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Susceptibility to Neuraminidase Inhibitors and M2 Blockers of Seasonal Influenza Strains Isolated in Bulgaria 2004-2007

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Abstract

M2 blockers and neuraminidase inhibitors (NAIs) are two classes of drugs currently approved for prophylaxis and treatment of seasonal influenza A virus infections. The frequency of antiviral drug resistance has increased dramatically over the last 20 years, therefore monitoring of susceptibility to licensed inhibitors should be an essential component of influenza surveillance and therapy in Bulgaria and worldwide.

Classical and molecular techniques were used to evaluate the susceptibility of influenza (H1N1) and (H3N2) strains isolated in Bulgaria 2004-2007 to neuraminidase inhibitors and M2 blockers. IC_{50} values of rimantadine were determined by CPE inhibition in cell cultures. IC_{50} of NAIs were evaluated fluorimetrically by neuraminidase susceptibility assay with MUNANA substrate. RT-PCR and sequencing were carried out on gene segments HA, NA and M2 with subsequent phylogenetics analysis using the neighbor-joining method and bootstrap analysis. Of the total 26 influenza strains (H1N1) and (H3N2) analyzed, 22 were sensitive and 4 (two H1N1 and two H3N2) were resistant to rimantadine hydrochloride in CPE inhibition assay. 17 isolates were subjected to a fluorescent assay, which showed the IC_{50} of zanamivir to range from 1.05 nM to 5.28 nM and that of osletamivir IC_{50} were - from 0.28 nM to 1.31 nM. Sequencing revealed S31N and V27T mutations in transmembrane region of the M2 protein to confer resistance to adamantanes in the A/Sofia/1250 (H3N2) strain. The virus remained susceptible to neuraminidase inhibitors. In all other viruses analyzed no mutations associated with resistance to either M2 blockers or NAIs were found.
Keywords: influenza, susceptibility, Bulgaria, rimantadine, oseltamivir, zanamivir

Abbreviations

H, HA – hemagglutinin, N, NA – neuraminidase, NAIs – neuraminidase inhibitors, nt-nucleotide, CPE – cytopathic effect, IC_{50} – inhibitory concentration 50, NCIPD - National Center of Infectious and Parasitic Diseases

Резюме

М2 блокерите и невраминидазните инхибитори (НАИ) са два класа антивирусни средства понастоящем одобрени за профилактика и лечение на сезонен грип А. Степента на резистентност към тези препарати е нарастнала драстично през последните 20 години и затова мониторингът на чувствителността към лицензираните препарати трябва да е съществен компонент в лечението на грипа в България и по света.

Използвахме класически и молекулярни техники за оценка на чувствителността на щамове грипни вируси (H1N1) и (H3N2), изолирани в България 2004-2007 г. към инхибитори на невраминидазата и М2 блокери. IC_{50} стойностите на римантадин се определяха чрез инхибиция на ЦПЕ в клетъчни култури. IC_{50} на НАИ бяха оценени флуорометрично чрез анализ на невраминидаза

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чувствителност с MUNANA субстрат. Бяха извършени RT-PCR и секвениране за анализ на генни сегменти HA, NA и M2 с последваща филогенетика, в която приложихме neighbor-joining метод и bootstrap анализ. От общо 26 грипни щама (H1N1) и (H3N2) 22 бяха чувствителни и 4 (два H1N1 и два H3N2) бяха резистентни към римантадин хидрохлорид в постановка на инхибиране на ЦПЕ. 17 изолата бяха подложени на флуоресцентен анализ като IC_{50} на занамивир вариераше от 1.05 nM до 5.28 nM, и за оселтамивир IC_{50} бяха от 0.28 nM до 1.31 nM. Секвенирането показа S31N и V27T мутации в трансмембранния регион на M2 протеин, отговорни за резистентност към адамантани в A/София /1250 (H3N2) щам. Вирусът бе със запазена чувствителност към НАИ. При всички други вируси не бяха открити мутации, свързани с резистентност към M2 блокери или инхибитори на невраминидазата.

Introduction

Seasonal influenza virus types A (H1N1) and (H3N2) are a major cause of respiratory infections with a significant number of deaths in annual epidemics. Despite the existence of two classes of antivirals - M2 blockers and neuraminidase inhibitors, resistance occurrs due to natural variability of the viruses or in response to the conducted treatment restricting their efficacy. M2 blockers or adamantane derivatives (amantadine and its derivative rimantadine) bind the M2 proton channel and block the influx of H^+ ions into the virion - a process essential for the uncoating stage of the viral replication cycle. Particular amino acid substitutions (mainly S31N) in the M2 protein significantly reduce its susceptibility to this class of drugs (Hay *et al*., 1985, 1986; Belshe *et al*., 1988). Worldwide, resistance to adamantanes has increased dramatically over the last 20 years, from 0.8% in 1995 to 100 % of S31N (H3N2) substituted mutants being resistant to this class of drugs in 2005/06 (Deyde *et al.,* 2007; Nelson *et al.,* 2009).

