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Neuraminidase Inhibitor Susceptibility of Influenza Viruses Circulating in Bulgaria during the Last Four Consecutive Epidemic Seasons (2011/12 to 2014/15)

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Abstract

Emergence of resistant influenza virus progeny to currently approved antiviral drugs determines the need for antiviral susceptibility monitoring. The aim of the present study is to analyze neuraminidase inhibitor susceptibility of influenza viruses circulating in Bulgaria during the 2011/2012, 2012/2013, 2013/2014 and 2014/2015 flu seasons. A phenotypic fluorescence-based assay with MUNANA substrate was conducted with 93 influenza A(H1N1)pdm09, A(H3N2) and type B isolates. Screening of 352 influenza A(H1N1)pdm09 viruses was carried out using a Real Time RT-PCR discrimination assay for detection of the H275Y oseltamivir resistance point mutation. Phenotypic (IC_{50}) evidence for resistance or reduced susceptibility to neuraminidase inhibitors was not found for any of the influenza A(H1N1)pdm09, A(H3N2) and type B viruses screened. Only one (0,3%) influenza A(H1N1)pdm09 virus carrying the H275Y substitution was detected. Real Time RT-PCR assay could be applied to screen large numbers of clinical A(H1N1)pdm09 influenza virus positive samples for oseltamivir resistance. The present study highlights the importance of continued influenza antiviral susceptibility monitoring in clinical specimens.

Keywords: Influenza virus; Resistance; H275Y mutation.

1. Introduction

Influenza viruses cause annual epidemics with significant morbidity and mortality, accounting for about 600 million illness episodes and 250 000-500 000 deaths globally [1].

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Vaccination remains the most effective means for preventing influenza. Treatment and chemoprophylaxis with antiviral drugs are another option for the control of the disease. There are two classes of antiviral drugs currently approved in many countries – M2 ion channel inhibitors (rimantadine and amantadine) and neuraminidase inhibitors (NAIs) (oseltamivir, zanamivir, peramivir, laninamivir – the last two NAIs are approved in Japan, South Korea and USA). The major problem associated with use of antiviral drugs is the emergence of resistant viral progeny among circulating influenza viruses due to subtype- and inhibitor-specific point mutations in the M2 or neuraminidase (NA) virus genes. Numerous resistance-associated amino acid substitutions have been identified in the different influenza virus types and sub-types that lead to the reduction or complete loss of drug effectiveness [2, 3, 4]. As adamantanes resistance among currently circulating influenza A viruses is widespread (>99%) and having in mind the ineffectiveness of adamantanes against influenza B viruses, these drugs nowadays are not strongly recommended for treatment or prevention of influenza. Antiviral resistance to neuraminidase inhibitors among circulating influenza viruses is currently low, but resistant virus progeny can always emerge and antiviral treatment could enhance the selection of resistant virus strains. Since resistant influenza viruses could rapidly spread and displace virus strains susceptible to antiviral drugs, the World Health Organization (WHO) encourages monitoring antiviral drug susceptibility of circulating influenza viruses. For this purpose, various methods have been used, both - phenotypic (functional) and genotypic [5, 6].

Phenotypic assays (fluorescent, chemiluminescent, etc.) measure the concentration of drug required to inhibit neuraminidase activity of the virus by 50% (ie inhibitory concentration, IC_{50}). These methods evaluate the impact of multiple mutations – either known or novel, leading to the emergence of a resistant phenotype. Phenotypic analysis is widely accepted as the “gold standard” for detecting influenza virus drug resistance, however, the assays are lengthy, labor-intensive; require high titer cultured viruses, expensive equipment and reagents. Genotypic techniques (Real Time RT-PCR, single-nucleotide polymorphism assays, Sanger sequencing, pyrosequencing) can be carried out directly with clinical specimens thus eliminating the need for virus propagation in cell culture. PCR based genotypic assays are fast, highly sensitive, lower cost assays which, however, can identify the presence of a single known molecular markers of NAI resistance. There are real-time RT-PCR assays specially designed for detection of the point mutation in the influenza A(H1N1) neuraminidase gene that results in a tyrosine to histidine substitution at amino acid position 275 (H275Y) and which results in clinical resistance to oseltamivir but not to zanamivir [7, 8, 9]. This is the most commonly detected oseltamivir-resistance mutation in N1-containing viruses.

