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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.

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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0168-1702/

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.

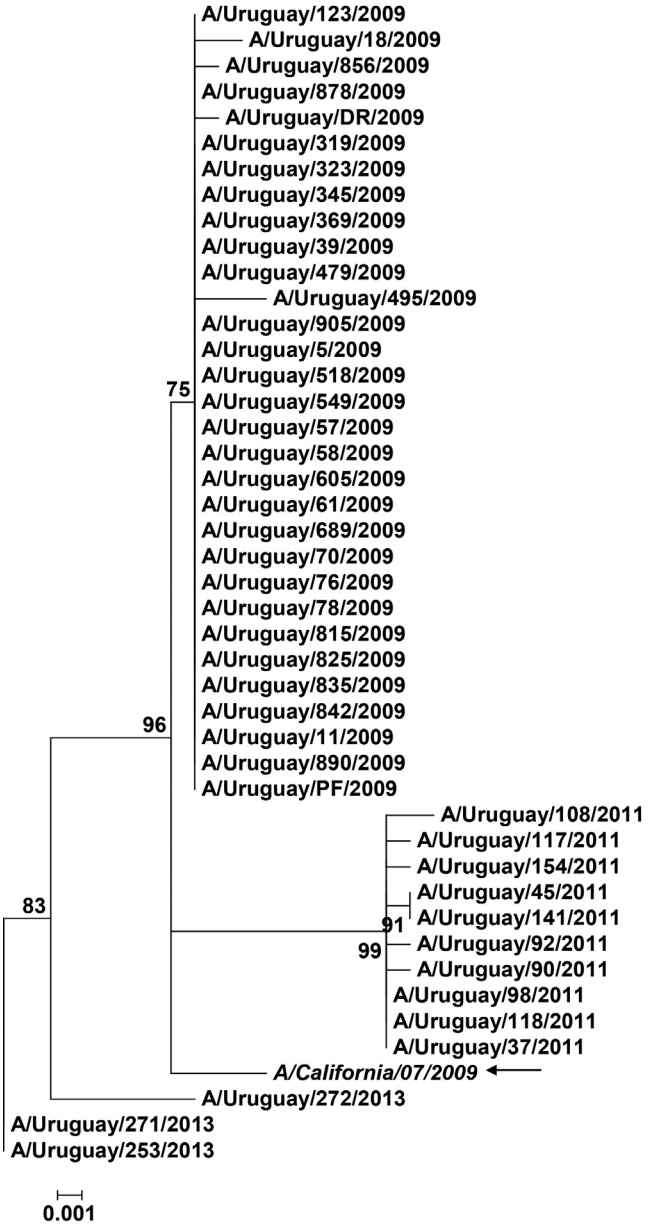

Fig. 1. Maximum-likelihood phylogenetic tree analysis of NA genes of H1N1pdm IAV strains circulating Uruguay. Strains in the tree are shown by name. Numbers at the branches show aLRT values. The bar at the bottom of the tree denotes distance. The 2010–2013 vaccine strain A/California/7/2009 is indicated by an arrow.

3. Results

3.1. Phylogenetic analysis of the NA gene from H1N1pdm IAV strains isolated in South America

In order to gain insight into the degree of genetic variability of H1N1pdm IAV isolated from Uruguayan patients we first study 44 NA sequences from H1N1pdm lineage isolated in Uruguay in 2009, 2011 and 2013. The region analyzed comprises amino acids 56–401, and includes the region encoding for the NA drug-binding pocket. Once aligned, phylogenetic trees were created using the maximum-likelihood method under the HKY model. The robustness of the nodes was assessed by an approximate likelihood ratio test (aLRT). The results of these studies are shown in Fig. 1.

All strains isolated in 2009 are clustered together; strains isolated in other years are cluster separately (see Fig. 1). These clusters were supported by high aLRT values. This result reveals that strains isolated in 2009 have a more close genetic relation among themselves and a more distant genetic relation with 2011 and 2013 strains. H1N1 vaccine strain A/California/7/2009, recommended for the Southern hemisphere seasons 2010–2013 (WHO, 2009c), has a distant genetic relation with strains isolated in Uruguay in all these seasons (Fig. 1). These studies also show the co-circulation of at least two different genetic lineages in Uruguay in 2013.

