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Research paper

Antigenic and genetic characterization of influenza viruses circulating in Bulgaria during the 2015/2016 season

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ABSTRACT

Influenza virological surveillance is an essential tool for early detection of novel genetic variants of epidemiologic and clinical significance. The *aim* of this study was to determine the antigenic and molecular characteristics of influenza viruses circulating in Bulgaria during the 2015/2016 season. The season was characterized by dominant circulation of A(H1N1)pdm09 viruses, accounting for 66% of detected influenza viruses, followed by B/Victoria-lineage viruses (24%) and A(H3N2) viruses (10%). All sequenced influenza A(H1N1)pdm09, A(H3N2) and B/Victoria-lineage viruses belonged to the 6B.1, 3C.2a and 1A genetic groups, respectively. Amino acid analysis of 57 A(H1N1)pdm09 isolates revealed the presence of 16 changes in hemagglutinin (HA) compared to the vaccine virus, five of which occurred in four antigenic sites, together with 16 changes in neuraminidase (NA) and a number of substitutions in proteins MP, NP, NS and PB2. Despite the many amino acid substitutions, A(H1N1)pdm09 viruses remained antigenically closely related to A/California/7/2009 vaccine virus. Bulgarian A(H3N2) strains (subclade 3C.2a) showed changes at 11 HA positions four of which were located in antigenic sites A and B, together with 6 positions in NA, compared to the subclade 3C.3a vaccine virus. They contained unique HA1 substitutions N171K, S312R and HA2 substitutions I77V and G155E compared to Bulgarian 3C.2a viruses of the previous season. All 20 B/Victoria-lineage viruses sequenced harboured two substitutions in the antigenic 120-loop region of HA, and 5 changes in NA, compared to the B/Brisbane/60/2008 vaccine virus. The results of this study reaffirm the continuous genetic variability of circulating seasonal influenza viruses and the need for continued systematic antigenic and molecular surveillance.

1. Introduction

Of the many viral respiratory infections in humans, influenza has the greatest clinical and epidemiologic importance. Each year 600 million influenza cases occur worldwide, with 3 million having severe disease and 250,000–500,000 resulting in death (WHO, 2014). Periodically, at intervals of 10 to 40 years since 1890, influenza type A viruses cause pandemics resulting from the emergence of a radically new subtype/variant influenza virus against which the human population has little or no preexisting immunity — a process called antigenic shift. The last influenza pandemic in 2009/2010 was caused by A(H1N1)pdm09 virus containing a complex combination of gene segments from swine, avian and human influenza viruses (Neumann et al., 2009). This virus completely replaced former seasonal A(H1N1) viruses and continues to circulate worldwide as a seasonal influenza virus together with A(H3N2) and type B viruses.

Influenza vaccines were developed to reduce the substantial influenza-associated morbidity and mortality but their effectiveness declines over time due to emerging genetic and associated antigenic differences between vaccine and circulating viruses. Influenza viruses are one of the most variable and rapidly evolving human viruses because of their high mutation rate, rapid replication, segmented genome (which facilitates the reassortment of genes between different influenza viruses) and zoonotic events for type A viruses. Evolution of influenza viruses proceeds by continuous replacement of genetic groups with new ones leading to increases in the antigenic distances from the current vaccine viruses.

The influenza virus surface glycoproteins, HA and NA are subjected to the strongest pressure by the host immune system resulting in a gradual accumulation of amino acid changes and altered antigenicity. This process, known as antigenic drift, enables circulating viruses to evade host immune responses leading to recurrent seasonal epidemics and reduction of vaccine effectiveness, necessitating updates of vaccine composition. Distinct antigenic sites in A/H1 (Sa, Sb, Ca 1/2, Cb), A/*H3* (A-E) and in type B viruses (120 loop, 150 loop, 160 loop, 190 helix) located on the globular head of the HA1 subunit are targets of neutralizing antibodies (Wiley and Skehel, 1987, Wilson and Cox, 1990). Amino acid substitutions within epitopes and the attachment of *N*-glycans to the globular head region of HA, shielding antigenic epitopes, can reduce

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the recognition of virus by neutralizing antibodies and thus resulting in the escape from pre-existing immunity (Skehel et al., 1984).

The World Health Organization (WHO) encourages National Influenza Centers (NICs) to conduct ongoing influenza virologic surveillance, to monitor spread of viruses and their continuous evolution to inform twice yearly recommendations on vaccine composition and assessing other risks associated with circulating influenza viruses. Combining data from phylogenetic and molecular analyses of influenza viruses is essential to detect virus variants that have undergone antigenic drift, variants with enhanced virulence or variants reduced sensitivity to antivirals. Such combined genetic, antigenic and phenotypic analyses provide improvements in the process of vaccine virus selection and inform patient treatment regimens. The *aim* of the present study was to analyse influenza virus circulation in Bulgaria during the 2015/2016 season and determine the genetic and antigenic characteristics of the detected viruses related to amino acid changes at antigenic, *N*-glycosylation and functionally significant sites of HA and NA.

2. Material and methods

2.1. Study population and specimen collection

From October 2015 to May 2016, patients, who were ambulatory treated or hospitalized either for influenza like illness (ILI) or acute respiratory illness (ARI), were enrolled in different regions of the country. Combined nasal and pharyngeal specimens from the enrolled patients were collected with the help of commercial polyester collection swabs (Deltalab, Spain). Swabs were stored at 4 °C for up to 72 h before shipment to the NIC. Specimens were processed immediately or stored at −80 °C before testing.