Neuraminidase inhibitors (NAIs) – zanamivir and oseltamivir (and its derivative peramivir) are structural analogues of the N-acetylneuraminic acid in cellular glycoprotein receptors. They bind the viral neuraminidase (N), thus blocking the cleavage and susequent release of the influenza progeny from the surface of the infected cell (Moscona, 2005a, b).

In the autumn of 2008, a H275Y mutation in the viral N1 neuraminidase protein emerged naturally, conferring resistance of A (H1N1) viruses to oseltamivir. It spread out quickly but fortunately with an impaired aptitude to being transmitted form person to person (Lackenby *et al*., 2008, Nelson *et al*., 2009). In 2009, the reassortant H1N1v virus resistant to M2 blockers, but sensitive to neuraminidase inhibitors was introduced into the human population from swine and began its circulation. However, a resistance of the virus to this group of inhibitors as well has been demonstrated in some countries (Australia, Singapore, Brunei), reaching as high as 30%, including the newly identified S247N mutation and double mutants H275Y + S247N (Harvala *et al.*, 2010, Lackenby *et al*., 2011, Hurt *et al.,* 2011). This determines the monitoring of resistance as a mandatory component in the control of influenza viruses circulating in Bulgaria, as well as globally.

Materials and Methods

Viruses

A total of 26 Influenza A (H1N1) and (H3N2) strains isolated and subtyped in the National Influenza and Acute Respiratory Diseases Reference Laboratory in Bulgaria (National Center of Infectious and Parasitic Diseases - NCIPD) in the period between 2004 and 2007 were kindly provided for further analyses of their susceptibility to antivirals. Viruses were propagated in 10-day-old chick embryos by several intra-allantoic passages. A/Aichi/2/68 (H3N2); A/Panama/2007/99 (H3N2); A/Puerto Rico/8/34 (H1N1) and A/New Caledonia/20/99 (H1N1) were used as reference sensitive strains.

Cells

Madin-Darby canine kidney (MDCK) cells were obtained from the ATCC (Manassas, VA, USA) and were grown in DMEM (Gibco BRL, Paisley, Scotland, UK), supplemented with 10% fetal bovine serum (Gibco BRL, Paisley, Scotland, UK), 3.7 mg/ml sodium bicarbonates, 10 mM HEPES buffer (AppliChem GmbH, Darmstadt, Germany), 100 IU/ml of penicillin, 100 μg/ml of streptomycin and 50 μg/ml of gentamycin in a 5% CO_2 incubator Thermo Scientific 311 (Thermo Fisher Scientific, USA).

Compounds

Rimantadine hydrochloride was provided by Olainfarm, SA, Riga, (Latvia). Oseltamivir car-

M	Oligonucleotides	Annealing T
1Mfw/+/1	5‘AGCAAAAGCAGGTAGATATTGA	Tm 48°C
2Mfw/+/237	5‘AGCGAGGACTGCAGCGTAG	Tm 62°C
3Mfw/+/651	5‘CAGATGGTGCAGGCAATGA	Tm 58°C
4Mfw/+/827	5‘GATATTGTGGATTCTTGATCG	Tm 46°C
1MRc/-/1027	5‘AGTAGAAACAAGGTAGTTTTTTACTC	Tm 47°C
2MRc/-/841	5‘GAATCCACAATATCAAGTGCA	Tm 48°C
3MRc/-/876	5‘CTCATTGCCTGCACCAT	Tm 52°C
4MRc/-/250	5‘TGCAGTCCTCGCTCACTG	Tm 58°C
H1		
A/H1/+/5	5’AAAGCAGGGGAAAATAAAAACAACC	Tm 57°C
A/H1/+/360	5’GGGTATTTCGCCGACTATGAGG	Tm 56°C
A/H1/-/793	5’GGTTCCAGCAGAGTCCAGTAGTA	Tm 52°C
A/H1/-/1117	5’ATCATTCCAGTCCATCCCCCTTCAAT	Tm 62°C
N1		
A/N1/+/2	5’GCAAAAGCAGGAGTTTAAAATGAA	Tm 54°C
A/N1/+/600	5’AACGGCATAATAACTGAAACC	Tm 45°C
A/N1/-/720	5’CACAGACACATTCAGACTCTTG	Tm 52°C
A/N1/-/1431	5’ACTTGTCAAT GGTGAATGGC AAC	Tm 55°C
H3		
A/H3/+/68	5’AAGCAGGGGATAATTCTATTAACC	Tm 51°C
A/H3/+/361	5’GCAACTGTTACCCTTATGATGTG	Tm 51°C
A/H3/-/694	5’GAGACTGTGACTCTCCCTGATG	Tm 50°C
A/H3/-/1197	5’CTGCTTGAGTGCTTTTAAGATCTG	Tm 52°C
N2		
A/N2/+/1	5’AGCAAAAGCA GGAGTGAAGATG	Tm 53°C
A/N2/+/299	5’ATTACAGGATTTGCACCTTTTTC	Tm 51°C
A/N2/-/972	5’GCACACATAACTGGAATCAATGC	Tm 54°C
A/N2/-/1459	5’CAAGGAGTTTTTTTCTAAAATTGCG	Tm 55°C

boxylate was obtained from Hoffman-La Roche (Switzerland). Zanamivir was kindly provided by Mark von Itzstein.

