SEQUENCE ANALYSIS OF RESISTANCE MARKERS IN PANDEMIC INFLUENZA A(H1N1)2009 VIRUSES

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Background:

In April 2009, a new influenza A(H1N1) virus was detected circulating in humans. Its rapid widespread transmission led to the declaration by the WHO of an influenza pandemic. This virus had origin in a reassortment between a North American swine lineage (already a triple reassortant, circulating in pigs since the late 1990's) and a Eurasian swine lineage (which contributed to the A(H1N1) 2009 with the neuraminidase (NA) and matrix (MP) gene segments).

Pandemic H1N1 viruses, as well as Eurasian swine viruses are resistant to adamantanes (amantadine and rimantadine), since they carry the S31N change in the M2 protein. The majority of A(H1N1) 2009 viruses were sensitive to NA inhibitors (NAI). At February 2010, a total of 225 oseltamivir/resistant cases were reported, sharing all these viruses the H275 mutation in the neuraminidase gene (WER, 2010).

The NA inhibition assay is the base test to determine the susceptibility to NA inhibitors (NAI), however it requires virus isolation, and the consequent detection of a resistant viral strain requires confirmation by sequencing of the NA gene segment present in the original biological product. Often, NA inhibition assay is not possible when there are no available viral isolates. This same isolation in cell cultures leads to a change in the ratios of sensitive and to resistant viral populations in analysis (Deyde *et al*., 2010). In this study, conventional sequencing was used to a rapid detection of NA resistance markers.

Material and Methods:

In Portugal, during the 2009 influenza pandemic, about 16500 clinical samples were tested by the National Influenza Reference Laboratory for the presence of influenza A(H1N1) 2009 virus. From near 8000 A(H1N1) 2009/positive samples, 153 were isolated in MDCK cells and of these 56 isolates were taken for sequence analysis of the NA and MP gene segments. Nucleotide sequences were aligned (ClustalW) and the phylogenetic trees constructed (Neighbour/joining, Kimura 2/parameter) using MEGA 4.0 software.

Among these 56 sequenced viruses, the NA inhibition assays were performed in 39 viral isolates from a total of 77 analysed by the WHO collaborating centre (London).

Results:

As all A(H1N1) 2009 viruses, sequenced Portuguese isolates carried the S31N change in the M2 protein, which confers resistance to the adamantanes.

The M2 proteins of isolated A(H1N1) 2009 viruses are 100% identical to the A/California/7/2009 M2 protein.

The most common mutation in N1 neuraminidase associated with resistance to oseltamivir is the histidine to tyrosine (H275Y) substitution. From our 56 sequenced isolates, only one carried this mutation: A/Lisboa/171/2009 (Table I and Figure 1).

This was from an 8/years/old child with chronic diseases such as a DiGeorge syndrome diagnosed in 2004 and Evans Syndrome with auto/immune haemolytic anaemia diagnosed in 2006. Initially, a sample collected from this child at November 24th was A(H1N1) 2009 positive but was wild type at NA position 275 (histidine). The patient was treated with an subtherapeutic level of oseltamivir, however, as the influenza/like syndrome persisted with a suspicion of viral pneumonia, led to the antiviral treatment dose review. A second sample collected at December 11th confirmed that the A(H1N1) 2009 infection persisted but during this time the patient influenza strain developed the H275Y mutation. The NA inhibition assay confirmed that this virus was resistant to oseltamivir with a IC_{50}=171,42.

The NA inhibition assays performed in our viral isolates and accordingly to our sequence data, revealed that the majority of Portuguese pandemic viruses were sensitive to NAI (data not shown).

All neuraminidases presented the V106I and N248D substitutions, except for A/Lisboa/35/2009, a viral strain from an early clade (at least, previous to clade 4). The NA amino acid change Y155H was observed in 17 viral strains. Other amino acid substitutions, such as E119V, I223V, Q136K, known to reduce the susceptibility to NAI, were not found in our isolates (Figure 1 and Table I).