2.2. Extraction of nucleic acids and real time RT-PCR

Virus RNAs were extracted automatically from the respiratory specimens using a commercial ExiPrep Dx Viral DNA/RNA kit (Bioneer, Korea) in accordance with the manufacturer's instructions. Detection and typing/subtyping of influenza viruses were carried out by a Real Time RT-PCR method with the use of a kit — SuperScript III Platinum ® One-Step qRT-PCR System (Invitrogen, USA). All samples were first tested for the presence of influenza A and B viruses. Those that were positive for influenza A were subsequently screened for A(H1N1)pdm09 and A(H3N2). The genetic lineage of detected influenza B viruses was also determined by Real Time RT-PCR. Primers, probes and positive controls were provided by WHO-CC, Atlanta. Amplification was performed with a Chromo 4 thermal cycler (Bio-Rad) in accordance with the protocol of WHO-CC, Atlanta (reverse transcription at 50 °C for 30 min, Taq inhibitor inactivation at 95 °C for 2 min, followed by 45 cycles of denaturation at 95 °C for 15 s and annealing/amplification at 55 °C for 30 s). A C_t value <38 was regarded as positive.

2.3. Virus isolation and antigenic characterization

All Real Time RT-PCR positive clinical specimens with C_t values <28 were inoculated onto Madin Darby canine kidney (MDCK) and MDCK-SIAT1 (that express increased levels of α2,6-sialyltransferase, Matrosovich et al., 2003) cell cultures. Cultures were incubated at 35 °C in a 5% CO_2 atmosphere and observed daily for 7 days for evidence of cytopathology. The presence of virus in culture was confirmed by haemagglutination assay following standard protocols using a 1% suspension of guinea pig red blood cells. Antigenic characterization of isolates was performed by the haemagglutination inhibition (HI) assay, in accordance with the WHO Manual, using vaccine viruses/antigens and their corresponding antisera provided by the WHO-CCs in London and Atlanta (WHO, 2011). More detailed HI assay of representative Bulgarian influenza isolates with panels of reference viruses and antisera were performed at the WHO-CCs in London and Atlanta.

2.4. Genetic characterization

Full-genome or HA and NA gene sequences of influenza viruses detected in Bulgaria during the 2015/2016 season were determined at WHO-CC, London. Full-genome sequencing was carried out at WHO-CC, Atlanta. Sequences have been deposited in the Global Initiative on Sharing All Influenza Data (GISAID) database (http://www.gisaid.org) with sequences of all but three viruses being derived from virus isolates that were characterized antigenically. For phylogenetic analyses all sequences, including those of reference viruses whose genetic group identities were known and viruses representing different countries of Europe during the 2015/2016 season, were retrieved from GISAID. Phylogenies for HA and NA genes were constructed using the maximum likelihood method within Molecular Evolutionary Genetics Analysis software (MEGA, version 6.0; http://www.megasoftware.net/). Best nucleotide substitution models were used: the Hasegawa-Kishino-Yano model with a gamma distribution (HKY + G) for HA; and the Tamura 3-parameter model with gamma distribution (T92 + G) for NA. Reliability of the tree topology was assessed by bootstrap analysis with 1000 replications. HA amino acid numbering was applied after removing the signal peptide. Amino acid identity was calculated using flusurver (http://flusurver.bii.a-star.edu.sg).

2.5. Prediction of N-glycosylation motifs

Putative N-glycosylation motifs in the HA and NA were predicted using the NetNGlyc 1.0 web Server (http://www.cbs.dtu.dk/services/NetNGlyc) to identify sequence motifs N–X–S/T (sequon), where X can be any amino acid except proline.

2.6. Antiviral susceptibility surveillance

Screening of A(H1N1)pdm09 viruses for the presence of point mutations conferring H275Y oseltamivir resistance was carried out using a Real Time RT-PCR assay that allowed discrimination of a single nucleotide difference between oseltamivir sensitive and resistant viruses. Two TaqMan probes differing in position 823 of the NA gene were used simultaneously: the first probe contained a cytosine at position 823 and was labeled with VIC (H275), while the second probe contained thymine in the same position and was labeled with FAM (275Y). Primer/probe sequences and protocol were kindly provided by Public Health England (formerly Health Protection Agency, England), London. Reference influenza viruses A/Denmark/524/2009 (sensitive, H275) and A/Denmark/528/2009 (resistant, 275Y) provided by WHO-CC, London were used as positive controls. A phenotypic analysis (MUNANA test) of influenza virus susceptibility to neuraminidase inhibitors (oseltamivir and zanamivir) was performed at WHO-CC, London.

2.7. Statistics

Age and gender of patients, the clinical features of their illness and the incidence of each virus were compared using the Chi square or Fisher's exact tests for categorical variables. p values of <0.05 were considered statistically significant.

3. Results

Bulgaria is a country with a total population of approximately 7.2 million people and an ARI surveillance system is used to monitor influenza. It comprises a national sentinel network of general practitioners and pediatricians working in 208 health care facilities situated in all 28 major cities — regional centers covering 5.3% of the population in the country. Primary care physicians report the weekly number of