



Short communication

Monitoring the susceptibility to oseltamivir of Influenza A(H1N1) 2009 virus by nested-PCR and pyrosequencing during the pandemic and in the season 2010–2011

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For the early detection of the H275Y mutation as a marker of oseltamivir resistance in A(H1N1) pandemic strains, a sensitive and specific pyrosequencing assay was developed. This assay analyses a region 99 nts long, encompassing the H275Y site, amplified by a nested PCR. Seventy-five respiratory specimens, obtained from 62 patients during the pandemic and in the 2010–2011 influenza season, in Tuscany, were tested. Resistant strains were demonstrated in 10 patients. In three other patients, resistant and sensitive variants were found. This pyrosequencing assay may be a useful method for monitoring the spread of resistant influenza H1N1 2009 strains.

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The H275Y mutation in the neuraminidase (NA) gene of the N1 subtype is known to confer resistance to the neuraminidase inhibitor (NAI) oseltamivir (Collins et al., 2009; Gubareva, 2004; Renaud et al., 2011). In the epidemic season 2007–2008, the rate of resistance of type A(H1N1) influenza viruses to oseltamivir, previously very low (<0.5%), started to increase, spreading globally. In the season 2008–2009, the majority of the seasonal H1N1 strains were resistant to oseltamivir (but still sensitive to zanamivir), independent of patient exposure to the drug (Renaud et al., 2011; Yang et al., 2011). Fortunately, the pandemic H1N1 2009 virus was sensitive to both oseltamivir and zanamivir. Nevertheless, the widespread use of these drugs, predominantly oseltamivir, for pandemic control in economically developed countries, was followed by great concern. Many efforts have been aimed at developing rapid molecular assays for the early detection of the H275Y mutation as a marker of oseltamivir resistance of pandemic strains. As an alternative to Sanger sequencing, many of the proposed assays were based on PCR genotyping (Renaud et al., 2011). In May 2009, the World Health Organization (WHO) suggested the use of a pyrosequencing method set up at the Centres for Diseases Control

(CDC) (Atlanta) (Deyde et al., 2010; World Health Organization Collaborating Centre, 2010), which targeted a region of the NA RNA 493 nts long.

In the current study, using the method suggested by WHO, taken as a reference method, a preliminary observation showed that only positive samples, with a threshold cycle not >28 at the diagnostic real time (slightly less than the 50% of the samples included in this study), were amplified.

With the aim of increasing the sensitivity of the reaction, and considering as well the performance of the instrument available for pyrosequencing in the laboratory, a nested PCR was developed which amplified a region 99 nts long, encompassing the H275Y mutation site. This was suitable for analysis via pyrosequencing using the Pyromark ID 1.0 (Biotage-Diatech, Iesi, An, Italy). In order to localize the target region and the primers for its amplification, published NA sequences of pandemic H1N1 viruses were aligned using BioEdit software (version 7.0.9; North Carolina State University). The primers for the nested PCR (Table 1) were designed using Primer3.

The extraction of viral RNAs directly from clinical samples was carried out using a commercially available kit (E.Z.N.A. Viral RNA kit, Omega bio-tek, Norcross, Georgia, USA) according to the manufacturer's instructions.

Superscript III Platinum One-step qRT-PCR System (Invitrogen, Carlsbad, California, USA) was used for RT-PCR according to the manufacturer's instructions, with the outer primers listed in

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Table 1
Selected primers for the nested PCR and amplicons length.

Nested PCR	Primers	Sequence	Length of the product
First round	IC1 F 724	ATGACCGATGGACCAAG	181 bp
	IC2 R 905	GGTCGATTCGAGCCAT	
Second round	801F	CGAAATGAATGCCCTAAT	99 bp
	900R biotinylated	ATTCGAGCCATGCCAGTTAT	

Table 1. In particular, after retrotranscription and denaturation, 30 cycles of amplification at 94 °C for 15 s, 62 °C for 30 s, 72 °C for 1 min were performed, followed by a final extension at 72 °C for 7 min. The reaction volume was 25 µl (12.5 µl of 2× reaction mix, 0.5 µl of Superscript III Platinum Taq mix, 0.5 µl of each primer [10 µM], 5 µl of extracted RNA and H₂O to reach the final volume). The second step consisted of denaturation for 5 min at 95 °C, followed by 35 cycles of amplification, each performed at 94 °C for 30 s, 62 °C for 30 s, 72 °C for 1 min, followed by final extension at 72 °C for 7 min. It was performed in a final volume of 20 µl using 1 µl of the first step product as a template. Applied Biosystems reagents were employed (Buffer 2× 2 µl, dNTPs [10 µM] 0.6 µl, MgCl₂ [25 µM] 1.6 µl, 1 µl of each primer 801F and 900R biotinylated [20 µM], TaqGold 100U 0.2 µl). Amplification reactions were performed on 2720 Thermal Cycler (Applied Biosystems, Milan, Italy). The sensitivity of this nested PCR was compared with that of the PCR performed with the primers used in the reference method and of a single step PCR with the internal primers used in the nested PCR. Using serial dilutions of a H1N1 2009 strain already isolated, cultured and titrated in our laboratory (Galli et al., 2010), the nested PCR was more sensitive of 2 and 1 log than the PCR of the reference method and than the single step PCR with the inner primers, respectively.