Primers for RT- PCR and sequencing

All primers used for sequencing were designed and constructed for the National Reference Laboratory, Region North, and France and supplied by Proligo® Reagents, Sigma-Aldrich).

Phenotypic assays:

Suscepitibility to M2 blockers

After multiplication in the allantoic fluid in 10-day-old chicken embryos, virus samples were centrifuged at 3000 rpm/min and 10-fold dilutions were prepared in DMEM culture medium supplemented with 3 µg/l trypsin and 1M HEPES - 2% by volume medium. 0,1 ml of each dilution was inoculated in a 24-hour monolayer of MDCK in 4 wells of 96-well plates . After incubation of 1h at room temperature for adsorption, the virus was removed and 0.2 ml of 3.2, 1, 0.32 , 0.1 µM rimantadine in maintenance medium DMEM (Gibco) with 0.5% fetal calf serum (Gibco) was added. The plates were incubated for 48-72 hours at 37^0C. The presence of cytopathic effect was analysed by an Olympus CK40 microscope. Infectious virus titer was calculated in $\log_{10}$ by end-point dilution of Reed and Muench (1938). The evaluation of the antiviral effect of the inhibitor on a given isolate was performed by comparison with a viral control set in parallel and to the effect of the compound on the corresponding sensitive reference strain. Strains in which the CPE was reduced by the compound at concentrations between 3.2 -0.1 µM were considered sensitive.

Suscepitibility to NAIs

For 16 of the viruses, including 7 strains subtype H3N2 and 9 strains subtype H1N1, a fluorescent assay determining neuraminidase activity with 2-(4-methylumbelliferyl)-D-N-acetylneuraminic acid-MUNANA (Sigma) (Potier *et al*., 1979) was carried out. Initially the standard viral dose was determined with 100 µM substrate and viral dilutions

of 1/2, 1/4, 1/8, 1/16, 1/32, 1/64, 1/128, 1/256. All dilutions were made up in a working solution of MES 33 mM, NaCl 120 mM, $CaCl_2$ 4 mM at pH 6.5, with the concentration of the inhibitor ranging from 0, 0.00128, 0.0064, 0.032, 0.16, 0.8, 4, 20, 100 to 1000 nM. After incubation at 37°C for 70 min, the reaction was terminated with a stop solution containing Glycine 0.1M, EtOH 25%, pH 10.7. IC_{50} for each isolate was determined by measuring the fluorescent signal of 40 μM substrate MUNANA by fluorimeter Twinkle LB970 (Berthold Technologies) using a software Microwin 2000®.

The results in fluorescent units were processed by Kaleidagraph (Synergy Software), NA activity was determined from the dose-response curve and IC_{50} was calculated by the following formulae:

IC_{50}= ((Km+[S])/Km)*Ki
For N1: [S]=40μM and Km (N1h)=20
IC_{50}=((20+40)/20)*m2=(60/20)*m2
IC_{50}=3*m2
For N2: [S]+=40μM и Km (N2)=30
C_{50}=((30+40)/30)*m2=(70/30)*m2
IC_{50}=2,3*m2 , where
[S]= MUNANA concentration
Km – Michaelis-Menten constant
Ki – inhibitory constant

Genetic analysis:

RNA extraction and RT-PCR

For 17 strains, RNA extraction was carried out with Qiamp Mini Spin®, Qiagen kit as per the manufacturer's instructions. RNA was stored at +4°C until RT-PCR-amplification of HA, NA and M gene segments with Superscript™ One-Step RT-PCR with Platinum® *Taq* (Roche). The amplification mix contained 6,5 μl dH_2O; 12,5 μl 2X reaction mix buffer; 0,2 μl Fw primer (50 μM); 0,2 μl Rc primer ,(50 μM); 0,12 μl RNAsin (Promega) 40 U/μl; 0,5 μl RT/Platinum Taq mix. To the 20 μl of amplification mix 5 μl RNA were added and 40 RT-PCR cycles (Veriti, Applied Biosystems) were performed. The amplified products were stored at +4°C and visualized by agarose gel electrophoresis (as described later). For samples where a weak signal was observed, nested PCR (30 cycles) with the internal primers was conducted.

Agarose gel electrophoresis

A volume of 7 μl of each sample was mixed with 3 μl loading buffer bromphenol blue (Sigma B8026) and loaded onto a 1% agarose gel (0.5 g agarose in 50 ml buffer with 1 μL 0.5xTBE and 10 mg/mL ethidium bromide) and 100 to 110 V electrophoresis was performed. The products of the nested PCR were visualized in a 2% agarose gel. In addition, a negative control (amplification mix where RNA volume is replaced by dH_2O) and RNA from a reference virus were loaded with bromphenol blue as well as a DNA ladder for the determination of fragment length. The products were visualized under UV light and photographed. The reaction was considered to have gone successfully if a signal was present in the samples and positive control and absent in the negative control.

