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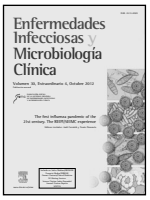

Influenza A(H1N1)pdm09 virus: viral characteristics and genetic evolution

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A B S T R A C T

From April 2009 to the present, the influenza A(H1N1)pdm09 virus has been evolving continuously, acquiring new amino acid changes that may alter its antigenic characteristics, virulence, and its antiviral drug susceptibility. Phylogenetic analysis of the hemagglutinin (HA) gene of A(H1N1)pdm09 viruses showed that it clustered into 8 genetic groups relative to A/California/7/2009, in addition to others reported by regional influenza surveillance networks. However, none were considered antigenically distinct from the vaccine virus A/California/7/2009, which was recommended for use during the 2012-2013 influenza season in the Northern Hemisphere. Amino acid substitution D222G in the HA1 subunit of HA was the first potential virulence marker of the influenza A(H1N1)pdm09 virus that was associated with severe clinical outcomes. The vast majority of influenza A(H1N1)pdm2009 viruses tested by the WHO-GISRS (World Health Organization-Global Influenza Surveillance and Response System) laboratories were sensitive to neuraminidase inhibitor (NAI) drugs, and during the 2011-2012 influenza season the resistance prevalence was low (1%) or undetectable in the United States and Europe. Resistance to NAIs was detected predominantly in patients with severe conditions, most of whom were immunosuppressed. The resistance was usually associated with the H275Y mutation in the NA protein sequence, although other amino acid substitutions were also reported to confer resistance or decreased susceptibility to 1 or more NAIs. Global virological surveillance should be strengthened for new influenza variants carrying new mutations or reassorted segments that may affect viral features such as virulence, transmission, or antiviral susceptibility.

Virus de la gripe A(H1N1)pdm09: características virales y evolución genética

Palabras clave:
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H275Y

R E S U M E N

El virus de la gripe A(H1N1)pdm09 ha estado evolucionando continuamente desde abril de 2009 hasta la actualidad. Los cambios experimentados en los aminoácidos del virus pueden alterar sus características antigénicas, su virulencia y su sensibilidad a los fármacos antivirales. Mediante el análisis filogenético del gen hemaglutinina (HA) del virus A(H1N1)pdm09 se han observado 8 grupos genéticos relacionados con el A/California/7/2009, además de otros informados por las redes regionales de vigilancia de la gripe, si bien ninguno de estos grupos genéticos ha sido considerado antigénicamente distinto del virus de la vacuna A/California/7/2009, cuyo uso se recomendó durante la estación de gripe 2012-2013 en el hemisferio norte. El primer marcador de virulencia potencial del virus de la gripe A(H1N1)pdm09 que fue asociado con una mayor severidad clínica de la enfermedad fue la sustitución del aminoácido D222G en la subunidad HA1 del HA. La inmensa mayoría de los virus de la gripe A(H1N1)pdm2009 estudiados por los laboratorios del WHO-GISRS (World Health Organization-Global Influenza Surveillance and Response System) eran sensibles a los fármacos inhibidores de la neuraminidasa (INA). Tanto en Estados Unidos como en Europa, el predominio de resistencia fue bajo (1%) o indetectable durante la temporada de gripe 2011-2012. Los pacientes con enfermedad grave, la mayoría de los cuales eran inmunodeprimidos, fueron principalmente los que presentaron resistencia a los INA. Habitualmente, la resistencia estaba asociada a la mutación de H275Y en la secuencia de la proteína NA, aunque también se apreciaron otras sustituciones de aminoácido que

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conferían resistencia o disminución de la sensibilidad a uno o más INA. Los autores recomiendan extremar la vigilancia virológica global para detectar nuevas variantes de la gripe debidas a nuevas mutaciones o reordenamientos genómicos que pueden afectar características virales como la virulencia, la transmisión o la sensibilidad a los antivirales.