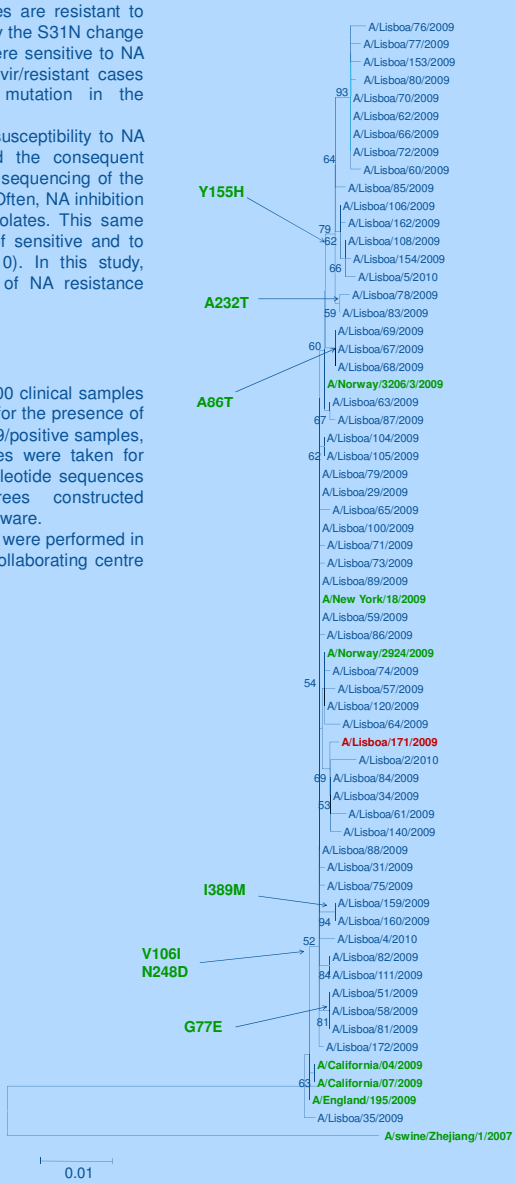

Figure 1 – Phylogenetic tree of NA nucleotide sequences from pandemic influenza A(H1N1)2009 strains isolated in Portugal (Neighbour/joining method; Kimura 2/parameter model; bootstrap values above 50 are shown – 2000 replicates). In green / reference strains, in red / resistant strain.

Table I – Amino acid substitutions in the neuraminidase protein of pandemic A(H1N1)2009 portuguese viral isolates comparing to the vaccine strain A/California/7/2009.