Sequencing

Amplified gene segments were subjected to complete sequencing in sequencer ABI PRISM 3730XL DNA analyzer, Applied Biosystems in Platform 8, Institut Pasteur Paris. Sequences were processed and analyzed for mutations contributing to the development of resistance. Phylogeny of the viruses through the construction of a dendrogram for NA and HA genes was carried out using the neighbor-joining method with CLC Main Workbench® and FigTree software. Sequences used for the alignment were published and retrieved from the NCBI Influenza Virus Sequence Database (http://www.ncbi.nlm.nih.gov/).

Results and Discussion

Phenotypic susceptibility to M2 blockers

From the total of 26 samples isolated from patients in the period 2004-2007 at NCIPD, 23 were studied for their sensitivity to rimantadine in MDCK cells. Strains A/Sofia/207/07 (H3N2), A/Sofia/490/06 (H1N1) A/Sofia/246/06 (H1N1) did not induce CPE and therefore it was not possible to determine their phenotypic susceptibility to rimantadine. As sensitive were determined those strains whose CPE was inhibited in comparison with the control virus by more than 1.66 Δlg. Strains A/Sofia/1246/06(H3N2), A/Sofia/1250/06(H3N2), A/Sofia/1244/06(H1N1) and A/Sofia/1248/06(H1N1) were characterized as phenotypically resistant since there was no inhibition of the cytopathic effect in the presence of rimantadine at the concentrations used (Table 1).

Due to the high levels of resistance to M2 blockers and the increasing ones to NAIs, specialists highlight monitoring of susceptibility as a primary factor in determining the appropriate treatment of influenza infections (Monto *et al.,* 2006; Deyde *et al.*, 2007; Ong and Hayden, 2007; Pabbaraju *et al*., 2007, etc.).

The majority of isolates we analysed - 85% of H3N2 and 80% of H1N1 were phenotypically sensitive to M2 blockers. The time of isolation of

Table1. Susceptibility of Influenza A (H3N2) and (H1N1) viruses isolated in Bulgaria 2004-2007 and reference strains to rimantadine hydrochloride assayed in MDCK cells

H3N2 strain	IC_{50} Rim (μM)	Susceptibility	H1N1 strain	IC_{50} Rim (μM)	Susceptibility
A/Sofia/747/04	0.01 ÷ 3.2	S	A/Sofia/240/05	0.01 ÷ 3.2	S
A/Sofia/684/04	0.01 ÷ 3.2	S	A/Sofia/361/05	0.01 ÷ 3.2	S
A/Sofia/649/04	0.01 ÷ 3.2	S	A/Sofia/422/05	0.01 ÷ 3.2	S
A/Sofia/1251/05	0.01 ÷ 3.2	S	A/Sofia/522/05	0.01 ÷ 3.2	S
A/Sofia/209/06	0.01 ÷ 3.2	S	A/Sofia/246/06	0.01 ÷ 3.2	S
A/Sofia/1246/06	> 3.2	R	A/Sofia/218/06	0.01 ÷ 3.2	S
A/Sofia/1250/06	> 3.2	R	A/Sofia/418/06	0.01 ÷ 3.2	S
A/Sofia/1247/06	0.01 ÷ 3.2	S	A/Sofia/179/06	0.01 ÷ 3.2	S
A/Sofia/374/07	0.01 ÷ 3.2	S	A/Sofia/126/06	0.01 ÷ 3.2	S
A/Sofia/206/07	0.01 ÷ 3.2	S	A/Sofia/490/06	0.01 ÷ 3.2	S
A/Sofia/413/07	0.01 ÷ 3.2	S	A/Sofia/1244/06	> 3.2	R
A/Sofia/427/07	0.01 ÷ 3.2	S	A/Sofia/1248/06	> 3.2	R
A/Sofia/319/07	0.01 ÷ 3.2	S	A/Puerto Rico/8/64	0.01 ÷ 3.2	S
A/Sofia/374/07	0.01 ÷ 3.2	S			
A/Aichi/2/68	0.01 ÷ 3.2	S			

the strain is an important factor in monitoring resistance, since in the beginning of each flu season the majority of circulating viruses are sensitive, and at the end - resistant to adamantanes both as a consequence of therapy and due to the fact that mutants are transmissible to the same extent as the wild type virus.

All samples analyzed had been isolated at the beginning and at the peak of the epidemic in each year, which partly explains the high percentage of H3N2 isolates susceptible to rimantadine. A sharp increase in circulating H3N2 adamantane-resistant strains in 2005-2006 was reported (Deyde *et al.,* 2007), however in our study such an event was not observed, although the small sample size should be kept in mind. Similar studies also show that for the same period both resistant and sensitive influenza viruses have been isolated (Yavarian *et al*., 2010). Low resistance in season 2006-2007 was associated with the emergence of adamantane-sensitive variants that had once again acquired the sensitive M segment through genetic reassortment of sensitive viruses close to the ones circulating before 2005-2006 (Furuse *et al.,* 2009a).

