceptible to oseltamivir, as indicated by the IC50 values. Some of the IC50 which were above the mean + 3SDs were genotyped but no NAI resistance inducing mutation was detected, rendering the isolate susceptible to oseltamivir. This finding attests the importance of genotyping in NAI susceptibility testing.

The amantadine/rimantadine resistance profiles assessed in România in the 2007-2009 time frame correlates with data from other European countries. Because all of the isolates from 2008-2009 season had amantadine/rimantadine resistance inducing mutation in the absence of treatment, M2 sequencing is no longer performed.

The oseltamivir susceptibility data of pandemic H1N1 revealed normal oseltamivir IC50 profiles of the isolates tested in the two epidemic seasons (2009-2011), with only slight inter-season baseline variation.

In the case of influenza B we observed a higher baseline for the 2010-2011, compared to the previous season 2008-2009 (in 2009-2010 there was limited influenza B circulation). The increase of baseline IC50 of the influenza B isolates is explained by the genetic evolution and the antigenic drift, explaining the necessity for yearly IC50 baseline retesting. The influenza B sequence although not presenting any of the known NAI inducing mutations had several changes compared

Table 4. GenBank genetic database accesion numbers for NA and M2 gene fragments of the strains sequenced in 2007-2011

Strain	NA - Accesion number	M2 - Accesion number
A/Iasi/47959/2010	CY090790	
B/Brasov/51666/2011	CY090772	
B/Prahova/54376/2011	CY090779	
B/Prahova/54378/2011	CY090784	
B/Prahova/54372/2011	CY090781	
B/Galati/54883/2011	CY090783	
B/Bucuresti/7114/2011	CY090786	
B/Iasi/2513/2011	CY090787	
A/Neamt/01/2010(H1N1)	CY078362	
A/Constanta/363/2009(H1N1)	CY078385	
A/Botosani/557/2009(H1N1)	CY078389	
A/Botosani/552/2009(H1N1)	CY078388	
A/Prahova/146/2010(H1N1)	CY078372	
A/Bistrita-Nasaud/06/2010(H1N1)	CY078364	
A/Prahova/404/2010(H1N1)	CY078386	
A/Ilfov/138/2010(H1N1)	CY078369	
A/Covasna/357/2010(H1N1)	CY078384	
A/Botosani/565/2010(H1N1)	CY078390	
A/Prahova/026/2010(H1N1)	CY078363	
B/Iasi/6927/2011	CY090773	
B/Dolj/50322/2011	CY090774	
A/Bucuresti/11/08(H1N1)		CY078367
A/Bucuresti/61/08(H1N1)	CY078393	CY078394
A/Bucuresti/71/08(H1N1)		CY078399
A/Bucuresti/81/08(H1N1)		CY078408
A/Bucuresti/91/08(H1N1)	CY078415	CY078408
A/Bucuresti/101/08(H1N1)		CY078366
A/Bucuresti/131/08(H1N1)		CY078368
A/Bucuresti/141/08(H1N1)		CY078371
A/Bucuresti/151/08(H1N1)	CY078373	CY078374
A/Bucuresti/161/08(H1N1)		CY078376
A/Bucuresti/171/08(H1N1)		CY078377
A/Bucuresti/181/08(H1N1)		CY078378
A/Bucuresti/191/08(H1N1)		CY078379
A/Bucuresti/201/08(H1N1)		CY078380
A/Bucuresti/211/08(H1N1)	CY078381	CY078382

to the reference strain: N199D, F73L, P51S, N329D (influenza B numbering).

The rapid genetic evolution of influenza viruses shows that continuation of antiviral susceptibility monitoring and increasing capacity to allow timely response in the emergence of a transmitting oseltamivir resistant strain, are mandatory.

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Table 4. (continuation)

Strain	NA - Accesion number	M2 - Accesion number
A/Bucuresti/221/08(H1N1)		CY078383
A/Bucuresti/103/09(H3N2)		CY078391
A/Bucuresti/273/09(H3N2)		CY078392
A/Bucuresti/289/09(H3N2)		CY078395
A/Giurgiu/320/09(H3N2)		CY078396
A/Bucuresti/230/09(H3N2)		CY078397
A/Bucuresti/339/09(H3N2)		CY078398
A/Bucuresti/721/09(H3N2)		CY078400
A/Bucuresti/731/09(H3N2)		CY078401
A/Bucuresti/741/09(H3N2)	CY078402	CY078403
A/Bucuresti/781/09(H3N2)	CY078404	CY078405
A/Bucuresti/791/09(H3N2)	CY078406	CY078407
A/Bucuresti/821/09(H3N2)	CY078409	CY078410
A/Bucuresti/478/09(H3N2)		CY078411
A/Bucuresti/502/09(H3N2)		CY078412
A/Bucuresti/871/09(H3N2)		CY078413
A/Bucuresti/891/D21(H3N2)		CY078414
A/Bucuresti/526/09(H3N2)		CY078417
A/Bucuresti/578/09(H3N2)		CY078418
A/Bucuresti/961/09(H3N2)		CY078419
A/Bucuresti/590/09(H3N2)		CY078420
A/Bucuresti/641/09(H3N2)		CY078365
A/Bucuresti/1391/09(H3N2)		CY078370
A/Bucuresti/1531/09(H3N2)		CY078370
A/Neamt/01/2010(H1N1)	CY078362	
A/Constanta/363/2009(H1N1)	CY078385	
A/Botosani/557/2009(H1N1)	CY078389	
A/Botosani/552/2009(H1N1)	CY078388	
A/Prahova/146/2010(H1N1)	CY078372	
A/Bistrita-Nasaud/06/2010(H1N1)	CY078364	
A/Prahova/404/2010(H1N1)	CY078386	
A/Ilfov/138/2010(H1N1)	CY078369	
A/Covasna/357/2010(H1N1)	CY078384	
A/Botosani/565/2010(H1N1)	CY078390	
A/Prahova/026/2010(H1N1)	CY078363	
A/Bucuresti/451/08(H1N1)	CY078387	

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