Introduction

The influenza A viruses belonging to the Orthomyxoviridae family are characterized by a negative-sense segmented RNA genome that consists of 8 gene segments that encode for 11 proteins. Influenza A viruses possess a lipid membrane derived from the host cell that harbors the hemagglutinin (HA) and the neuraminidase (NA), which are used to subtype them.[1,2]

Influenza viruses continuously undergo genetic changes, either through progressive amino acid substitutions (called minor changes or antigenic drift) or by complete segment swapping (called major changes, reassortment or antigenic shift), to evade the immunological pressure of the host immune system and to adapt to novel hosts. The lack of proofreading ability of the viral RNA-dependent RNA polymerase facilitates antigenic drift.[3] Therefore, mutant viruses with higher fitness become dominant and rapidly spread widely through the human population. Amino acid changes at critical positions on HA and NA proteins can alter, among other things, their antigenic characteristics and their antiviral drug susceptibility. Influenza pandemics may occur when an influenza virus acquires an HA protein through reassortment, which is then efficiently transmitted from human to human and emerges in the human population with little or no existing immunity.[4]

In April 2009 a new influenza A(H1N1) virus, with transmission capacity between humans, was isolated from influenza-like illness patients in Mexico and the United States.[5] The influenza A(H1N1) pdm09 virus contained a combination of gene segments previously not reported in swine or human viruses. It was the result of multiple reassortment events over several years that brought together genomic segments from classical swine H1N1 influenza virus, human seasonal H3N2 influenza virus, North American avian influenza virus, and Eurasian swine influenza viruses.[6-8] The segments coding for the HA came from North American triple reassortant swine viruses and for the NA and matrix proteins (M1 and M2) from Eurasian swine viruses, which were known to carry adamantane-resistant mutations on the M2 protein.[8,9]

In addition to antigenic divergence within the various triple reassortant H1 viruses in the swine population, seasonal A(H1N1) viruses intermittently circulated in humans starting in 1918, accumulating substantial antigenic drift in humans away from the 1918 pandemic A(H1N1) virus. This significant antigenic gap between classical swine H1 and human seasonal H1 viruses[8] led to a reduced immune protection in the population and to global spreading.

Evolution and genetic diversity

Influenza virus populations circulating among humans vary genetically from season to season and even within a season in different regions of the world. Surveillance for circulating influenza viruses through the World Health Organization-Global Influenza Surveillance and Response System (WHO-GISRS) is critical to monitor the unpredictability of viral mutations and their impact on the future severity of infection, vaccine composition and susceptibility of the virus to antiviral drugs. Phylogenetic analysis based on the nucleotide changes that have led to amino acid substitutions in the HA and in the NA coding sequence are usually performed for routine influenza surveillance. Phylogenetic analysis provides information on how the viruses are disseminated, and it discriminates between virus lineages that have indistinguishable antigenic properties.[10] Both phylogenetic and antigenic analysis are used by the WHO Collaborating Centres for Reference and Research on Influenza to make recommendations on appropriate strains to be included in annual seasonal influenza vaccines for the Northern and Southern hemispheres.

The first concatenated whole genome-based phylogenetic analysis from globally isolated samples during the early phase of the pandemic (April 1st-July 9th, 2009) revealed that the early influenza A(H1N1) pdm09 virus had diversified into 7 clades (1-7).[11] Within each gene segment, the high (99.9%) identity among the viruses sequenced suggested that introduction into humans was probably a single event, without ruling out the possibility of multiple events of genetically similar viruses.[8] Among the circulating viruses, those belonging to clade 7, characterized by amino acid substitutions V100I in NP, V106I and N248D in NA, S203T in HA and I123V in NS1, spread widely and became the predominant variant.[11,12]

High evolutionary rates were found in the influenza A(H1N1) pdm09 virus. During the early outbreak period of the pandemic (April – May 2009), the mean evolutionary rate varied from 2.34 to 3.67×10^{3} substitutions/site/year.[7] But throughout an extended pandemic period (April 2009-March 2010) the mean substitution rates for each segment sequence were relatively higher (3.65 to 6.17×10^{-3} substitutions/site/year).[13] The influenza A(H1N1)pdm09 virus had high non-synonymous to synonymous ratios in all 8 segments during this pandemic period, suggesting that a strong purifying selection was active on all segments.[7,13-15] Interestingly, the highest values were associated with HA and NS, key proteins for virus-host interactions, suggesting adaptation to the new host specie.[13-14] In addition, selection-pressure analysis revealed that most of the positively selected sites were located within the antigenic regions, involved in glycosylation and receptor-binding ability. Amino acid substitutions at these positively selected sites may help the virus to adapt and evolve in the human host.[16-19]

According to the last ECDC influenza virus characterization report, published last March 2012,[20] phylogenetic analysis of the HA gene of influenza A(H1N1)pdm09 viruses clusters into 8 genetic groups, defined by specific amino acid substitutions compared to the vaccine strain A/California/7/2009, as shown in Figure 1. Nevertheless, none of these genetic groups are considered antigenically distinct from the vaccine virus.