Phenotypic susceptibility to NAIs (zanamivir and oseltamivir)

A total of 16 H3N2 (n = 7) and H1N1 (n = 9) strains were assayed to determine the sensitivity of viral neuraminidase to oseltamivir and zanamivir by fluorescent MUNANA substrate. All strains analyzed were determined as sensitive to oseltamivir (IC_{50} ranging from 0.24 to 1.7 nM) and zanamivir (IC_{50} from 0.86 to 5.16 nM) which is consistent with published data on viruses isolated in other regions in this period and also characterized as sensitive. Studies on the sensitivity of the influenza virus to neuraminidase inhibitors indicate that resistant variants of both subtypes show values as high as 600 -1000 nM (Ramaix-Welti *et al*., 2008, Correia *et al.*, 2010; Puzelli *et al.,* 2011), however such values were not measured in this study. As is apparent from the results shown in Table 2 (A) and (B), the total sensitivity to oseltamivir was higher as compared with that to zanamivir. All H3N2 sub-

Table 2. Average susceptibility of viral neuraminidase of Influenza A (H3N2) and (H1N1) viruses from Bulgaria 2004-2007 to NAIs oseltamivir carboxylate and zanamivir as analyzed by fluorescence assay with MUNANA substrate.(A) NA of clinical isolates, compared to the reference virus (B).

(A) Clinical isolate	IC_{50} Zanamivir (nM) ± SD	IC_{50} Oseltamivir (nM) ± SD
A/Sofia/747/04 (H3N2)	3.58 ± 1.62	0.47 ± 0.27
A/Sofia/684/04 (H3N2)	1.69 ± 1.21	0.31 ± 0.11
A/Sofia/649/04 (H3N2)	1.69 ± 0.91	0.55 ± 0.46
A/Sofia/1247/06 (H3N2)	2.33 ± 1.45	1.04 ± 0.53
A/Sofia/1250/06 (H3N2)	5.28 ± 2.48	0.55 ± 0.09
A/Sofia/206/07 (H3N2)	1.32 ± 0.91	0.28 ± 0.16
A/Sofia/413/07 (H3N2)	3.58 ± 0.76	1.03 ± 0.09
A/Sofia/361/05 (H1N1)	1.27 ± 0.11	1.15 ± 0.34
A/Sofia/422/05 (H1N1)	1.29 ± 0.37	1.02 ± 0.16
A/Sofia/522/05 (H1N1)	1.05 ± 0.73	0.93 ± 0.17
A/Sofia/240/05 (H1N1)	2.06 ± 0.39	1.59 ± 0.24
A/Sofia/218/06 (H1N1)	1.16 ± 0.59	1.09 ± 0.29
A/Sofia/418/06 (H1N1)	1.31 ± 0.62	1.01 ± 0.15
A/Sofia/179/06 (H1N1)	1.23 ± 0.31	1.13 ± 0.32
A/Sofia/126/06 (H1N1)	1.27 ± 0.84	0.98 ± 0.69
A/Sofia/490/06 (H1N1)	2.42 ± 1.36	1.31 ± 0.26

(B) Reference strain	IC_{50} Zanamivir (nM) ± SD	IC_{50} Oseltamivir (nM) ± SD
A (H3N2) $_{average}$	2.78 ± 1.33	0.6 ± 0.24
A/Aichi/2/68 (H3N2)	1.25 ± 0.3	0.27 ± 0.12
A $(H1N1)_{average}$	1.45 ± 0.59	1.13 ± 0.29
New Caledonia/20/99 (H1N1)	1.27 ± 0.45	0.51 ± 0.46

SD – standard deviation

types show higher sensitivity to oseltamivir (IC_{50} = 0.6 nM) compared with H1N1 (IC_{50} = 1.13 nM), but lower with respect to zanamivir (IC_{50} = 2.78 nM for H3N2 and IC_{50} = 1.45 nM in H1N1 type), confirming data from previous studies (McKim-Breshkin *et al*., 2003). No trend towards change in IC_{50} with the year/season of isolation (season 04/05, 05/06 and 06/07) was observed as evidenced by the individual values for each of the isolates submitted. Differences in the sensitivity of the N2, N1 and B neuraminidase are described by a number of authors and are determined by phylogenetic differences in their structure. Most sensitive to oseltamivir carboxylate is neuraminidase N2, followed by N1, and the least sensitive is virus type B. Unlike the M2 blockers amantadine and rimantadine, which demonstrate cross-resistance due to the same mutation in all virus types, resistance to neuraminidase inhibitors is drug-specific and type-specific, which will be discussed in details in the analysis of gene segments.

