Genomic signatures and antiviral drug susceptibility profile of A(H1N1)pdm09

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ABSTRACT

Background: Genetic changes in influenza surface and internal genes can alter viral fitness and virulence. Mutation trend analysis and antiviral drug susceptibility profiling of A(H1N1)pdm09 viruses is essential for risk assessment of emergent strains and disease management.

Objective: To profile genomic signatures and antiviral drug resistance of A(H1N1)pdm09 viruses and to discuss the potential role of mutated residues in human host adaptation and virulence.

Study design: A(H1N1)pdm09 viruses circulating in Portugal during pandemic and post-pandemic periods and 2009/2010 season. Viruses were isolated in MDCK-SIAT1 cell culture and subjected to mutation analysis of surface and internal proteins, and to antiviral drug susceptibility profiling.

Results: The A(H1N1)pdm09 strains circulating during the epidemic period in Portugal were resistant to amantadine. The majority of the strains were found to be susceptible to oseltamivir and zanamivir, with five outliers to neuraminidase inhibitors (NAIs) identified. Specific mutation patterns were detected within the functional domains of internal proteins PB2, PB1, PA, NP, NS1, M1 and NS2/NEP, which were common to all isolates and also some cluster-specific.

Discussion: Modification of viral genome transcription, replication and apoptosis kinetics, changes in antigenicity and antiviral drug susceptibility are known determinants of virulence. We report several point mutations with putative roles in viral fitness and virulence, and discuss their potential to result in more virulent phenotypes. Monitoring of specific mutations and genetic patterns in influenza viral genes is essential for risk assessing emergent strains, disease epidemiology and public health implications.

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1. Background

The emergence of virulent influenza phenotypes is a consequence of genetic changes altering the function of individual proteins or their functional compatibility.

Genetic changes in the viral surface proteins can impact on virus binding, entry, assembly, release, induction of the host's immune response and antiviral drug resistance.

Within the internal proteins, genetic changes can impact on virulence and infectivity if they alter the ability of the virus to replicate or induce cellular apoptosis.¹ The viral replication process is multi-genetic and interactive, involving the replication complex and the structural and functionally associated M1 and NS2/NEP proteins.^{1,2} The gene segments coding for the proteins involved in replication, code for additional proteins associated with the induction of

cellular apoptosis during viral infection. The (+1)ORF of PB1 encodes PB1-F2 with pro-apoptotic function in the host cells and the NS gene encodes NS1 protein associated with the activation of anti-apoptotic mechanisms, permitting viral replication to occur.^{3–5} The cassette of internal genes therefore controls the major processes by which viral fitness is determined: replication and apoptosis. An increased replication rate or stimulation of anti-apoptotic mechanisms, produces higher viral loads that can overcome host immune response, enhance infectivity and result in a more severe and transmissible disease. The most severe epidemiological situations caused by seasonal influenza viruses occurred in 1947 and 1951 as a consequence of genetic changes within internal proteins involved in replication of A(H1N1) viruses. The strains spread worldwide and caused exceptionally high mortality.⁶

Monitoring evolutionary trends in the genes encoding influenza virus internal proteins, and profiling antigenic proteins and antiviral drug susceptibility, are therefore essential to risk assessing influenza virulence, disease epidemiology and public health implications.

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Fig. 1. Phylogenetic analysis of the aminoacid sequences of HA1 subunit of hemagglutinin (1A) and neuraminidase (1B) genes of influenza A(H1N1)pdm09 strains circulating in Portugal.

2. Objectives

With the aim of risk assessing emergent strains, the main objectives of the study were to profile genomic signatures and antiviral drug susceptibility of A(H1N1)pdm09 viruses and discuss the potential putative role of mutated residues in human host adaptation and virulence.

