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Influenza A H1N1pdm 2009 Virus in Paraguay: Nucleotide Point Mutations in Hemagglutinin and Neuraminidase Genes are not Associated with Drug Resistance

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Abstract: Influenza virus is associated with upper respiratory tract infections. The fourth influenza pandemic was declared in 2009. The aim of this study was to determine the genetic variability of the 2009 H1N1 pandemic virus circulating in Paraguay. Nasal swabs were collected from 181 patients with flu symptoms managed at the Hospital of the Medical School in Asunción, Paraguay, between August and October 2009. Virus detection was carried out by real-time reverse transcription-polymerase chain reaction, followed by sequencing of the hemagglutinin and neuraminidase genes, and phylogenetic analysis. H1N1pdm09 was detected in 14.9% (27/181) of the suspected cases. Analysis of 13 samples showed that these viruses the clustered in a single genetic group. Neither the mutation related to exacerbation of disease (D239G in hemagglutinin) nor that related to antiviral resistance (H275Y in neuraminidase), both detected in neighboring countries, were found. This genetic analysis of H1N1pdm09 will help to understand the spread of the disease.

Keywords: Hemagglutinin, pandemic influenza H1N1 2009, neuraminidase, respiratory disease, swine flu.

INTRODUCTION

Influenza A viruses, which infect a wide variety of mammals and birds, belong to the *Orthomyxoviridae* family. These enveloped viruses have a genome composed of eight segments of single-stranded, negative-sense RNA. Two antigenic proteins are anchored in the virus envelope: Hemagglutinin (HA) and Neuraminidase (NA). These proteins, which exhibit higher antigenic variability than the other virus proteins, are the main determinants of pathogenicity [1]. Eighteen HA subtypes (H1-H18), and eleven NA subtypes (N1-N11) have been found [2], and several combinations of these proteins due to genetic reassortment can be detected in nature [1].

The fourth influenza pandemic (H1N1) was declared on June 11 (2009) [3], after the cases of 1918 (H1N1), 1957 (H2N2), and 1968 (H3N2) [1]. It is thought that the new 2009 H1N1 pandemic virus (henceforth, H1N1pdm09) has emerged through at least four reassortment and transmission events among swine, avian and human H1N1 lineages, probably in Asia and North America [4]. Particularly, the HA segment of H1N1pdm09 was originated from the American swine lineage, whereas the NA segment derives from the European swine lineage [5, 6].

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In South America, information about H1N1pdm09 diversity is scarce. However, circulation of antiviral resistant strains has been reported in countries neighboring Paraguay. In Argentina, a study carried out in 2009 isolated six oseltamivir-resistant strains containing the NA H275Y mutation, from 262 cases with mild to severe forms of the disease; none of the mutations in HA or NA were related to fatal cases [7]. In Brazil, another study carried out in 2009 found one oseltamivir-resistant strain out of 305 cases from a patient with scarce medical record [8].

In Paraguay, the first confirmed case was declared on May 19, 2009 and by the end of that year, 8,284 H1N1pdm09 suspected cases were reported, including 987 confirmed cases and 46 deaths (May to December 2009) [9]. Approximately 50% of the suspected cases were reported in July 2009, 60% of whom were female, 30% ranged from 20 to 39 years of age, and 60% were inhabitants of the Central Department and Asunción. The severity of the disease, and mortality related to H1N1pdm09 infection were associated with the presence of pre-existing medical conditions, such as obesity, pregnancy, diabetes mellitus, and cardiovascular disease, as well as with being male, older than 60 years, and not vaccinated against seasonal influenza virus during 2009 [10].

The aim of this study was to determine the genetic variability of the H1N1pdm09 viruses circulating in the Central Department of Paraguay during the pandemic phase. Nasal swabs were obtained from 181 children and adults with clinical symptoms of influenza-like illness or severe

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acute respiratory infection (suspected cases), without data of antiviral treatment, managed at the Hospital of the Medical School, National University of Asunción (UNA), Paraguay, between August and October 2009 [10]. This Hospital provides medical care to low-income families residing in the Central Department, which has a population of around two million (~25% of the Paraguayan population). Samples were collected by the hospital personnel using a synthetic swab (Dacron) and stored in 2 mL of viral transport medium (0.5% BSA, 100 U/mL penicillin, 100 U/mL gentamicin, diluted in PBS). Samples were collected within two-five days of the appearance of symptoms. These samples were maintained at 4ºC for up to three days and then sent to the Molecular Biology Laboratory of the *Instituto de Investigaciones en Ciencias de la Salud*, UNA (IICS-UNA). The samples were fractionated and stored at -80ºC until analysis. Samples were codified to maintain confidentiality of patients.