The purpose of the present study is to determine the frequence of pandemic A(H1N1)pdm09 viruses harbouring the resistance H275Y mutation and characterization of the phenotypic NAI susceptibility of influenza viruses circulating in Bulgaria during the last four consecutive epidemic seasons (2011/12 to 2014/15).

2. Materials and Methods

2.1. Patients and specimens

Clinical specimens (nasal and throat swabs) were collected from patients presenting with symptoms of influenza-like illness (ILI) or acute respiratory illness (ARI) in different regions of the country during the 2011/2012, 2012/2013, 2013/2014 and 2014/2015 winter seasons.

2.2. Real Time RT-PCR for detection and typing/subtyping of influenza viruses

Viral nucleic acids were automatically extracted from the respiratory specimens using a commercial ExiPrep Dx Viral DNA/RNA kit (Bioneer, Korea) according to the manufacturer's instruction. Detection and typing/subtyping of influenza viruses was carried out by the Real Time RT-PCR method with the use of a kit - SuperScript III Platinum ® One-Step Quantitative RT-PCR System (Invitrogen). All specimens were initially tested for the presence of influenza A and B viruses, and those positive for influenza A were subsequently tested for A(H1N1)pdm09 and A(H3N2). Primers, probes and positive controls for influenza A/B viruses were provided by the US CDC-Atlanta. Amplification was performed with a Chromo 4 thermal cycler (Bio-Rad) according to the protocol of CDC [10]. A Ct value <38 was regarded as positive.

2.3. Isolation of influenza viruses

Isolation of influenza viruses from PCR-confirmed clinical specimens was carried out on MDCK (Madin-Darby Canine Kidney) and MDCK-SIAT (over-expressing SA2, 6Gal receptor) cell cultures following standard techniques [11]. Clinical samples were inoculated onto cell cultures and passaged twice to reach sufficient titers for NA inhibition testing. Propagation of influenza viruses in cell cultures was confirmed by implementation of the hemagglutination reaction in culture supernatants.

2.4. Neuraminidase inhibition assay

Neuraminidase activity and drugs susceptibility were determined in the World Health Organization Collaborating Centre (WHO-CC), London by phenotypic fluorescence-based method. The assay is based on biochemical NA inhibition using small synthetic sialic acid conjugate as a substrate–2'-O-(4-methylumbelliferyl)-N-acetylneuraminic acid (MUNANA) resulting in a fluorescent signal upon cleavage by the viral NA surface protein. The assay determines the concentration of NAI required to inhibit NA activity by 50% as compared to a mixture containing virus but no inhibitor (IC_{50} value) [11]. Phenotypic NAI susceptibility of influenza A viruses was evaluated based on the fold-change in IC_{50} values compared to that in susceptible reference virus from the same NA subtype/lineage: "normal inhibition" (<10-fold increase); "reduced inhibition" (10–100-fold increase); "highly reduced inhibition" (>100-fold increase); for influenza B a similar criterion was used but interpretation values were as follows: <5-fold, 5–50-fold and >50-fold increases, respectively [4, 12]. Briefly, isolated viruses were incubated in the presence of a range of different oseltamivir or zanamivir concentrations at 37^0C for 30 min. 2-(4-Methylumbelliferyl)-a-D-N-acetylneuraminic acid (MUNANA; Sigma–Aldrich) was added at a final concentration of 100 μM and the mixture was incubated at 37^0C for 60 min. The reaction was stopped by the addition of 150 μL stop solution. The plates were read using 96 well fluorescence plate reader (Biotek, Synergy 2 Multi-Mode Microplate Reader) at 355 nm (excitation wavelength) and 460 nm (emission wavelength) and the IC_{50} of each virus was determined. The mean IC_{50} value was calculated from two independent experiments in duplicates.