During the 2010-2011 influenza season, an additional genetic group characterized by amino acid substitutions E172K, S203T, K308E in HA, often with V47I mutation, was identified in Spain,[21] indicating the importance of performing regional influenza surveillance.

Changes in antigenic sites: A(H1N1)pdm09 immune escape mutants

The HA of influenza viruses is the viral surface glycoprotein responsible for binding to the host cell receptor and subsequent membrane fusion and viral entry.[22] The HA plays, through its 5 immunodominant antigenic sites (Sa, Sb, Ca1, Ca2, and Cb), an important role in host immune responses, stimulating the production of neutralizing antibodies responsible for protective immunity.[23-25] Amino acid changes occurring either in the antigenic sites or on the surface of the HA molecule may have an important effect on antibody

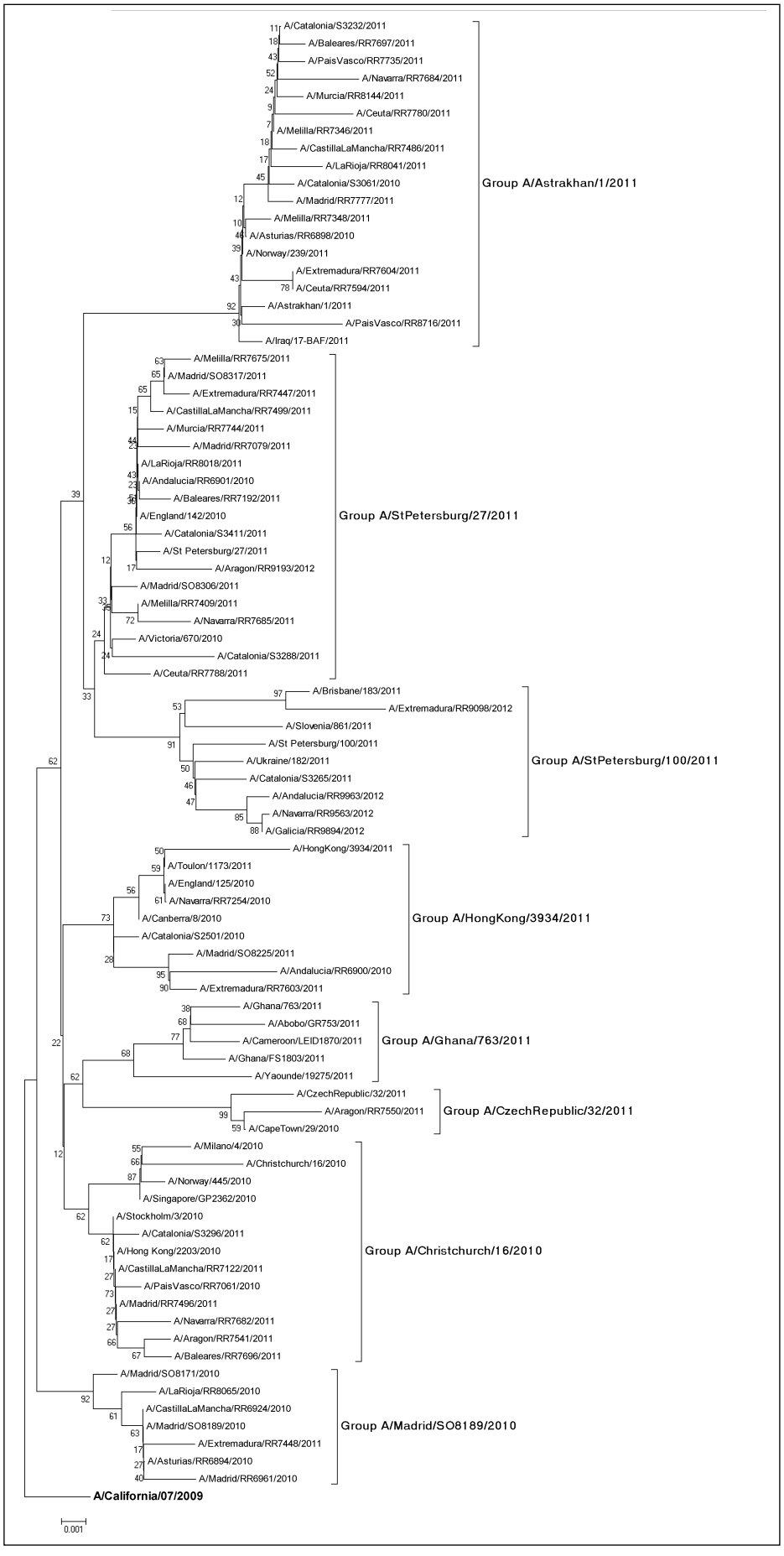

Figure 1. Phylogenetic analysis of the HA1 coding region of the HA gene of representative influenza A(H1N1)pdm09 viruses circulating in Spain during the 2010-2011 and 2011-2012 seasons. The tree was rooted with A/California/07/2009 (vaccine strain) as the outgroup.

recognition, enabling the virus to escape immune responses. The annual influenza epidemics are attributed primarily to these changes. Therefore HA is continually under selection pressure driven by the host immune response, induced by vaccination or natural infection.[23,26,27]

Structural characterization and sequence analysis revealed that influenza A(H1N1)pdm09 was closer to the 1918 A(H1N1) viruses than to seasonal A(H1N1) influenza viruses, and experimental studies demonstrated that influenza A(H1N1)pdm09 was antigenically similar to viruses that circulated between 1918 and 1956.[28-30] Epidemiological data reported that the major impact of the influenza A(H1N1)pdm09 pandemic was on younger age groups, while the elderly population was underrepresented among cases, with a pattern of morbidity and mortality different from that observed in seasonal influenza epidemics, due to preexisting immunity.[29,31-35] Since vaccination with prior non-adjuvanted or adjuvanted seasonal influenza A(H1N1) vaccine induced little or no cross-reactive antibody response to A(H1N1)pdm09 in any age group, large segments of the human population throughout the world lacked protective immunity against the novel influenza A(H1N1)pdm2009 virus and they were thus susceptible to the infection.