Total RNA was extracted from 200 µL of sample with the AxyPrep Body Fluid Viral DNA/RNA Miniprep Kit (Axygen Biosciences, CA, USA), following the manufacturer's recommendations, and then eluted in 60 µL of nuclease-free water.

Real time reverse transcription-polymerase chain reaction (RT-PCR) analysis was carried out following standard procedures [11]. Briefly, the reaction contained 5 µL of total RNA, 0.5 µL of primers and TaqMan probes (Influenza A 2009 H1N1 Assay Sets v1.0, Applied Biosystems, CA, USA), 0.5 µL of one-step enzymes (AgPath-ID One-Step RT-PCR Kit, Ambion, CA, USA), and 12.5 µL of 2X buffer in a final volume of 25 µL. Each sample was analyzed in four different tubes, depending on the amplified gene target: matrix (InfA, 106-bp), swine nucleoprotein (swInfA, 195-bp), swine HA type 1 (swH1, 116-bp), and human RNase P (internal control, 65-bp). A 7500 Real-Time PCR System (Applied Biosystems) was used. The mixture was incubated at 50ºC for 30 min, followed by incubation at 95ºC for 2 min, and 40 cycles of amplification, each consisting of incubations at 95ºC for 15 sec and 55ºC for 30 sec. A sample was considered positive if both the InfA and the respective subtype (swInfA or swH1) reaction curves crossed the threshold (Ct) line within the first 40 cycles [11].

The HA and NA genes were amplified by RT-PCR. The reaction for cDNA synthesis contained 10 µL of total RNA, 200 ng of random primers (Invitrogen, USA), 0.25 mM dNTPs mix (Invitrogen), 80 U of RNAseOUT (Invitrogen), 200 U of M-MLV Reverse Transcriptase (Promega, USA), and 8 µL 5X buffer (250 mM Tris-HCl pH 8.3, 375 mM KCl, 15 mM $MgCl_2$, 50 mM DTT), in a final volume of 40 µL. The mixture was incubated at 25ºC for 10 min, followed by incubation at 37ºC for 4 h, and a final incubation of 5 min at 85ºC.

Three overlapping fragments of the HA and NA genes were amplified by PCR: HA (635-bp, 864-bp, and 813-bp), and NA (584-bp, 681-bp, and 473-bp). The amplification reaction contained 5 µL of cDNA, 0.15 µM of each primer (primers used are listed in Table **1**), 0.25 mM dNTPs mix (Invitrogen), 1.5 U of DFS-Taq DNA polymerase (Bioron, Germany), and 5 µL 10X buffer II (500 mM KCl, 100 mM Tris-HCl pH 8.8, 0.1% Tween-20, 15 mM $MgCl_2$), in a final volume of 50 µL. The cycling conditions were as follows: denaturation at 95ºC for 2 min, and 45 cycles of amplification, each consisting of denaturation at 95ºC for 30 sec, primer annealing at 45ºC for 30 sec, and primer extension at 72ºC for 5 min, followed by a final extension at 72ºC for 7 min. The PCR products were analyzed in 1.8% agarose gels, stained with ethidium bromide, and visualized under UV light. Standard procedures to avoid any type of contamination with amplicons were performed in different rooms.

The HA and NA PCR products were purified from 1.8% agarose gels, using the AxyPrep DNA Gel Extraction Kit (Axygen Biosciences), and directly sequenced (both strands, 3X coverage each) in an ABI PRISM 310 DNA analyzer (Applied Biosystems). Nucleotide sequences were manually edited with BioEdit v.7.0.5 [12], and aligned with

Table 1. Primers used for complete amplification of the HA and NA coding sequences.

Primer	Gene	Position*	Sense	Sequence (5' → 3')
ha 635	HA	-40 to -22	Forward	ATACGACTAGCAAAAGCAGGGG
ha 635'	HA	574 to 595	Reverse	GATGGTGAATGCCCCATAGCAC
ha 864	HA	514 to 532	Forward	GGAAATTCATACCCAAAGC
ha 864'	HA	1,356 to 1,377	Reverse	CACATTTGAATCGTGGTAGTCC
ha 813	HA	938 to 962	Forward	ATCCGATCACAATTGGAAAATGTCC
ha 813'	HA	1,727 to 1,750	Reverse	GTGTCAGTAGAAACAAGGGTGTTT
na 584	NA	-20 to -6	Forward	AGCAAAAGCAGGAGT
na 584'	NA	545 to 564	Reverse	GATGCCATCATGACAAGCAC
na 681	NA	466 to 485	Forward	CGAACCCTAATGAGCTGTCC
na 681'	NA	1,126 to 1,146	Reverse	CCCAGTCCATCCGTTCGGATC
na 473	NA	966 to 988	Forward	CGGAGACAATCCACGCCCTAATG
na 473'	NA	1,424 to 1,438	Reverse	AGTAGAAACAAGGAG

*Nucleotide positions are based on the H1N1pdm 09 vaccine strain A/California/07/2009.