	NA aa	Q	T	M	A	Q	Q	T	Q	T	G	A	S	V	Y	G	V	A	N	V	N	H	S	T	I	I	I	S
Vaccine and reference influenza	A/California/7/2009																											
	A/California/4/2009																											
	A/England/195/2009																											
	A/Auckland/3/2009														I				D									
	A/Norway/2924/2009														I				D									
	A/Norway/3206/2009														I				D									
	A/Norway/3487-2/2009														I				D									
Portuguese influenza isolates A/H1N1 pdm	A/Lisboa/29/2009														I				D									
	A/Lisboa/31/2009														I				D									
	A/Lisboa/34/2009														I				D									
	A/Lisboa/35/2009				I																	G						
	A/Lisboa/51/2009									E					I				D									
	A/Lisboa/57/2009														I				D									
	A/Lisboa/58/2009									E					I				D									
	A/Lisboa/59/2009														I				D									
	A/Lisboa/60/2009				H										I	H			D									
	A/Lisboa/61/2009														I				D									
	A/Lisboa/62/2009														I	H			D									
	A/Lisboa/63/2009														I				D									
	A/Lisboa/64/2009							R							I		E		D									
	A/Lisboa/65/2009					K									I				D									
	A/Lisboa/66/2009														I	H			D									
	A/Lisboa/67/2009											T			I				D									
	A/Lisboa/68/2009											T			I				D									
	A/Lisboa/69/2009											T			I				D									
	A/Lisboa/70/2009														I	H			D									
	A/Lisboa/71/2009														I				D									
	A/Lisboa/72/2009														I	H			D									
	A/Lisboa/73/2009													N	I				D									
	A/Lisboa/74/2009														I				D									
	A/Lisboa/75/2009														I				D									
	A/Lisboa/76/2009			T											I	H			D		D		I					
	A/Lisboa/77/2009														I	H			D									
	A/Lisboa/78/2009												S		I	H		T	D									
	A/Lisboa/79/2009														I				D									
	A/Lisboa/80/2009														I	H			D									
	A/Lisboa/81/2009									E					I				D									
	A/Lisboa/82/2009														I				D									
	A/Lisboa/83/2009														I	H		T	D									
	A/Lisboa/84/2009														I				D									
	A/Lisboa/85/2009														I	H	M		D									
	A/Lisboa/86/2009														I				D									
	A/Lisboa/87/2009														I				D									
	A/Lisboa/88/2009														I				D									
	A/Lisboa/89/2009														I				D									
	A/Lisboa/100/2009														I				D									
	A/Lisboa/104/2009														I				D									
	A/Lisboa/105/2009														I				D									
	A/Lisboa/106/2009														I	H			D									
	A/Lisboa/108/2009														I	H			D									
	A/Lisboa/111/2009														I				D									
	A/Lisboa/120/2009														I				D									
	A/Lisboa/140/2009														I				D									V
	A/Lisboa/153/2009								A						I	H			D									
	A/Lisboa/154/2009														I	H			D									
	A/Lisboa/159/2009														I				D								M	
	A/Lisboa/160/2009														I				D								M	
	A/Lisboa/162/2009		I												I	H			D									
	A/Lisboa/171/2009														I				D			Y						
	A/Lisboa/172/2009														I				D					M				
	A/Lisboa/2/2010					K									I				D	I								I
	A/Lisboa/4/2010					L									I				D									
	A/Lisboa/5/2010														I	H			D									

Conclusions:

From the sequenced viral isolates (36,6% from all isolates) and those where the NA inhibition assay was performed (50,3%) we can conclude that the majority of isolated viral strains were sensitive to oseltamivir.

The unique case of NI resistance detected in Portuguese viral strains apparently was developed during the oseltamivir treatment in a landscape of other clinical complications.

The association between the oseltamivir therapy and the development of the H275Y mutation has been widely documented (Wang *et al*., 2010; Harvala *et al*., 2010; MMWR, 2009). At February 2009, these cases represented 38% of all oseltamivir/resistant pandemic influenza cases (WER, 2010).

We didn't detected in the studied strains other mutations, involved in resistance to NAI, such as E119V, I223V, Q136K.

Conventional sequencing can provide a rapid tool for monitoring the molecular markers of antiviral resistance in influenza A virus and for an early detection of antiviral resistant strains.

Bibliography:

Deyde V., Sheu T., Trujillo A., Okomo/Adhiambo M., Garten R., Klimov A., Gubareva L. Detection of molecular markers of drug resistance in 2009 pandemic influenza A(H1N1) viruses by pyrosequencing. 2010. Antimicrobial agents and chemotherapy. 54(3):1102/1110

Harvala H., Gunson R., Simmonds P. et al. 2010. The emergence of oseltamivir/resistant pandemic influenza A(H1N1)2009 viruses amongst hospitalised immunocompromised patients in Scotland, November/December, 2009. Euro Surveillance 15(14)

Oseltamivir/Resistant 2009 Pandemic Influenza A (H1N1) Virus Infection in Two Summer Campers Receiving Prophylaxis / North Carolina, 2009. 2009. MMWR 58(35): 969/972

UIpdate on oseltamivir/resistant pandemic A(H1N1)2009 influenza virus: January 2010. 2010. Weekly Epidemiological Record 85 (6):37/48

Wang B., Dwyer D., Blyth C. Et al. 2010. Detection of the rapid emergence of the H275Y mutation associated with oseltamivir resistance in severe pandemic influenza virus A/H1N1 09 infections. Antiviral Research 87:16/21

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