Although the several genetic groups identified for circulating influenza A(H1N1)pdm09 viruses already showed common amino acid substitutions in the HA gene located within the antigenic sites Sa (N125D, K163T and S162H), Sb (S185T and A186T), Ca1 (S203T and R205K) and Ca2 (A141S),[23-25] these were not antigenically different from vaccine strain A/California/07/2009 in hemagglutination inhibition assays with ferret antiserum.[36,37] Therefore, the initial pandemic A/California/07/2009 virus strain remained the WHO-recommended vaccine candidate for the 2012–2013 vaccine to be used in the Northern Hemisphere.[36] However, amino acid substitutions in the antigenic sites and in other positions susceptible to alterations of the antigenic features of influenza A(H1N1)pdm09 should be carefully monitored.

Viruses carrying the amino acid substitution S203T became the predominant strain, and may be responsible for the global success of clade 7 viruses.[11,38] It is unclear whether the predominance of S203T could be attributed to immune selection or whether is a consequence of further adaptive changes to the human host or optimization of viral fitness. Substitution S203T, located within antigenic site Ca, has the potential to contribute to antigenic drift.[39] However, its buried position on the HA structure, near the monomer-monomer interface, leaves uncertainty about its real effects.[23,25]

During the last 2009-2010 Northern Hemisphere season, influenza A(H1N1)pdm09 viruses carrying double mutations N125D and E374K were found, with increased frequency during the 2010 Southern Hemisphere season,[40,41] although these amino acid changes did not result in significant antigenic changes that might make the current vaccine less effective.[40] Furthermore, mutation E374K is located in the HA oligomerization interface and is also part of a known highly conserved antigenic site in the HA stem region.[42] Mutation E374K may alter salt bridge patterns and stability in a region of the HA oligomerization interface with a possible role in membrane fusion,[43] and changes in the antigenicity of this epitope would not necessarily be reflected in HI assays.[39] However, the significance of E374K alone or in concurrence with other mutations needs further study.