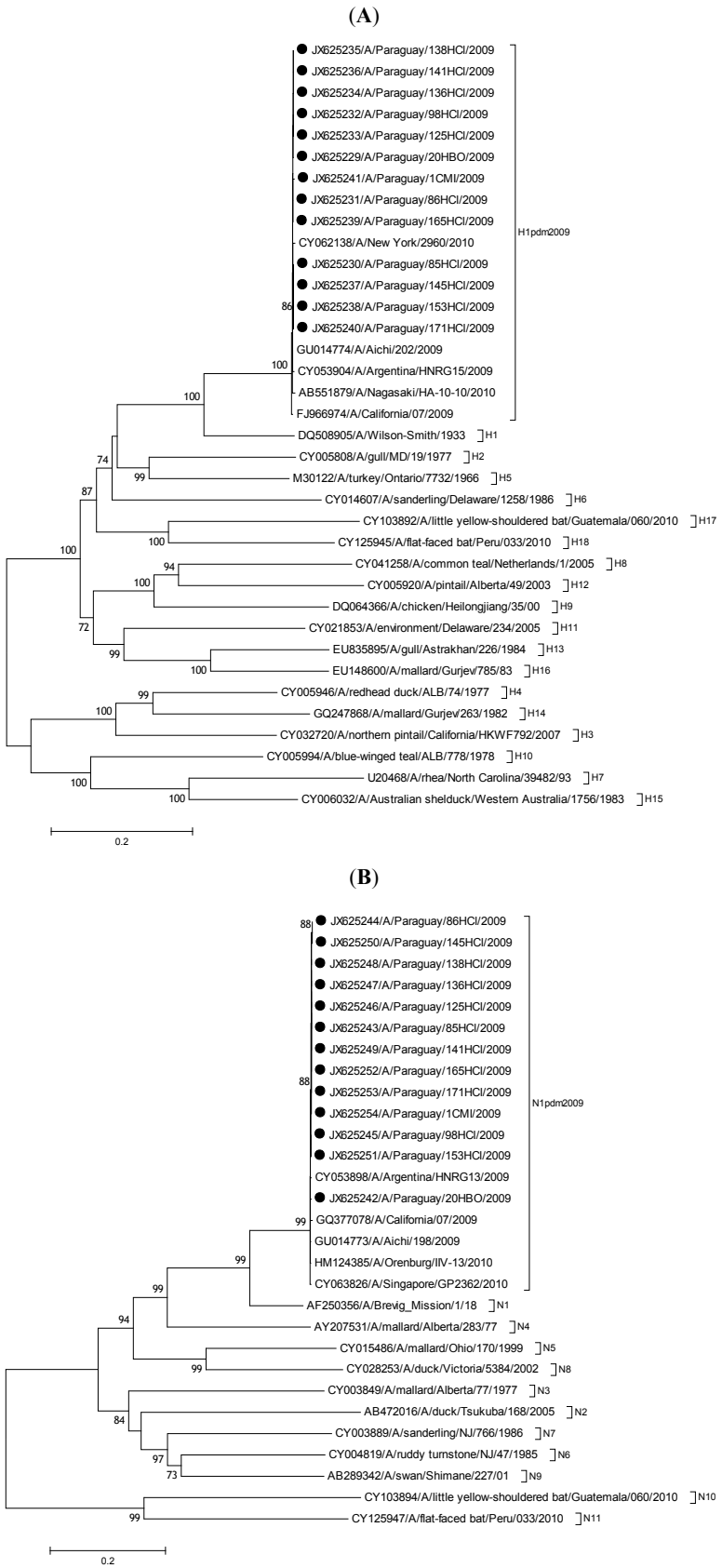

Fig. (1). Phylogenetic trees corresponding to complete coding nucleotide sequences for the (**A**) HA and (**B**) NA genes, respectively, each comparing all known subtypes, four worldwide H1N1pdm09 strains (randomly selected), 13 Paraguayan isolates, and the vaccine strain A/California/07/2009. Each sequence is indicated with its GenBank accession number, host of origin (omitted for human strains), geographical origin, name of isolate, and year of isolation. Bootstrap values greater than 70% are shown at branch nodes. Paraguayan isolates are shown with a filled circle. Branch distances are indicated by a scale bar (0.02 nt substitution per site) at the bottom of each tree.

