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Phylogenetic analysis of the neuraminidase gene of pandemic H1N1 influenza A virus circulating in the South American region

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ABSTRACT

Molecular characterization of circulating influenza A viruses (IAV) in all regions of the world is essential to detect mutations potentially involved in increased virulence, anti-viral resistance and immune escape. In order to gain insight into these matters, a phylogenetic analysis of the neuraminidase (NA) gene of 146 pandemic H1N1 (H1N1pdm) influenza A virus strains isolated in Argentina, Brazil, Chile, Paraguay, Peru and Uruguay from 2009 to 2013 was performed. Comparison of vaccine strain A/California/7/2009 included in the influenza vaccine recommended for the Southern hemisphere from 2010 through 2013 influenza seasons and strains isolated in South America revealed several amino acid substitutions. Mapping of these substitutions revealed that most of them are located at the surface of the protein and do not interfere with the active site. 3.4% of the strains enrolled in these studies carried the H275Y substitution that confers resistance to oseltamivir. Strains isolated in South America differ from vaccine in two predicted B-cell epitope regions present at positions 102–103 and 351–352 of the NA protein. Moreover, vaccine and strains isolated in Paraguay differ also in an epitope present at position 229. These differences among strains isolated in South America and vaccine strain suggests that these epitopes may not be present in strains isolated in this region. A potential new N-linked glycosylation site was observed in the NA protein of an H1N1pdm IAV strain isolated in Brazil. The results of these studies revealed several genetic and antigenic differences in the NA of H1N1pdm IAV among vaccine and strains circulating in South America. All these findings contribute to our understanding of the course of genetic and antigenic evolution of H1N1pdm IAV populations circulating in the South American region and, consequently, contribute to the study and selection of future and more appropriate vaccines and anti-viral drugs.
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1. Introduction

Influenza A virus (IAV) is a member of the family *Orthomyxoviridae* and contains eight segments of a single-stranded RNA genome with negative polarity (Neumann et al., 2004). IAV causes 300,000–500,000 deaths worldwide each year, and in pandemic years, this number can increase to 1 million (in 1957–1958) or as high as 50 million, as was seen in 1918–1919 (Nguyen-Van-Tam and Hampson, 2003). IAV exhibits a rapid evolution and complex molecular dynamics patterns due to its wide host range, high substitutions rates and rapid replication (Holmes, 2010). Hemagglutinin (HA) and neuraminidase (NA) are the two envelope glycoproteins that are responsible for attaching the virions to the host receptors, determining pathogenicity, and releasing newly produced viral particles (Li et al., 2011). Amino acid substitutions on these glycoproteins can modify virus replication and impact over the potential spread in the human population (Pizzorno et al., 2012; Abed et al., 2006). The NA is also playing an important role as a target of the single calls of available anti-influenza drugs, e.g. NA inhibitors.

The first influenza pandemic of this century was declared in April of 2009, with the emergence of a novel H1N1 IAV strain (H1N1pdm) in Mexico and the USA (CDC, 2009; WHO, 2009a,b,c). This virus rapidly spread to the South American region, where it was

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first detected in May 2009 (Baker et al., 2009). This was in the typical winter season for influenza transmission for countries from temperate regions of the Southern Hemisphere, where a full epidemic of H1N1pdm IAV was observed and the pandemic strain became the predominant circulating influenza virus, replacing seasonal strains in many countries (WHO, 2009b).

Understanding the evolution of H1N1pdm strains within the South American region is essential for studying global diversification and anti-viral resistance of H1N1pdm IAV strains circulating in this region of the world, as well as determining the genetic and antigenic relationships among South American H1N1pdm IAV strains and vaccine strains included in the influenza vaccine recommended for the Southern Hemisphere.

In order to study the genetic and antigenic variability of this H1N1 lineage in the South American region, we performed a phylogenetic analysis of the NA gene from 146 H1N1pdm IAV strains isolated in this region from 2009 to 2013.

2. Material and methods

2.1. Human samples

Nasal swabs from 44 Uruguayan patients with clinical symptoms of influenza wereavailable at the Asociación Española Primera de Socorros Mutuos Hospital and National Influenza Center, Ministerio de Salud Pública, in Montevideo, Uruguay. All ethical procedures were approved by Dirección de la Asociación Española Primera de Socorros Mutuos Hospital and Ministerio de Salud Pública, Uruguay. World Health Organization's ethical norms were observed.