Among other relevant mutations, amino acid substitutions A134T, A186T and S185T within the receptor binding site (RBS), and S183P, A197T and I216V near RBS may affect the interaction of HA with its receptor.[44] Amino acid substitutions S183P and I191L, immediately adjacent to the sialic acid binding site, may affect cell attachment, substrate specificity and growth characteristics. These amino acid substitutions seem to improve replication in cell culture and eggs.[45] The amino acid substitution S162N resulted in the acquisition of a N-glycosylation site that falls into antigenic site Sa.

D222G, the first potential virulence marker of influenza A(H1N1) pdm09 viruses

Influenza A(H1N1)pdm09 infection is mostly a mild, self-limiting upper respiratory tract illness. The spectrum of clinical presentation varies from asymptomatic cases to primary viral pneumonia resulting in respiratory failure, acute respiratory distress, multi-organ failure and death.[46] The pathogenicity of the influenza virus is dependent on the function of viral proteins and on host immune responses,[47] including innate and acquired immunity, indicating the importance of both viral factors and the host immune system for influenza pathogenesis.

Previous studies on highly pathogenic influenza viruses have contributed to the identification of numerous molecular markers such as a multibasic HA cleavage site,[48] and key amino acids substitutions in PB2,[49-51] in NS1,[52] and the presence of full-length PB1-F2,[53] associated with high pathogenicity, increased transmissibility or virulence, in avian or mammalian species. But the presence of these specific molecular markers in the A(H1N1)pdm09 viruses was discarded early, after the first A(H1N1)pdm09 genomic analysis.[8,54,55]

The specificity and affinity of the viral HA for host receptor is one of the crucial determinants of host tropism[55] and transmission. Since the RBS at the membrane distal end of each HA monomer shows specificity for sialic acid of the host cell receptor and the nature of the linkage between sialic acid and galactose residue, it determines the host range-restriction.[28,44].

Human influenza virus strains preferentially bind receptors that possess sialic acid linked to galactose by an α2,6-linkage (SA α2,6 Gal), mainly found on the epithelial cell surface of the human upper respiratory tract, while avian influenza viruses prefer SA α2,3 Gal, localized on human lung alveolar cells, almost equally distributed with SA α2,6 Gal, and on the digestive and respiratory tracts of birds.[56-60] Influenza A(H1N1)pdm09 viruses preferentially recognize α2-6 sialylated receptors,[28,44,61], and have low affinity for α2-3-linked receptor analogs.[28,62,63]

Amino acid changes in and around the RBS dramatically alter the receptor binding preference of influenza viruses. Mutation D222G in HA was the first potential virulence marker of A(H1N1)pdm09 viruses associated with severe clinical outcomes.[64-72]. Changes at this residue[44,73] cause a reduction in the binding avidity to SA α2,6 Gal receptors and an increase in the binding to SA α2,3 Gal receptors.[60,74]. The presence of SA α2,3 Gal receptors in the alveolar region of human lungs may explain the severe pneumonia, as in human cases of H5N1 infection, indicative of viral replication in the lower respiratory tract.[56,57,75] A study using cultures of human tracheobronchial epithelial cells showed that mutant D222G A(H1N1)pdm09 viruses could infect ciliated bronchial cells.[76]

Since replication in the upper airway may be required for efficient transmission and initiation of a productive infection in humans,[56] the slight shift in receptor specificity then would affect cell tropism and possibly the virulence of these viruses, but it also may reduce its ability to transmit, as reflected in its vastly reduced prevalence.[28] In experimental inoculation of pigs with mixed virus populations carrying residues D222 and G222, the D222 variant was found in nasal secretions, while the G222 variant was found in the lower respiratory tract, providing potential clues to the existence and biological significance of viral receptor-binding variants with D222 and G225.[77]