In order to study if the different clusters observed in the Uruguayan strains were related to amino acid substitutions in the NA sequences, we translated in silico all the sequences involved in the previous analysis and aligned them to vaccine strain A/California/7/2009 using software from the MEGA 5.05 program (Tamura et al., 2011). The results of these studies are shown in Table 1. All Uruguayan strains isolated in 2011 share S299A and N369S substitutions (Table 1).

To observe if these substitution can be found in H1N1pdm strains isolated in other South American countries, all these sequences were aligned with all available and comparable NA sequences from strains isolated in Argentina, Brazil, Chile, Peru and Paraguay, for a total of 146 NA sequences (see Supplementary Material Fig. S1). These studies revealed that NA substitution S299A and N369S was only observed in strains isolated in Uruguay in 2011 (Supplementary Material Fig. S1).

Supplementary Fig. S1 related to this article can be found, in the online version, at http://dx.doi.org/10.1016/j.virusres.2014.11.008.

Recent studies have shown that a remarkable heterogeneity was accumulated in the NA gene worldwide from 2009, with the presence of at least five different clusters worldwide (Piralla et al., 2013).

In order to gain insight into the diversification of the NA gene in the South American region, the sequences of the strains isolated in Uruguay were aligned with corresponding sequences of NA strains isolated elsewhere, for whom their assignment to a particular NA cluster was previously established. A phylogenetic tree analysis was carried out (Supplementary Material Fig. S2) and the result of this analysis revealed that all strains isolated in Uruguay in 2011 belong to NA cluster II (Piralla et al., 2013).

Supplementary Fig. S2 related to this article can be found, in the online version, at http://dx.doi.org/10.1016/j.virusres.2014.11.008.

Moreover, NA sequences from strains isolated in South America differ from the NA sequence of vaccine strain A/California/7/2009 in other different amino acid substitutions (Supplementary Material Fig. S1). All the South American NA sequences included in this study differed from the NA sequence of the vaccine strain by three amino acid changes, namely V106I, N248D and Y351F (Supplementary Material Fig. S1). These substitutions were observed to appear early and spread worldwide during the global pandemic (Graham et al., 2009). Substitution N248D was also recently found to be involved in the low-pH stability of H1N1pdm NA, which might have contributed to the rapid worldwide spread and adaptation to humans of these strains during the early stage of the 2009 pandemic (Takahashi et al., 2013). Substitution A232V was only observed in strains isolated in Paraguay. Substitutions at that position were also previously observed in an Italian patient with severe disease (A232T) (Piralla et al., 2013). Substitutions N369K has only been observed in strains isolated in Brazil in 2012 and in Uruguay in 2013, while substitution V241Ihas only been observed in Brazil. Substitution H275Y, which confers resistance to Oseltamivir (Abed et al., 2006), were found in 3.4% of the strains enrolled in these studies (Supplementary Material Fig. S1). Strains carrying this substitution have been previously observed in strains isolated in Brazil and Argentina (Barrero et al.,

Table 1
Mapping of amino acid substitutions in NA protein of H1N1pdm IAV strains isolated in Uruguay.