Genetic markers of resistance of H3N2 and H1N1 viruses to the M2 blockers and neuraminidase inhibitors (oseltamivir and zanamivir)

M segment

The length of the M segment is 1027 nucleotides, which encode two possible products rendered by alternative splicing - M1 matrix protein from position 26 to 784 and M2 ion channel (97 amino acids) - from position 26 to 51 and 740 to 1007 (Lamb *et al*., 1981). The nucleotide sequences of the M gene of the 17 isolates were translated into M2 protein (from 9 to 69 AA) and compared to each other and to those of the reference strains for the respective period (Fig. 1).

Among the analyzed M segments, one isolate - A/Sofia/1250/2006 (H3N2) - was found

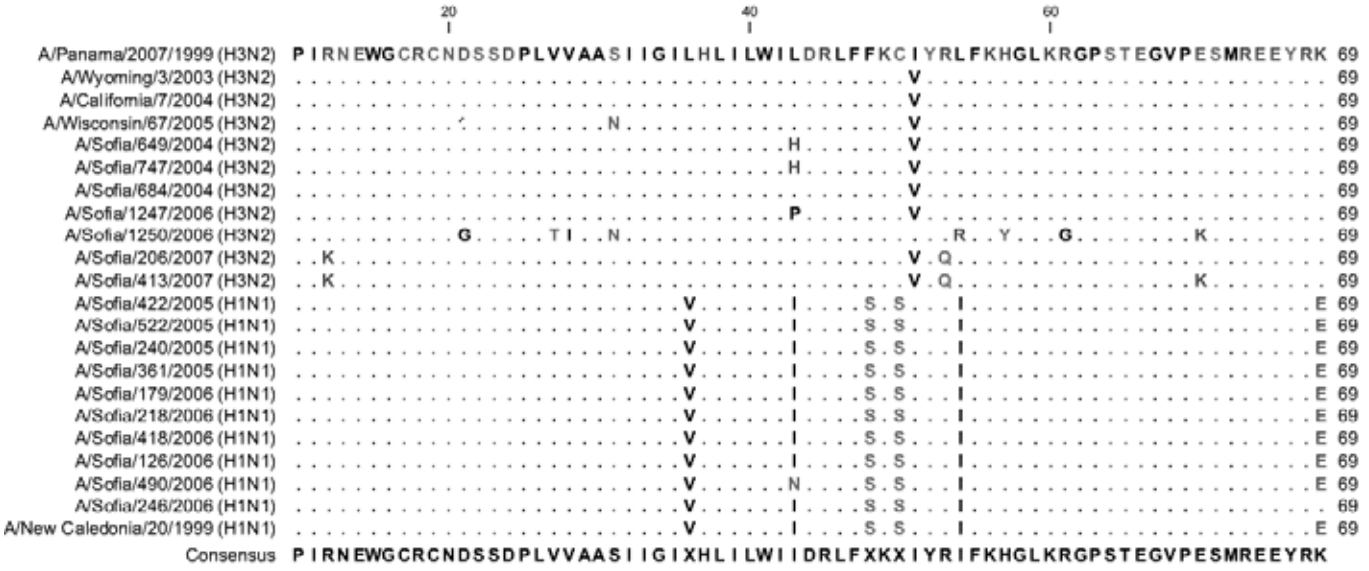

Fig. 1. Overall alignment of M2 protein (9-69) of Influenza A strains (H3N2 and H1N1) from Bulgaria 2004-2007 with reference strains.

bearing the S31N mutation in the transmembrane region of the M2 protein which confers resistance to adamantanes. In over 90% of all described cases of resistance to M2 blockers, this particular amino acid change has been detected. The second most frequent mutation is the V27A substitution (Furuse *et al.,* 2009b). The A/Sofia/1250/2006 (H3N2) isolate above contained a V27T substitution, which is also known to contribute to the development of resistance (Durrant *et al.,* 2015). The same strain exhibited phenotypic resistance in cell cultures as well. It should be noted that this particular isolate was the only one with numerous genetic variations in all sequenced segments. While this could suggest errors and/or improper sequence reading during the computational analysis , the phenotypic analysis confirmed the resistance pattern. None of the other strains contained any mutations known to determine resistance to rimantadine and amantadine.

Phylogenetic analysis of the M gene showed close relationship of the majority of H1N1 isolates with the reference strain A/New Caledonia/20/99 (H1N1). H3N2 viruses showed considerable variation regarding the M segment - some were similar to A/Wyoming/3/2003 and A/California/7/2004, however the A/Sofia/1250/2006 (H3N2) strain with its S31N mutation did not cluster with the resistant A/Wisconsin/67/2005. The reason for this is the additional mutations in both the transmembrane and other regions of the ion channel of this strain.

NA segment

Sequencing and genetic analysis of the NA segment (1413 nt) encoding neuraminidase showed no presence of any known substitutions in translated proteins responsible for resistance to oseltamivir (H275Y for H1N1; E119V, R292K, I222V and N294S for H3N2) or zanamivir (R136Q, I223V). S247N which was found by Sheu *et al*. (2008) and Hurt *et al*. (2011), to contribute to reduced sensitivity to both inhibitors when present in the neuraminidase of seasonal and pandemic H1N1 viruses, was also not registered in our samples. H1N1 viruses A/Sofia/240/2005, A/Sofia/361/2005 and A/Sofia/490/2006, which showed phylogenetic differences to other isolates from Bulgaria regarding the M segment, were also clustered into different groups by their NA gene. Common mutations are indicated on the tree Fig. 2 (A and B). For strain A/Sofia/413 (H3N2), the NA segment was not successfully amplified and sequencing and further analysis for the detection of genetic markers of resistance to NAIs were not possible.