Although amino acid D222G falls into antigenic site Ca2, it was reported that it does not affect the antigenic properties of A(H1N1) pdm09, as determined by HI assays. These data suggest that currently available pandemic influenza vaccines would provide similar protection to the wild-type or D222G mutant viruses, and that vaccination could potentially limit the impact of viruses carrying D222G in the future.[75]

Other amino acid substitutions, D222E and D222N,[67,72,78] were also detected among pandemic viruses. Viruses carrying D222N in HA

have also been associated with severe infections,[72,78,79] but no particular pathogenicity has been associated with the D222E variants since they are present at the same frequency in severe and mild infections.[64,67]

Antiviral resistance: H275Y mutation and others

Influenza vaccination is the most important and effective approach to protect against influenza infection, to reduce morbidity and mortality, and to reduce viral transmission within the population. However antiviral drugs may be the only option available not only for treatment but also for prevention of influenza upon emergence of a novel strain.

Antiviral resistance in influenza virus evolves through mutations or by reassortment, resulting in acquisition of mutations that confer antiviral resistance.[80] The influenza A(H1N1)pdm09 virus is resistant to M2 inhibitors, amantadine and rimantadine, because it carries the Eurasian swine M gene with the genetic marker (S31N in *M2*) related to resistance to adamantanes.[8,9] The vast majority of influenza A(H1N1)pdm2009 viruses tested in the WHO-GISRS laboratories were sensitive to neuraminidase inhibitor drugs (NAIs), oseltamivir, zanamivir or peramivir.[81]. However the emergence and spread of drug-resistant variants are matters of serious concern, and continuous monitoring is advocated to track the mutations associated with resistance to NAIs.[82]

Although NAI resistance in the influenza A(H1N1)pdm09 virus remained rare, from September 2010 to March 2011 about 1.5% of tested strains in the WHO Collaborating Centres were resistant to oseltamivir.[81] All these resistant viruses were associated with a H275Y mutation in the NA coding sequence that confers resistance to oseltamivir and peramivir, but not to zanamivir. The similarity in the binding of oseltamivir and peramivir in the NA active site may count for this cross-resistance.[83] However zanamivir resistance has been rarely documented in surveillance viruses,[84] perhaps because of the less frequent use of inhaled zanamivir than oral oseltamivir. Moreover, zanamivir often retains full or partial in vitro inhibitory activity against oseltamivir-resistant variants, including viruses harboring H275Y.[84] Based on TESSY/EuroFlu reporting, from September 2010 to March 2011 the National Influenza Centres in Europe reported 2.95% of samples to be resistant to oseltamivir.[81] During the 2011-2012 influenza season, the resistance to neuraminidase inhibitors has been low (1%) or undetectable in the United States and Europe, respectively, but this data may be biased due to the low number of samples tested because of the scarce global circulation of A(H1N1)pdm09 viruses.[85] Most cases of resistance were reported from people with severe conditions on antiviral therapy, such as immunocompromised patients undergoing oseltamivir treatment. Only a small number of cases of resistance were detected in individuals who were neither receiving oseltamivir nor in known contact with others receiving treatment,[82,86] suggesting low-level community transmission of resistant viruses.[82,86,87]

Current in vitro and in vivo studies of the fitness of resistant influenza A(H1N1)pdm09 strains are conflicting. Results on the effect of the H275Y substitution in different A(H1N1)pdm09 viruses ranged from no change in viral fitness and virulence[88-90] to reduced respiratory droplet transmissibility and replication.[91,92] Studies are ongoing to better understand the role of other potential resistance mutations or likely compensatory amino acid substitutions mainly in NA[93] and HA[94] proteins that can counteract the loss of fitness conferred by specific drug resistance-conferring substitutions.[95] For resistant seasonal A(H1N1) viruses carrying H275Y mutation in NA, a compensatory role was assigned to the NA amino acid changes V234M, R222Q and D344N,[93,96] restoring the initial loss of NA activity due to the NAI resistance mutation (H275Y) and facilitating the global spreading of the H275Y change unrelated to drug usage. The recent rapid global spread of oseltamivir resistance should serve as a reminder to maintain constant surveillance for similar trends in currently circulating subtypes and in any future novel subtypes. During the 2010-2011 Southern Hemisphere influenza season an oseltamivir-resistant A(H1N1)pdm09 virus was community-transmitted in Australia, although it did not spread globally.[87] These community-clustered viruses were highly similar genetically, suggesting the spread of a single variant, and they contained 2 other NA substitutions, V62I and N386S, which were absent from most of the oseltamivir-susceptible A(H1N1)pdm09 viruses, in addition to V241I and N369K, also in oseltamivir-susceptible strains. Computational analysis predicted that neuraminidase substitutions V241I, N369K and N386S in these viruses may offset the destabilizing effect of the H275Y substitution, retaining viral fitness.[97]