2.2. Real-time PCR

In order to detect and assign the IAV strains isolated from Uruguayan patients to H1N1pdm lineage, a real-time RT-PCR assay was performed using a specific rRT-PCR reagent kit, provided by the Center for Disease Control and Prevention (CDC), Atlanta, GA, USA, according to instructions given by the providers.

2.3. RNA extraction and RT-PCR amplification

RNA extraction and PCR amplification of the NA gene were done as previously described (Goñi et al., 2012). PCR products were analyzed by gel electrophoresis on a 1.2% agarose gel and then purified using a QIAquick Gel Extraction Kit (QUIAGEN) according to the manufacturer's instructions prior to sequencing.

2.4. Sequencing reactions

The sequence reaction was carried out using a BigDye DNA Sequencing Kit on a 3730 XL DNA Sequencer Apparatus, both from PerkinElmer at Institut Pasteur-Montevideo facility. The NA sequences obtained from Uruguayan patients were deposited in the EMBL Database under accession numbers HE804101 through HE804131 and HG764555 through HG764574.

2.5. Neuraminidase sequences

All 146 NA sequences from H1N1pdm IAV strains isolated in South America were obtained from the Influenza Virus Resource at the National Center for Biotechnology Information (Bao et al., 2008).

2.6. Sequence alignment

The NA sequences were aligned using software from the MEGA 5.05 program (Tamura et al., 2011).

2.7. Evolutionary model analysis

Once aligned, the Datamonkey webserver (Delport et al., 2010) was used to identify the optimal evolutionary model that best fitted our sequence data. Akaike information criteria (AIC) and the log of the likelihood (ln L) revealed that the HKY model was the best fit to the data (AIC of 2843.47 and ln L of 0.093207).

2.8. Maximum-likelihood phylogenetic tree analysis

Maximum-likelihood phylogenetic trees were constructed under the HKY model using software from the PhyML program (Guindon et al., 2005). As a measure of the robustness of each node, we used an approximate likelihood ratio test (aLRT), which demonstrates that the branch studied provides a significant likelihood against the null hypothesis that involves collapsing that branch of the phylogenetic tree but leaving the rest of the tree topology identical (Anisimova and Gascuel, 2006). The aLRT value was calculated using a Shimodaira–Hasegawa-like procedure (SH-like) (Shimodaira, 2003; Shimodaira and Hasegawa, 2001).

2.9. Mapping of amino acid substitutions in a 3D structure of NA

Amino acid substitutions present in the H1N1pdm IAV strains were mapped with respect to vaccine strain A/California/7/2009, included in the influenza vaccine for the 2009 through 2013 seasons of the Southern Hemisphere. A 3D structure model of the NA protein from 2009 H1N1 IAV was obtained from Maurer-Stroh et al. (2009) from the Bioinformatic Institute, A*STAR's Biomedical Sciences Institutes, Singapore.

2.10. Epitope predictions

In order to identify linear B-cell epitopes (i.e. contiguous amino acids in an antigen, here NA) that are recognized by the antibodies of the human immune system, we used BepiPred approach (Abdussamad and Aris-Brosou, 2011; Larsen et al., 2006). This machine learning method is based on the combination of a hidden Markov model with a propensity scale method (Larsen et al., 2006). For each amino acid position in an alignment, a prediction score is calculated, and site assignment to a linear B-cell epitope is made when the score is above a certain threshold. Different thresholds give different sensitivities (Sn) and specificities (Sp). We have used BepiPred online server (available at: http://www.cbs.dtu.dk/services/BepiPred) with a default threshold of 0.35 that correspond to Sn = 0.49 and Sp = 0.75 (Abdussamad and Aris-Brosou, 2011).

2.11. Prediction of N-linked glycosylation sites

Potential N-linked glycosylation sites were predicted using the NetNGlyc 1.0 Server (Gupta et al., 2004). The NetNglyc server predicts N-glycosylation sites in proteins using artificial neural networks that examine the sequence context of Asn-Xaa-Ser/Thr sequences. A threshold value of >0.5 average potential score was set to predict glycosylated sites.

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