HA segment

The HA segment (1778 nt) encodes the two domains of the hemagglutinin HA1 (987 nt, 329 aminoacids) and HA2 (223 aminoacids). In the current study, a portion of 1080 nt was amplified, which represents a HA1 hemagglutinin monomer. It is known that certain amino acid substitutions in a number of viral proteins affect the pathogenicity and virulence of the virus in the new host. Mutations in M1 (D232N, A41V and T139A), NS (S23N), PB2 (D701N) and HA1 (G218W) are some examples of this effect (Ward, 1995; Ping *et al.,*

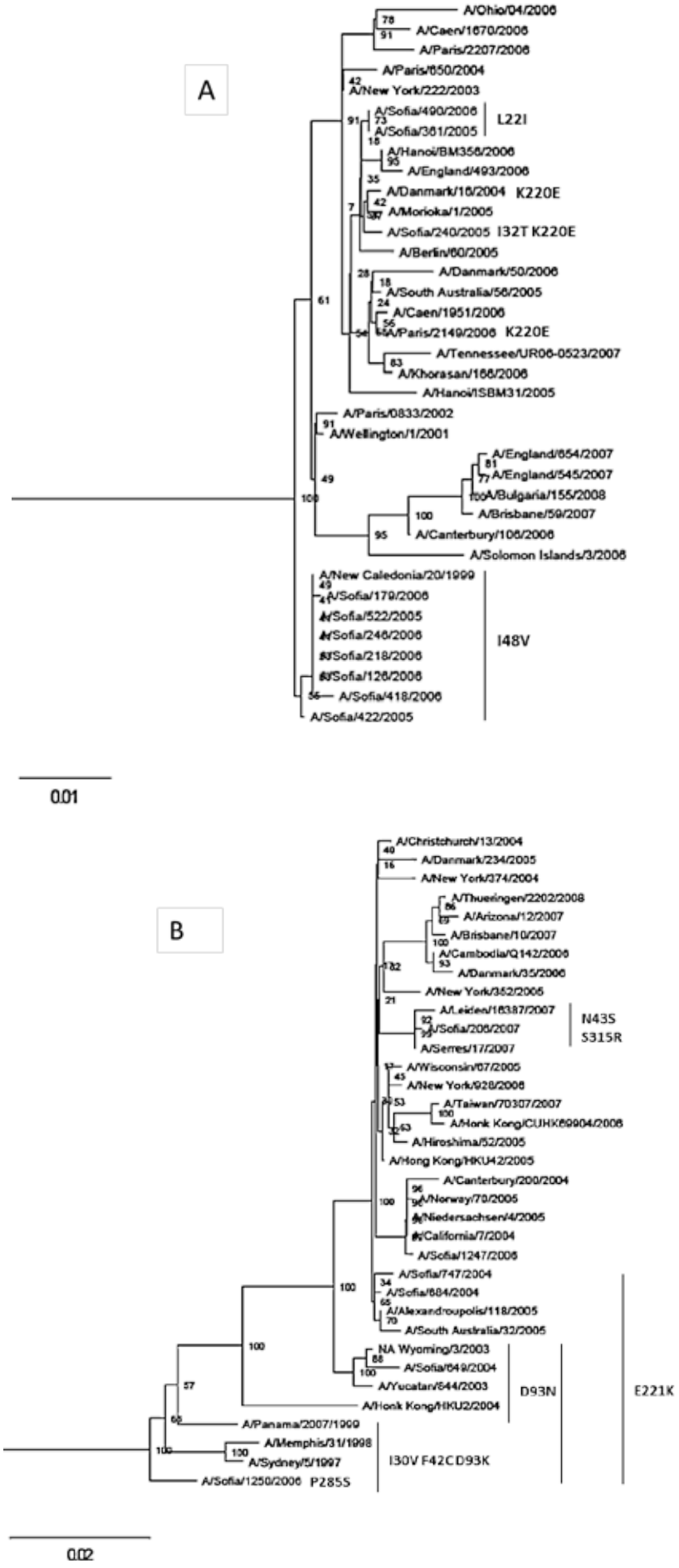

Fig. 2. Phylogenetic relationships between N1 (A) and N2 (B) genes of influenza A viruses isolated in Bulgaria, 2004-2007