2.5. Real Time RT-PCR for detection of H275Y point mutation

Clinical samples containing A(H1N1)pdm09 viruses that failed to be cultured were tested for the presence of

H275Y mutation using Real Time RT-PCR. In this assay two probes differing in position 823 of the NA gene were simultaneously used: the first probe contains a cytosine at position 823 and was labeled with VIC (H275), while the second probe contains thymine in the same position and was labeled with FAM (275Y). Primer/probe sequences and protocol were kindly provided by Health Protection England (HPE), London. The A(H1N1)pdm09 H275Y assay alloweds discrimination of a single nucleotide difference between oseltamivir sensitive and resistant viruses using TaqMan probes. Influenza viruses A/Denmark/524/2009 (sensitive, H275) and A/Denmark/528/2009 (resistant, 275Y) received from WHO-CC in London were used as positive controls.

3. Results

Clinical specimens (throat and nasal swab) of 3717 patients collected during the last four winter seasons (2011/2012 to 2014/2015), were tested for influenza viruses using Real Time RT-PCR. A total of 1194 (32%) specimens proved positive for influenza viruses, of which 884 (74%) were typed as influenza A and 310 (26%) as influenza B. Of the sub-typed seasonal influenza A viruses, 352 (41%) were influenza A(H1N1)pdm09 and 508 (59%) were influenza A(H3N2) virus. Co-circulation of all seasonal influenza types/ subtypes had been registered during each season, with the exception of A(H1N1)pdm09 virus in 2011/12 season.

During seasons included in the study, 56 influenza A(H1N1)pdm09, 24 A(H3N2) and 13 type B viruses were isolated on cell cultures and submitted to the WHO-CC, London. The number of A(H3N2) isolates was less due to the emergence of A(H3N2) viruses that failed to efficiently agglutinate guinea-pig red blood cells. Frequency of A(H3N2) strains with low hemagglutination activity has increased since 2011/12 season.

3.1. Phenotypic susceptibility of influenza viruses to NAIs

A total of 93 influenza viruses - 56 A(H1N1)pdm09, 24 A(H3N2) and 13 type B were examined in the fluorescent NA inhibition assay. The NAI susceptibility of influenza viruses isolated in Bulgaria is shown in Table 1. All tested viruses displayed normal inhibition by oseltamivir and zanamivir (IC50-fold increase <10 in comparison to susceptible reference virus of the same NA subtype/lineage). Mean IC50 values of oseltamivir and zanamivir among type A viruses - A(H1N1)pdm09 and A(H3N2) were low. Zanamivir had lower mean IC50 value for A(H1N1)pdm09 isolates as compared to values for A(H3N2) viruses. Oseltamivir IC50 values for type B viruses were significantly higher ranging between 6,95 and 36,16 nM.

3.2. Screening of influenza A(H1N1)pdm09 viruses for H275Y point mutation

During the 2012/2013, 2013/2014 and 2014/2015 winter seasons a total of 348 influenza A(H1N1)pdm09 viruses were tested using a Real Time RT-PCR method for detection of single point mutation in the NA virus gene that results in H275Y amino acid substitution. Only one sample from 2013/2014 season contained this mutation (Table 2). In this sample a positive signal was obtained with both a fluorescent dye-labeled probes which meant that the viral population comprised a mixture of sensitive and resistant viruses. The H275Y harbouring virus was detected in a 4 months old immunocompetent child diagnosed with meningitis treated with oseltamivir. The clinical specimen was obtained after the second drug dose. The resistant mutant virus failed to

be cultured and therefore no IC50 value was determined.

Table 1: NAI susceptibility of influenza viruses determined by the fluorescent NA inhibition assay

Viruses	IC_{50} values of NAIs							
	Oseltamivir				Zanamivir			
	No of isolates analyzed	Mean IC_{50}± SD*, (nM)	Min IC_{50} (nM)	Max IC_{50} (nM)	No of isolates analyzed	Mean IC_{50}± SD, (nM)	Min IC_{50} (nM)	Max IC_{50} (nM)
A(H1N1)pdm09	56	1,96±1,42	0,35	7,72	56	0,38±0,24	0,09	1,08
A(H3N2)	24	1,18±0,30	0,81	1,86	24	1,00±0,31	0,54	1,45
Type B	13	21,40±6,68	6,95	36,16	13	1.16±0,43	0,6	2,25