Strain	Amino acid position[a]														
	71	107	200	241	248	278	296	299	321	339	351	369	382	386	401
A/California/07/2009	S	V	N	V	N	E	W	S	I	S	Y	N	G	N	G
A/Uruguay/PF/2009	–	L	–	–	D	–	–	–	–	–	F	–	–	–	–
A/Uruguay/DR/2009	–	L	–	–	D	–	–	–	–	–	F	–	–	–	–
A/Uruguay/905/2009	–	L	–	–	D	–	–	–	–	–	F	–	–	–	–
A/Uruguay/890/2009	–	L	–	–	D	–	–	–	–	–	F	–	–	–	–
A/Uruguay/878/2009	–	L	–	–	D	–	–	–	–	–	F	–	–	–	–
A/Uruguay/856/2009	–	L	–	–	D	K	–	–	–	–	F	–	–	–	–
A/Uruguay/842/2009	–	L	–	–	D	–	–	–	–	–	F	–	–	–	–
A/Uruguay/835/2009	–	L	–	–	D	–	–	–	–	–	F	–	–	–	–
A/Uruguay/825/2009	–	L	–	–	D	–	–	–	–	–	F	–	–	–	–
A/Uruguay/815/2009	–	L	–	–	D	–	–	–	–	–	F	–	–	–	–
A/Uruguay/78/2009	–	L	–	–	D	–	–	–	–	–	F	–	–	–	–
A/Uruguay/76/2009	–	L	–	–	D	–	–	–	–	–	F	–	–	–	–
A/Uruguay/70/2009	–	L	–	–	D	–	–	–	–	–	F	–	–	–	–
A/Uruguay/689/2009	–	L	–	–	D	–	–	–	–	–	F	–	–	–	–
A/Uruguay/61/2009	–	L	–	–	D	–	–	–	–	–	F	–	–	–	–
A/Uruguay/605/2009	–	L	–	–	D	–	–	–	–	–	F	–	–	–	–
A/Uruguay/58/2009	–	L	–	–	D	–	–	–	–	–	F	–	–	–	–
A/Uruguay/57/2009	–	L	–	–	D	–	–	–	–	–	F	–	–	–	–
A/Uruguay/549/2009	–	L	–	–	D	–	–	–	–	–	F	–	–	–	–
A/Uruguay/518/2009	–	L	–	–	D	–	–	–	–	–	F	–	–	–	–
A/Uruguay/5/2009	–	L	–	–	D	–	–	–	–	–	F	–	–	–	–
A/Uruguay/495/2009	–	L	–	–	D	–	G	–	–	–	F	–	–	–	–
A/Uruguay/479/2009	–	L	–	–	D	–	–	–	–	–	F	–	–	–	–
A/Uruguay/39/2009	–	L	–	–	D	–	–	–	–	–	F	–	–	–	–
A/Uruguay/369/2009	–	L	–	–	D	–	–	–	–	–	F	–	–	–	–
A/Uruguay/345/2009	–	L	–	–	D	–	–	–	–	–	F	–	–	–	–
A/Uruguay/323/2009	–	L	–	–	D	–	–	–	–	–	F	–	–	–	–
A/Uruguay/319/2009	–	L	–	–	D	–	–	–	–	–	F	–	–	–	–
A/Uruguay/18/2009	–	L	–	–	D	–	–	–	–	–	F	–	–	–	R
A/Uruguay/123/2009	–	L	–	–	D	–	–	–	–	–	F	–	–	–	–
A/Uruguay/11/2009	–	L	–	–	D	–	–	–	–	–	F	–	–	–	–
A/Uruguay/90/2011	I	I	–	–	D	–	–	A	–	–	F	S	–	–	–
A/Uruguay/98/2011	–	I	–	–	D	–	–	A	–	–	F	S	–	–	–
A/Uruguay/117/2011	–	I	–	–	D	–	–	A	–	–	F	S	–	–	–
A/Uruguay/118/2011	–	I	–	–	D	–	–	A	–	–	F	S	–	–	–
A/Uruguay/141/2011	–	I	–	–	D	–	–	A	–	–	F	S	–	–	–
A/Uruguay/154/2011	–	I	–	–	D	–	–	A	–	–	F	S	R	–	–
A/Uruguay/45/2011	–	I	–	–	D	–	–	A	–	–	F	S	–	–	–
A/Uruguay/92/2011	–	I	–	–	D	–	–	A	–	–	F	S	–	–	–
A/Uruguay/37/2011	–	I	–	–	D	–	–	A	–	–	F	S	–	–	–
A/Uruguay/108/2011	–	I	–	–	D	–	–	A	–	–	F	S	–	D	–
A/Uruguay/253/2013	–	–	S	I	D	–	–	–	V	–	F	K	–	–	–
A/Uruguay/271/2013	–	–	S	I	D	–	–	–	V	–	F	K	–	–	–
A/Uruguay/272/2013	–	–	S	I	D	–	–	–	–	L	F	K	–	–	–

[a] Identity to vaccine strain A/California/07/2009 is indicated by a dash.