3. Study design

A total of 110 specimens from laboratory-confirmed cases of pandemic influenza were collected in Portugal from July 2009 to January 2010. Strains were isolated in MDCK-SIAT1 cell culture. Antigenic surface glycoproteins and M1 and M2 proteins were genetically characterized and antiviral drug susceptibility was profiled. Genetic characterization of internal proteins was performed on viruses isolated from subsets of pregnant women, outliers to

NAIs and a 14 years old deceased patient with no risk factors. Cycle sequencing was performed based on adaptation of a protocol from CDC, recommended by WHO.⁷ Genetic mutation analysis and phylogenetic analysis were performed with LasergeneV.4.05-DNASTAR and MEGA4.0, with comparison to sequences of the A(H1N1)pdm09 2010/2011 vaccine strain (A/California/7/2009) and published reference A(H1N1)pdm09 strains circulating worldwide within pandemic and post-pandemic periods. Phenotypic evaluation of antiviral drug susceptibility was performed by fluorescent assay with MUNANA substrate as previously described.⁸ Minor and major phenotypic outliers were identified through the establishment of lower and upper IC₅₀ cut offs values.⁸ Genotypic evaluation of antiviral drug susceptibility to NAIs and to amantadine was performed by analysing NA and M2 protein sequences, respectively. In the M2 sequence the 5 well-defined molecular markers of resistance to amantadine were analysed: L26F/I; V27A/D; A30T; S31N and G34E.

Fig. 2. Phenotypic evaluation of A(H1N1)pdm09 virus susceptibility to NAIs by fluorescence assay.

4. Results

Virus isolation was performed for all 110 A(H1N1)pdm09 cases. Of these, 37 were genetically characterized for antigenic glycoproteins and for M protein. Antiviral drug susceptibility profile was phenotypically evaluated for 103 of the 110 isolates. Ninety-eight were further sequenced for genotypic evaluation of NAIs susceptibility and 96 for amantadine susceptibility. Genetic characterization of internal proteins PB2, PB1, PB1-F2, PA, NP, NS1 and NS2/NEP was performed for 13 of the 37 isolates, including viruses isolated from cases in the subsets described above.

Within the HA1 domain of the HA, all 37 strains were shown to contain the mutations P83S and I321V (Fig. 1A). Thirty-three of the 37 strains had the substitution S203T, and 19 also contained D222E. Additional point mutations were observed, including A73T and S185N in individual strains, which are located in the putative antigenic sites Cb and Sb, respectively. The substitution K119N which results in the creation of an N-X-S motif, was observed in 2 strains. In the NA gene, 34 of the 37 strains analysed contained N248D and V106I substitutions (Fig. 1B). Fifteen strains had Y155H and two contained I223V in the NA gene.

The genetic characterization of MP of 37 strains identified M128L, E201D and M203I substitutions in individual isolates compared to the A(H1N1)pdm09 2010/2011 vaccine strain MP sequence, located within the M1 coding region.

Phenotypic evaluation of NAIs susceptibility revealed three minor and two major outliers to oseltamivir, as shown in Fig. 2. The three minor outliers exhibited reduced susceptibility of approximately two-fold and the two major outliers of approximately 3- and 4-fold, compared to baseline level. The two major outliers were also found to be minor outliers to zanamivir (MjOosel/MnOZana), with reduced susceptibility of approximately two-fold compared to baseline level. In the NA sequence of these two MjOosel/MnOZana strains, the mutation I223V was observed. Genotypic evaluation of susceptibility to amantadine revealed that all 96 strains analysed had an asparagine (N) at position 31 in the M2 coding region.

The genetic profile of the internal proteins of 13 A(H1N1)pdm09 isolates analysed revealed substitutions P224S in PA, V100I and L122Q in NP and I123V in NS1, which were common to all 13 isolates (highlighted in bold and #, Fig. 1), as shown in Table 1. PB1-F2 was present in the truncated non-functional form of 11 amino acids.

Table 1

Non-synonymous mutations detected per gene/protein. Strains are ordered according to epidemiologic week of beginning of symptoms.