*Standard Deviation

Table 2: Results of Real Time RT-PCR for detection of H275Y point mutation

Seasons	Number of A(H1N1)pdm09 viruses tested	Number of H275Y viruses detected
2011/2012	-	-
2012/2013	60	0
2013/2014	261	1 (0,4%)
2014/2015	31	0
Total	352	1 (0,3%)

4. Discussion

Testing influenza virus susceptibility to available antiviral drugs is important and recommended by WHO because the probability of occurrence and possible widespread of resistant virus strains with easy transmissibility, and capable of displacing sensitive strains, thus becoming dominant. The latter could lead to exhaustion of the potentialities for specific influenza treatment, which at present is limited to the use of neuraminidase inhibitors, i. e. oseltamivir, zanamivir, peramivir and laninamivir.

In the present study, the susceptibility of influenza A(H1N1)pdm09, A(H3N2) and type B viruses to NAIs (oseltamivir and zanamivir) is described and examined using a phenotypic fluorescence-based method and Real Time RT-PCR during the last four consecutive influenza seasons (from 2011/2012 to 2014/2015). Phenotypic (functional) approaches are well-established tools, which allow determination of the effect of different

mutations (both known and novel) on the sensitivity of different influenza virus types and subtypes to NAIs. All 93 newly isolated influenza viruses tested by the fluorescence-based phenotypic method were sensitive to oseltamivir and zanamivir. Our findings are in accordance with surveillance data in other countries [13, 14, 15]. The need to obtain high titer viral cultures in order to apply the procedure, restricts the opportunity for a wider use of phenotypic methods. Furthermore, emergence of influenza A(H3N2) viruses displaying poor hemagglutination activity has been observed since 2011/12 season. The inefficient hemagglutination activity of A(H3N2) strains further hampers the obtaining of virus isolates with sufficiently high titer.

As it is known, H275Y mutation causes high-level clinical resistance to oseltamivir but not to zanamivir in influenza A(H1N1) viruses. The vast majority of oseltamivir–resistant influenza A(H1N1) viruses harbors the mutation. Human A(H1N1) influenza viruses carrying H275Y substitution suddenly emerged in 2007/2008 winter season in the Northern hemisphere and quickly became the dominant circulating strains in many countries [16]. At present, incidence of H275Y mutation among pandemic A(H1N1)pdm09 viruses that completely replaced seasonal influenza viruses of the same subtype is very low. Global surveillance reveals that the frequency of oseltamivir-resistant A(H1N1)pdm09 viruses is about 1% and 3% in 2012/2013 and 2013/2014 influenza seasons, respectively [17, 18]. Although the overall frequency of NAI-resistance markers among A(H1N1)pdm09 viruses is very low, oseltamivir resistance could emerge due to both mutations in the NA gene, or to reassortment within the resistant virus. Therefore, oseltamivir susceptibility monitoring of pandemic viruses is very important especially given the fact that oseltamivir nowadays is the most commonly prescribed antiviral drug against influenza. The extensive screening of 348 A(H1N1)pdm09 viruses by Real Time RT-PCR presented here, identified only one virus that possessed the H275Y mutation. The H275Y virus was detected in a patient receiving oseltamivir treatment, which suggests that the drug was responsible for the selection of this mutant virus. Detection of H275Y mutants in specimens collected during or after drug therapy is also observed in other reports [19]. These findings emphasize the importance of H275Y mutation monitoring during the treatment course with a view to possible change of antiviral drug. The Real Time RT-PCR method used here allowed the rapid resistance testing of a large numbers of clinical samples with a short turnaround time (3-4 h) and at a relatively low cost. The assay is capable detecting 3% H275Y mutants in a mixed influenza A(H1N1)pdm09 virus population.

5. Conclusion

To summarize, frequency of influenza A(H1N1)pdm09 viruses harboring the H275Y mutation was very low. No resistance or reduced susceptibility to oseltamivir and zanamivir was found among isolated influenza A(H1N1)pdm09, A(H3N2) and type B viruses. Continuous surveillance of influenza virus susceptibility to NAIs is necessary in parallel with assessment of clinical effectiveness of each NAI.

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