2011). Interestingly, we have found substitution H275Y in strains isolated in Brazil in 2012 and 2013 (Supplementary Material Fig. S1).

3.2. Mapping of amino acid substitutions found in the NA protein of H1N1pdm IAV isolated in South America in a 3D NA protein model

In order to observe if the amino acids substitutions found in the NA genes of the 146 H1N1pdm IAV isolated in South America were associated to previously identified antigenic regions or the active site of the NA protein (being the latter the binding cavity of NA inhibitors drugs), we mapped these substitutions in an homology-based 3D structure model of the NA protein of H1N1pdm strains (Maurer-Stroh et al., 2009).

The results of these studies are shown in Fig. 2.

Substitution N248D maps at one of the antibody binding sites, as previously described (Goñi et al., 2009). Most of the substitutions, with the exception of H275Y, are located at the surface of the protein and do not interfere with the active site of the NA protein, in agreement with previous results (Goñi et al., 2009, 2012; Maurer-Stroh et al., 2009). Importantly, these substitutions do not appear to be close enough to affect the drug binding pocket (i.e. residues within 3 Å of the drug molecule that binds to the active site) (see Fig. 2).

3.3. Characterization of South American H1N1pdm N1 epitopes

In order to observe if amino acid substitutions found in the NA of the 146 H1N1pdm IAV circulating in South America may affect the antigenic properties of the NA protein, we set out to predict B-cell epitopes in the NA proteins from strains isolated in this region of the world and included in these studies. Fig. 3 shows the B-cell epitopes predicted for 2010–2013 Southern hemisphere vaccine strain (A/California/7/2009) and different South American isolates from different South American countries bearing different amino acid substitutions, with each peak above the 0.35 threshold indicating the presence of an epitope. Specifically, while most of the substitutions found in the NA proteins studied do not show significant antigenic variation among vaccine and South American isolates, strains isolated in this region differ from vaccine in two epitope regions present at positions 102–103 and 351–352

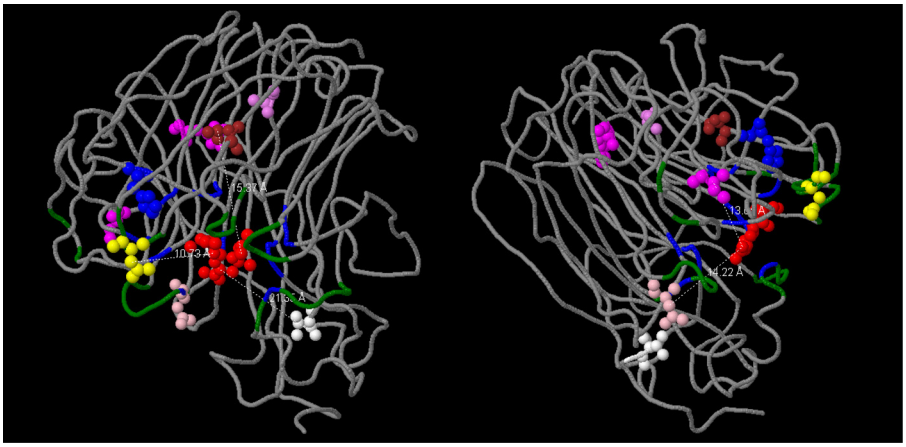

Fig. 2. Mapping of naturally occurring amino acid substitutions in South American H1N1pdm NA protein. The 3D structure model of the NA protein from H1N1pdm IAV shown in the figure was obtained by Maurer-Stroh et al. (2009) (Bioinformatic Institute, A*STAR's Biomedical Sciences Institute, Singapore). The structure is shown complexed with oseltamivir. Antibodies binding sites are shown in green in the backbone of the structure. Amino acids involved in the NA active site are shown in blue (Takahashi et al., 2013). Substitutions found at positions 106, 232, 241, 248, 275, 299, 351 and 369 in South American strains are shown in white, violet, brown, yellow, blue, magenta, fuchsia and pink, respectively, in space filling representation. oseltamivir atoms are shown in red. Dotted lines show distances in Å. Two views of the molecule, rotated on the *y*-axis are shown. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