Strain	Epidemiological week	Gene/protein				NS1	NS2/NEP	Observations
		PB2	PB1	PA	NP			
A/Portugal/17/2009	30/2009	V480I	-	P224S	V100I; L122Q	I123V	-	MjO Osel; MnO Zana
A/Portugal/82/2009	32/2009	V480I; D680E	-	P224S	V100I; L122Q; K400R	I123V	-	MjO Osel; MnO Zana
A/Portugal/2/2009	34/2009	-	-	P224S	V100I; L122Q	I123V; F103C	-	n.o.
A/Portugal/7/2009	34/2009	-	-	P224S	V100I; L122Q	I123V	-	Pregnancy
A/Portugal/8/2009	39/2009	R54K; I463M	S633G	P224S	V100I; L122Q	I123V	-	Pregnancy
A/Portugal/13/2009	39/2009	R54K	-	P224S; D347N; M561I	V100I; L122Q	I123V	S93G	Pregnancy
A/Portugal/9/2009	43/2009	R54K; I354V	S384L	P224S; D347N; C95G; N409S	V100I; L122Q	I123V	D54N	Pregnancy
A/Portugal/10/2009	44/2009	R54K; I354V	S384L	P224S; D347N	V100I; L122Q	I123V; A155V; R211K	D54N; G22E	Pregnancy
A/Portugal/11/2009	44/2009	R54K	S384L	P224S; D347N	V100I; L122Q	I123V; A155V; R211K	D54N	Pregnancy
A/Portugal/18/2009	45/2009	R54K; E249G	S384L	P224S; D347N	V100I; L122Q	I123V; P144H	-	MnO Osel; ICU
A/Portugal/12/2009	49/2009	-	-	P224S; S296N	V100I; L122Q	I123V	-	Deceased
A/Portugal/6/2009	50/2009	R54K	-	P224S; S364N; N614S	V100I; L122Q	I123V	-	Children
A/Portugal/1/2010	04/2010	E191K	-	P224S; Q193K; P400L	V100I; L122Q	I123V	-	Pregnancy
A/Portugal/1/2010	04/2010	K340N	S678N	P224S; I94L; N675H	V100I; L122Q; V217I; V313I	I123V; A155V	-	Pregnancy

MjO, major outlier; MnO, minor outlier; Osel, oseltamivir; Zana, zanamivir; ICU, intensive care unit; n.o., no observations.

Viruses isolated from the subset of pregnant women, were observed to have R54K substitution in PB2 in 6 cases, 5 of which also contained D347N in PA. Of these, 4 also contained S384L in PB1 and a small cluster of 3 isolates was characterized by the additional substitutions A155V and R211K in NS1, and D54N in NS2/NEP.

The mutation V480I was found only in the PB2 sequence of the two strains exhibiting a reduced susceptibility profile to both NAIs (MjOOsel/MnOZana).

5. Discussion

A(H1N1)pdm09 viruses characterized by the HA substitution S203T and both N248D and V106I in NA, were found to be the dominant circulating strain throughout the epidemic period (2009–2010) in Portugal. Previous studies have also reported dominant circulation of this strain worldwide and suggested this is a consequence of enhanced viral fitness.^{9,10}

Within HA1, there was no evidence of evolutionary trends, in agreement with the genetic and antigenic homogeneity of A(H1N1)pdm09.¹¹ The location of amino acid substitutions observed in HA1 however, suggests these could result in phenotypic changes; the D222E substitution is located within a loop of the receptor-binding site, A73T and S185N within the putative antigenic sites Cb and Sb, respectively, and K119N has been reported to result in creation of a potential N-glycosylation site.^{9,12} Correlation of these mutations with particular phenotypes, binding specificity or clinical outcome of infection however, needs further investigation.