Apart from the H275Y substitution, a few other amino acid substitutions in A(H1N1)pdm09 viruses isolated from patients receiving treatment have been shown to confer resistance to NAIs in phenotypic assays.[82]. N295S is also associated with resistance to oseltamivir and peramivir, but not zanamivir.[98] NAI susceptibility also depends on the occupying amino acid residue, and for example E119V confers resistance to both oseltamivir and zanamivir, as opposed to E119G, which is related to resistance to both zanamivir and peramivir.[98] Other amino acid changes are related to decreased susceptibility to 1 or more NAIs, such as I117V,[99] D199G,[98], I223K/R/V[98,100-103] and S247N.[104] Surprisingly, some of these substitutions detected in combination with H275Y cause even higher levels of resistance to oseltamivir and peramivir,[98-100,104,105] even without compromising fitness.[103].

It should be noted that detecting particular mutations by genotypic methods will not detect antiviral resistance due to substitutions other than those currently recognized. As a result, the lack of known mutations associated with antiviral resistance is not a guarantee of susceptibility to the corresponding drug. Thus, only viruses for which the IC50 values are determined by phenotypic NA enzyme-inhibitor assays can be designated resistant or sensitive to the neuraminidase inhibitors.[84]

The variation in prevalence of resistance to the limited antiviral medications available for influenza would have important public health and clinical implications in the future.[106] Therefore continuous monitoring of A(H1N1)pdm09 viruses for antiviral resistance is highlighted.

Global circulation and risk of reassortment

For segmented viruses, reassortment can introduce drastic genomic and phenotypic changes by allowing a direct exchange of genetic material between co-infecting strains.[107] The global circulation and occasional introductions of the A(H1N1)pdm09 virus in humans and in pigs[108,109] may allow this virus to reassort with other influenza viruses,[110] and produce variants with transmissibility and altered virulence for humans. There is evidence that the A(H1N1)pdm09 virus has re-infected swine herds in several countries and reassorted with swine-origin viruses, but most of these swine reassortants did not have high pathogenicity.[111-116] In fact, A(H1N1)pdm09 proved by itself that swine-origin influenza viruses can cause widespread infection in humans. Among the human cases of swine-origin influenza infections[117] there have been recent sporadic human infections in North America since August 2010, with possible human-to-human transmission, of a new swine-origin triple-reassortant influenza A(H3N2) virus that contained genes originating from swine, avian, and human viruses, including the M gene from influenza A(H1N1)pdm09 highlighted.[118-120] The systematic influenza surveillance in swineherds for novel viruses should at least be increased for the possible eventual adverse health impact on humans.

Conclusions

The influenza A(H1N1)pdm09 virus serves as a stark reminder of the inherently unpredictable nature of influenza viruses. The fact that most of the A(H1N1)pdm09 infections cause a mild respiratory disease with low mortality rate may be due to the absence of specific molecular markers associated with high pathogenicity, as well as a degree of preexisting cross-reactive immunity in the elderly population.

Although the genetic diversity observed among influenza A(H1N1) pdm09 viruses has been wide, the circulating viruses remained antigenically similar to the vaccine virus, also recommended to be used during the next 2012-2013 influenza season in the Northern Hemisphere. However the effects of the high evolutionary rate of A(H1N1)pdm09 have been demonstrated, particularly in the acquisition of some amino acid substitutions associated with more severe clinical outcomes or with antiviral resistance. Therefore, global virological surveillance should be strengthened for new influenza variants carrying new mutations or reassorted segments that may affect viral features such as virulence, transmission, or antiviral susceptibility.

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Conflicts of interest

All authors declare that they have no conflicts of interest in this article.

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