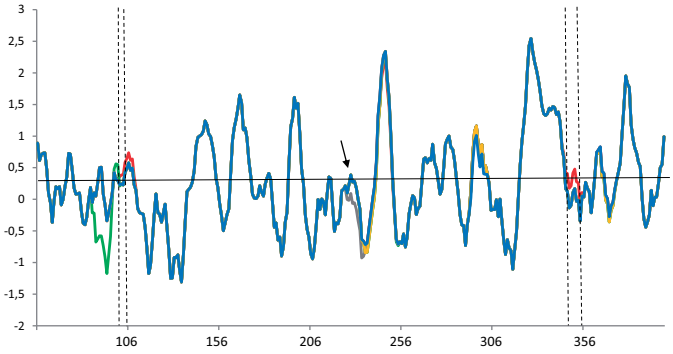

Fig. 3. Predicted B-epitopes of the NA protein. Comparison of 2010–2013 N1 vaccine (A/California/7/2009) vs. NA proteins of H1N1pdm IAV isolated in South America is shown. The BepiPred score is represented as a function of the amino acid position along the protein. Scores above the 0.35 threshold is shown by a horizontal line. Comparison of scores found for vaccine strain (red), A/Argentina/07–09/2009 (brown), A/Bahia/124/2009 (pale green), A/Para/110264/2012 (green), A/Paraguay/138HCl/2009 (gray), A/Rio Grande do Sul/678/2012 (yellow), and A/Uruguay/118/2011 (blue) are shown. Epitope differences at positions 102–103 and 351–352 between vaccine and South American strains are indicated by dotted vertical lines. Difference among vaccine and strains isolated in Paraguay at position 229 is indicated by an arrow. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

of the NA protein (see Fig. 3). Moreover, vaccine and strains isolated in Paraguay differ also in an epitope present at position 229 (Fig. 3).

3.4. Characterization of potential N-linked glycosylation sites

Glycosylation can potentially affect the antigenic properties of IAV (Li et al., 1993). For these reasons we analyzed the possible changes in the N-linked glycosylation sites of the NA proteins of the 146 H1N1pdm IAV isolated in South America. The results of these studies are shown in Table 2.

All NA sequences analyzed sheared seven conserved potential N-glycosylation sites by comparison with vaccine strain A/California/7/2009. Interestingly, a potential new glycosylation site was observed at position 143 of the NA protein of strains isolated in Minas Gerais, Brazil (A/Minas Gerais/21/2009). Moreover, N-glycosylation sites present in vaccine strain A/California/7/2009 at positions 12 and 32 of the NA protein where absent in strains isolated in Uruguay and Brazil (A/Uruguay/90/2011 and A/Santa Catarina/223/2012, respectively) (see Table 2).

4. Discussion

The antigenic variability of IAV is the basis for recurring epidemics each year (De Jong et al., 2000). IAV evades host immunity by accumulation of substitutions in the major surface glycoproteins, HA and NA, or as a result of genetic reassortment of

Table 2
Potential N-linked glycosylation sites in H1N1pdm IAV strains circulating in the South American region ($n = 146$).

NA position	Amino acid sequence	Potential N-Gly score[a]	Jury agreement[b]	Potential N-Gly result[c]	Sequence name
58[d]	NNT	0.6548	9/9	++	All
63[d]	NQT	0.7114	9/9	++	All
68[d]	NIS	0.7508	9/9	+++	All except A/Uruguay/90/2011
88[d]	NSS	0.7526	9/9	+++	All except A/Santa Catarina/223/2012
143	NHS	0.6017	8/9	+	A/Minas Gerais/21/2009
146[d]	NGT	0.6722	9/9	++	All
235[d]	NGS	0.7286	9/9	++	All
386[d]	NFS	0.2780	9/9	−−−	All