In the internal genes, the V100I and L122Q substitutions observed in NP, I123V in NS1 and P224S in PA characterize the genetic profile of all isolates analysed, reflecting the dominant circulation of this A(H1N1)pdm09 genetic variant in Portugal within the period analysed. Residues 100 and 122 in NP are located within the body domain of the protein and are thought to be involved in PB2 and PB1–NP interaction, which is crucial for RNA replication. The amino acid substitution V100I was associated with the raising of the pandemic alert from phase 4 to 6, and is a human to avian signature change known to translate into enhanced viral fitness probably by increasing viral transmissibility or infectivity.^{10,13} I123V in NS1 is located in the effector domain, a functional region regulating cellular apoptosis, and therefore suspected to play a role in adaptation to the human host and to increase virulence.¹⁰ P224S in PA is located in the N-terminal domain, which has endonuclease activity. Although no phenotypic outcome for this mutation has been established to date, endonuclease activity is critical for initiating transcription and therefore, genetic changes in this region might be expected to alter replication kinetics.¹⁴ Although the phenotypic outcome of L122Q has not been established, this mutation has been previously reported as a virulence factor in H5N1 by greatly increasing replication.¹⁵

In the viruses from pregnant women, in addition to the mutations common to all isolates analysed, a unique profile of 6 genetic signatures in the functional domains of internal proteins defined a specific cluster. Considering their genetic location, they present a high potential for translating into phenotypic changes in fitness and virulence. Both R54K in PB2 and D347N in PA are located within functional domains for binding PB1.^{7,18} Since the function of the polymerase proteins depends on the formation of a heterodimer for optimal viral RNA replication and transcription, these substitutions together with P224S in PA, may have impacted on polymerase subunit binding to PB1 and have had an unpredictable effect on replication in these strains.^{16,17} Within NS1, the cluster is defined by A155V and R211K substitutions, both located within the C-terminal of the effector domain, as is I123V, which has previously been found to regulate cellular apoptosis.^{5,18} Replication

and apoptosis are the major mechanisms that define viral fitness. The occurrence of different point mutations as occurred in strains in this cluster, could be compensatory or have had a cumulative effect leading to a different phenotype. These mutations will continue to be monitored in non-pregnant patients to evaluate whether they are subset exclusive, resulting from enhanced function of individual proteins or increasing functional compatibility of proteins.

The virus isolate from the deceased patient contained a distinctive set of mutations which could have contributed to this more severe clinical outcome. In addition to N248D and V106I in NA, S203T in HA, V100I in NP and I123V in NS1, this isolate contained L122Q in NP, S364N and N614S in PA, and R54K in PB2, all located in PB1 binding domains of the respective proteins.¹⁷ A direct association of PB1 binding to the other polymerase subunits and to NP with a more virulent phenotype has not been established to date. However, the formation of PB2–PB1–PA heterodimer and the binding of PB2 and PB1–NP into ribonucleoproteins both determine vRNA transcription and cRNA synthesis for genome replication and therefore, have the potential to greatly affect the viral replication cycle.¹⁷

Within the subset of outliers to NAIs, the two MjOOsel/MnOZana were defined by I223V in NA and V480I in PB2. Amino acid substitutions at position 223 in NA have previously been shown to affect virus susceptibility to both oseltamivir and zanamivir, in recent A(H1N1)pdm09 and seasonal viruses.^{19,20} Residue 223 is located in the framework of the NA active site, and thus interacts with the catalytic residues to which antiviral drugs bind.²¹ The contribution of V480I in PB2 to the reduced susceptibility profile observed is unclear. In addition, no phenotypic impact was observed in A(H1N1)pdm09 viruses containing NA mutation Y155H, previously observed in seasonal A(H1N1) viruses resistant to both NAIs.²²

Correlating specific mutations with virulent phenotypes in the human host is not straightforward. Animal models differ in their immune response to infection and multiple other factors interfere with clinical and epidemiology outcomes of infection. Also, the genetic background of specific mutations is divergent among influenza strains and can have different phenotypic outcomes. Several mutations have however, been recognized as virulence markers. Although some mechanisms remain unclear, the modification of genome transcription, replication, apoptosis and antigenicity are determinants of virulence. Additionally to known molecular markers of virulence, we have placed selected residues within functional domains of internal proteins that may enhance viral fitness or increase virulence under genomic surveillance.

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Competing interest

None declared.

Ethical approval

No human subjects were used in this research study. Specimens were accessed through anonymous banks, reviewed and exempt for specific issue document from the Ethic Commission of the National Institute of Health, Lisbon, Portugal.

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