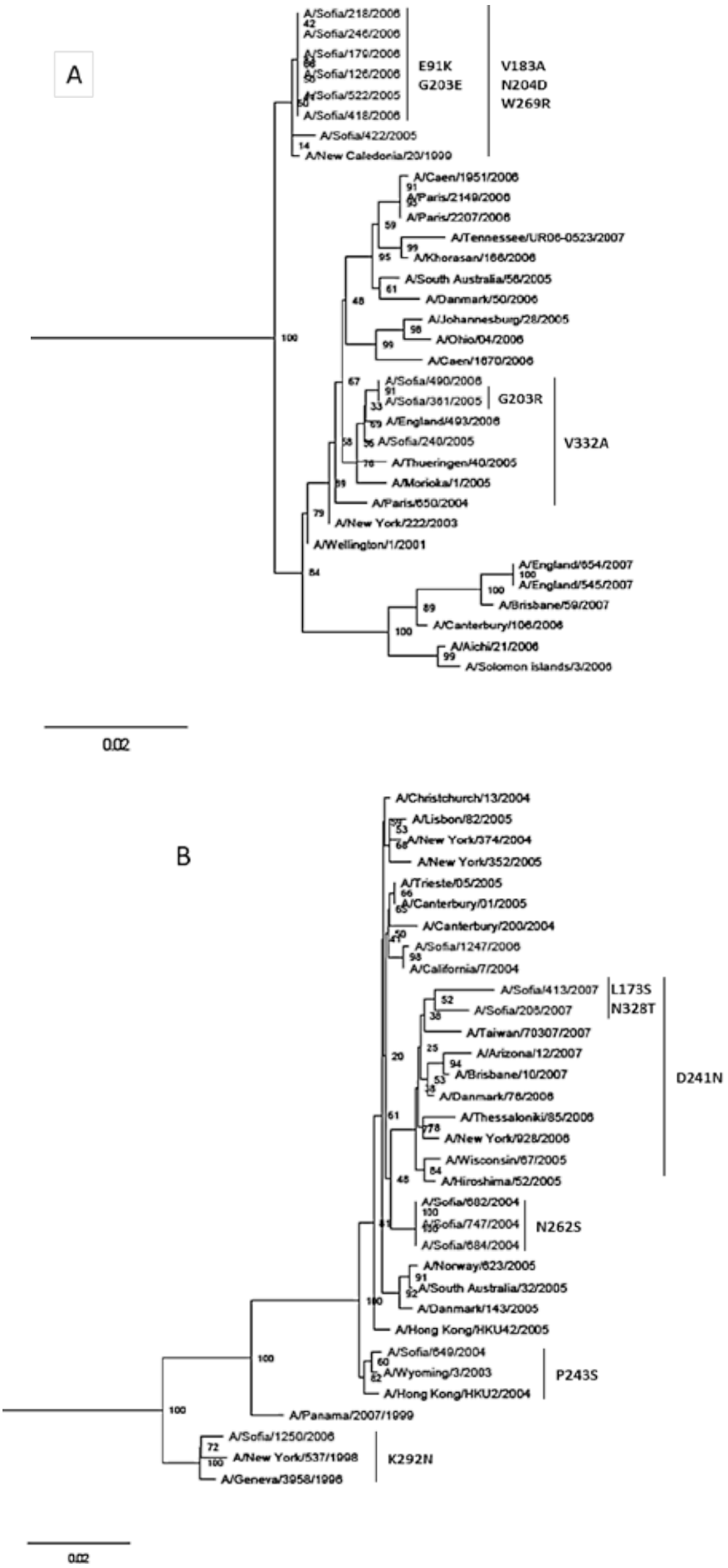

Fig. 3. Phylogenetic relationships between H1 (A) and H3 (B) genes of influenza A viruses isolated in Bulgaria, 2004-2007

2010). In the sequences of HA1 protein analyzed in the present study, the G218W mutation, which is an adaptive mutation that greatly enhances replication of the virus in the bronchial epithelium of mice experimentally infected with recombinant mutant virus type H3N2 (Kaleta *et al.,* 2008), was not detected. HA1 of strains H1N1A/Sofia/240/2005, A/Sofia/361/2005 and A/Sofia/490/2006 were also sequentially remote from A/New Caledonia/20/99 (H1N1) and from other isolates analyzed, which defines these viruses as phylogenetically different. Isolates type H3N2 were divided into various clusters and related to different reference viruses and this diversity is attributable to the higher degree of variability of the H3 and N2 subtype glycoproteins in comparison with H1 and N1 (Fig. 3 A and B).

All gene sequences of successfully amplified segments were deposited in the international database of NCBI [http://www.ncbi.nlm.nih.gov/genomes/FLU/FLU.html] and can be seen under the access numbers from CY103741 to CY103790.

Conclusions

From the 26 clinical isolates of Influenza A virus H3N2 and H1N1 circulating in Bulgaria during 2004-2007, 4 strains were phenotypically resistant to M2 blockers when tested in cell cultures. No resistant variants or ones with reduced susceptibility were present under evaluation of IC_{50} with MUNANA fluorogenic substrate in the presence of oseltamivir and zanamivir. Sequencing data revealed that out of the 16 analysed strains one H3N2 strain was double mutant in the transmembrane region and carried the S31N mutation conferring resistance to M2 blockers. No genetically resistant variants to NAIs were found among the 16 studied viruses.

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