[a] The potential scores shown are the averaged output of nine neural networks.
[b] Jury agreement indicates how many of the nine networks support the prediction.
[c] N-Glyc results are indicated (+) for a potential N-glycosylation site >0.5 threshold, (++) for a potential N-glycosylation site >0.5 threshold and jury agreement of 9/9 and (+++) for a potential N-glycosylation site >0.75 and jury agreement of 9/9. (−−−) indicates a potential N-glycosylation score <0.5 threshold.
[d] Potential N-linked glycosylation sites in NA sequences of vaccine strain A/California/07/2009.

segments from different IAV strains coinfecting the same cell (Nicholson et al., 2003). For that reasons, the characterization of the epidemic variants in all regions of the world is extremely important for improving the influenza vaccine formulation for both Northern and Southern hemispheres, since the closer the vaccine strain is to the dominant variant, the more effective the vaccine (Nelson et al., 2008). Moreover, molecular analysis of the NA gene is essential to address the emergence of viruses resistant to NA-inhibitors (i.e. oseltamivir and zanamivir) (Piralla et al., 2013). In contrary to Northern hemisphere countries, the emergence of H1N1pdm IAV overlapped with the Southern hemisphere annual peak of respiratory virus infections (Barrero et al., 2011), where a full epidemic of H1N1pdm influenza was observed and the pandemic strain quickly became the predominant circulating influenza virus strain, replacing the seasonal strains (WHO, 2009b). To understand the evolution of pandemic IAV of this origin over time is also important to predict its future impact on human populations (Nelson et al., 2009).

In this study, we performed a phylogenetic analysis of the neuraminidase (NA) gene from 146 H1N1pdm IAV strains isolated in the South American region from 2009 to 2013. Co-circulation of different genetic lineages has been found in 2013 strains isolated in Uruguay (Fig. 1), revealing a significant genetic distance among the NA gene of these strains and the NA gene of vaccine strain A/California/7/2009, recommended for the Southern hemisphere seasons 2010–2013 (Fig. 1). Interestingly, two amino acid substitutions, S299A and N369S, have been found in Uruguayan strains with respect to vaccine strain (Table 1). Unexpectedly, this substitution has not been found in all other strains isolated in other Latin American countries and included in these studies (strains isolated in Argentina, Brazil, Chile, Peru and Paraguay). Phylogenetic analysis revealed that these strains can be assigned to NA cluster II strains (Supplementary Material Fig. S2). Strains belonging to that cluster have been very recently isolated in Italy during the same year (Piralla et al., 2013).

Mapping of substitutions found in the NA protein region studied of the 146 H1N1pdm strains isolated in the South American and enrolled in these studies revealed that 3.4% of the strains carried the H275Y substitution conferring resistance to Oseltamivir (Barrero et al., 2011; Abed et al., 2006) (see Supplementary Material Fig. S1). The global circulation of Oseltamivir resistant H1N1pdm strains is approximately 1% (Hurt et al., 2012; Fry and Gubareva, 2012). Interestingly, all strains enrolled in these studies and carrying the H275Y substitution were isolated in Brazil in 2012 and 2013 (Brazilian states of Parana, Rio Grande do Sul, Rio de Janeiro and Santa Catarina; see Supplementary Material Fig. S1).

As recently suggested (Souza et al., 2013), the southern states of Brazil (such as Rio Grande do Sul, Santa Catarina and Parana) generally have a temperate climate and a more marked seasonality of influenza than other Brazilian states. As a result of that, the mortality rate during the pandemic of 2009 for Brazil as a whole was 1.1% (1.1 case per 100,000 inhabitants), whereas the ratio for the southern region was 3.0% (Ministerio da Saúde, 2010). This may explain, at least in part, that high influenza circulation in these states could lead to a more rapid emergence of resistant virus that may possess the ability for sustained transmission, in agreement with the results found in this work (Souza et al., 2013).