CLUSTAL W [13]. The HA and NA sequences obtained were compared with 2,038 HA and 1,273 NA coding sequences of H1N1pdm09 reported worldwide during 2009-2010 [14]. Phylogenetic relationships were reconstructed by the neighbor-joining method with Kimura's two-parameter as the model of nucleotide substitution and bootstrap analysis of 1,000 replicates, as incorporated in MEGA v5 [15]. The sequences selected from subtypes H1-H18 and N1-N11 were obtained from GenBank. The nucleotide sequences for the HA and NA genes obtained in this study were deposited in GenBank, under the following accession numbers: HA (JX625229– JX625241), and NA (JX625242–JX625254).

Written informed consent was obtained from parents or guardians of all participating individuals. This study was approved by the Ethics Committee of the IICS-UNA, under code M13/10.

Genomic RNA of H1N1pdm09 was detected by real-time RT-PCR in 14.9% (27/181) of the suspected cases. However, it was possible to amplify and sequence the DNA of the HA and NA genes of 13 samples, with Ct values <30. High percentage of genetic identity, ranging from 99.7% to 100% for the HA gene, and 99.5% to 100% for the NA gene, was observed among the viruses analyzed. This is in agreement with our previous report showing that the HA and NA gene sequences have a high percentage of identity with the H1N1pdm09 viruses reported worldwide during 2009-2010 [14]. The high percentages of nucleotide identity are in agreement with the single clustering of Paraguayan samples and those from the 2009-2010 worldwide circulation, as shown in the phylogenetic trees (Fig. **1**). The high percentages of HA and NA nucleotide identity are also in agreement with published serological data, which show that the new pandemic viruses are antigenically very similar [6].

When the deduced HA and NA amino acid sequences of Paraguayan H1N1pdm09 viruses were compared with the early vaccine strain A/California/07/2009 (isolated in California, USA, and sequenced and published by the CDC on April 27) [16], several amino acid mutations were found. Two amino acid substitutions, S220T (100%), and E391K (30.8%), were observed in the HA protein of the Paraguayan viruses (Table **2**). The amino acid S220 is localized within the HA antigenic site designated Ca (site C, subsite a) as well as at the receptor binding domain (RBD); thus, S220T could affect the transmissibility and infectivity of H1N1 in humans. The substitution E391K found in this study has been previously identified as part of a highly conserved epitope in the 1918 H1N1 virus, with a possible role in membrane fusion [17]. In the HA protein, we did not find the S101N mutation, which is thought to be an adaptation to the human host, or D239E/G, which has been associated with severe clinical outcomes [18] and exacerbate forms of respiratory disease [19], or N387H, localized at a glycosylation site that could potentially affect the antigenic properties of influenza viruses [20].

The NA protein of the Paraguayan viruses showed two amino acid substitutions, V106I (100%) and N248D (100%). This is in agreement with the observation that both mutations were present in respiratory samples at increasing numbers through the early pandemic phase (April to December 2009) [21]. V106I was reported in H1N1 cases of the 20th century (in 1918 [pandemic] and 1977), whereas N248D was also reported in 1977. Since the residue at position 248 is located at the drug target domain (DTD), a mutation at this point could potentially affect the sensitivity of antiviral drugs. We did not find the NA D199N mutation associated with an increase in oseltamivir resistance published in both seasonal and H5N1 virus strains [22], or the I223R mutation associated with resistance to oseltamivir, zanamivir and peramivir [23], or H275Y, located at the DTD and related to oseltamivir resistance especially in immunocompromised or severely ill people [24]. In countries neighboring Paraguay, such as Argentina and Brazil, however, some studies reported the circulation of oseltamivir-resistant strains [7, 8]. Thus, we cannot discard the possibility of circulation of H1N1pdm09 drug-resistant strains during the pandemic phase in Paraguay. The lack of detection could be related to the small number of sequenced viruses from confirmed cases in the country during the study period.

Table 2. Expected and observed amino acid mutations for the HA and NA genes found in this study.

Mutation*	Gene	Percentage (Expected)**	Percentage (Observed)
S101N	HA	0.2%	0.0%
S220T	HA	76.7%	100%
D239E	HA	5.5%	0.0%
D239G	HA	2.6%	0.0%
N387H	HA	1.7%	0.0%
E391K	HA	15.6%	30.8%
V106I	NA	85.1%	100%
D199N	NA	0.3%	0.0%
I223R	NA	0.2%	0.0%
N248D	NA	85.9%	100%
H275Y	NA	2.0%	0.0%

*Amino acid positions are based on the H1N1pdm 09 vaccine strain A/California/07/2009.

**Expected percentage of mutations for the HA and NA genes, based on published worldwide data [14].

CONCLUSION

In conclusion, the genetic analysis of H1N1pdm09 circulating in our region will help to understand the antigenicity and transmissibility of this virus associated with amino acid mutations, and will foster national surveillance systems.

CONFLICT OF INTEREST

The authors confirm that this article content has no conflict of interest.

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