No N294S substitution, also conferring resistance to Oseltamivir, was observed in the strains enrolled in these studies (Supplementary Material Fig. S1). Recently, a novel NA substitution, I223R, was identified in an A/H1N1 virus showing cross-resistance to the NA inhibitors oseltamivir, zanamivir and peramivir (van der Vries et al., 2012). This substitution was not found in the 146 strains isolated in South America and enrolled in these studies. Nevertheless, a substitution I223K was observed in a strain isolated in Chile (A/Santiago/21579/2009). Whether this substitution confers resistance to neuraminidase inhibitors is unknown (See also Supplementary Material Fig. S1). Substitution N369K, only been observed in strains isolated in Brazil, was recently reported to cause change in protein stability in patients also carrying H275Y substitution (Hurt et al., 2012). In addition, substitution S247N, recently associated with increased IC_{50} values for oseltamivir and zanamivir (Hurt et al., 2011), were not detected in these studies. Of note, substitution V116A, reported in H5N1 influenza strains with increased IC_{50} values for zanamivir and oseltamivir (Hurt et al., 2007), was also not observed in these studies. Nevertheless, a substitution V116I was observed in a Brazilian strain isolated in Rio de Janeiro in 2012 (A/Rio/Grande/Do/Sul/617/2012) (Supplementary Material Fig. S1).

These results of these studies reveal that most of the substitutions found in the NA protein region studied are located at the surface of the protein and do not interfere with the active site, in agreement with previous and recent results (Goñi et al., 2012; Maurer-Stroh et al., 2009).

In order to observe if the sites where substitutions were found can affect the antigenic properties of the NA protein, we predicted the B-cell epitopes of 2010–2013 Southern Hemisphere vaccine strain (A/California/7/2009) and H1N1pdm strains isolated in South America and enrolled in these studies (Fig. 3). Interestingly, strains isolated in this region differ from vaccine strain in two predicted 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 (Fig. 3). These results suggest the possibility that these epitopes may not be present in these strains (see Fig. 3). Nevertheless, substitutions S299A and N369S, only found in strains isolated in Uruguay, do not seem to affect the antigenicity of the protein (Fig. 3).

A potential new N-linked glycosylation site was observed in the NA protein of an H1N1pdm IAV strain isolated in Brazil (see Table 2). Recent reports suggest a potential immune escape mechanism in order to explain this fact (Sun et al., 2011).

Continuous surveillance is needed to monitor the emergence of novel influenza viruses with reduced susceptibility to the neuraminidase inhibitors or amino acid substitutions that may facilitate the emergence of circulating multi drug resistant influenza viruses (van der Vries et al., 2011). Toward that goal, characterization of H1N1pdm IAV strains isolated in the South American region are much needed, as well as establishing antigenic and genetic relations among that strains and vaccine strains included in the influenza vaccine recommended for the Southern Hemisphere.

Clinical studies have shown that licensed seasonal vaccines contain immunogenic amounts of NA, but the contribution of this immunity to vaccine efficacy is currently unknown (Eichelberger and Wan, 2014). Until now, the basic premise in influenza vaccination has been that adequate delivery of dominant influenza virus glycoprotein antigens, HA and NA, to vaccine recipients is sufficient for adequate vaccine efficacy (Yang et al., 2013). Nevertheless, very recent studies revealed that deliberate reduction of HA and NA expression in influenza virus leads to a protective live vaccine in mice (Yang et al., 2013). More studies will be needed to address these important issues as well as the roll of NA in influenza vaccines.

5. Conclusions

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 in the NA gene. Interestingly, substitution S299A, hallmark of NA cluster II lineage, has only been found in Uruguay. Nevertheless, this substitution does not seem to affect the antigenicity of the protein. Mapping of substitutions found in the NA protein of the

146 H1N1pdm strains isolated in South American and enrolled in these studies revealed that 3.4% of strains carried the H275Y substitution. Most of the substitutions found in the NA protein of these strains are located at the surface of the protein and do not interfere with the active site, in agreement with previous and recent results (Goñi et al., 2012; Maurer-Stroh et al., 2009). Strains isolated in South America differ from vaccine strain in two predicted B-cell epitope regions present at positions 102–103 and 351–352 of the NA protein. Besides, strains isolated in Paraguay differ from vaccine strain in an epitope present at position 229 of the NA protein. These findings suggest the possibility 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 the need of continuous surveillance of the emergence of novel influenza viruses in all regions of the world.

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