After Ethical Committee approval and patients' informed consent, a total of 75 clinical samples (70 throat swabs and 5 endo-tracheal aspirates), were obtained from 62 patients, either during the pandemic or during the influenza season 2010–2011. These samples that were confirmed to be positive for H1N1 2009 pandemic virus through a real-time RT-PCR (Galli et al., 2010), were analyzed by pyrosequencing, after nested PCR amplification. At the diagnostic real time the threshold cycle (Ct) for these samples varied between 20 and 37 (median 32, mean 30.54). Thirty patients were children (≤15 years) and 32 were adults. Altogether, 31 specimens were from children and 44 from adult patients. Seventeen children were hospitalized in the onco-hematology unit (Meyer Children's Hospital, Florence, Italy) with severe influenza, while 13 were outpatients with a mild form of the disease. Twenty of the 32 adult patients were hospitalized in the Intensive Care Unit (ICU) (Careggi Hospital, Florence, Italy), while 12 outpatients had a mild form of the disease. From outpatients, who did not receive oseltamivir treatment, only one sample was available. From all hospitalized patients a sample was taken at the moment of hospital admission, before starting antiviral therapy. From each out of 13 hospitalized patients (one child and 12 adults) a second positive sample was obtained 5–12 days after the first administration of oseltamivir.

Several precautions were taken in order to avoid cross contamination, which is a potential drawback of the nested PCR. In addition to the precautions normally taken for all the PCRs performed in our laboratory, additional precaution were taken throughout this study: no more than 10 samples were processed in each nested-PCR session and a negative control (water, instead of sample) was inserted every two samples.

For pyrosequencing analysis, single-strand biotinylated DNA from the nested PCR product, 99 bp long, and encompassing the polymorphic site C823T was purified using the PyroMark Vacuum

Table 2
Number of resistant and sensitive strains revealed by pyrosequencing.

		H1N1 strains		
		Sensitive	Resistant	Mixed
Children	Inpatients	10	5	2
	Outpatients	13	0	0
Adults	Inpatients	14	5	1
	Outpatients	12	0	0

Prep Workstation (Biotage-Diatech, Iesi, An, Italy). 30 µl of the PCR biotinylated product were captured using streptavidin sepharose beads (Streptavidin Sepharose™ High Performance, GE Healthcare Bio-Science AB, Uppsala, Sweden).

The primer used to perform sequencing (5'-AAATGAATGCCCTAAT-3') was designed using PSQ Assay Design Software Version 1.0.6 2004 Biotage AB and utilized in a 2 µM final concentration.

For pyrosequencing, the Pyromark ID 1.0 Biotage-Diatech, Italy, was used with the PyroMarkGold reagents. The results were analyzed using the PyroMark ID 1.0 software (Biotage-Diatech, Iesi, An, Italy).

In order to confirm the results of pyrosequencing, the nested PCR products were cloned and analyzed by dideoxy Sanger sequencing, as reference method for nucleotide sequence determination. The reverse inner primer 900R used in the second PCR was not biotinylated for this use. The product of the PCR was purified using QIAquick Gel Extraction Kit (Qiagen, Valencia, CA, USA). The cloning was performed according to the standard protocol of pGEM-T Easy Vector System (Promega, Madison, Wisconsin, USA). The plasmid DNA was purified by QIAprep Spin Miniprep Kit (Qiagen, Valencia, CA, USA).

Sequencing was carried out on an ABI Prism 377 automatic sequencer (Applied Biosystems, Milan, Italy) using the ABI Prism Dye Terminator cycle sequencing Ready Reaction kit.

In Fig. 1A and B the pyrograms of a sensitive and a resistant sequence are shown. In three cases, a pyrogram like that in Fig. 1C was obtained. This indicated the presence of a mixed population containing sensitive and resistant variants in the sample. The analysis by Sanger sequencing of 10 clones for each of the three samples demonstrated that in one sample 5 clones had the wild type condition with histidine at position 275, while the other 5 had tyrosine at the same position. In the second sample, 4 clones had H and 6 Y at position 275. In the third sample 9 clones had H and 1 Y. The cloning and subsequent sequencing by Sanger method of PCR products from the samples with a pyrogram like those shown in Fig. 1A or B showed only clones with H275 or H275Y, respectively.

The presence of resistant strains was demonstrated in five out of 17 children hospitalized during the second wave of the pandemic in autumn and winter 2009. Moreover, in two of the remaining 12 children, mixed populations with sensitive and resistant variants were present. As regards adult patients, a resistant strain of influenza A(H1N1)2009 was present in a respiratory sample from 2 of the 9 patients hospitalized in the ICU in November 2009. In addition, a resistant strain was detected in 3 of 11 ICU patients during the epidemic season 2010–2011. In another adult ICU patient, hospitalized during this last epidemic season, a mixed population was demonstrated. No resistant strains have been observed in the outpatients, either children or adults, affected by mild form of disease and not treated with oseltamivir (Table 2).

Regarding the 10 resistant strains found in this study, the resistant strain was already present in the specimen obtained at the onset of symptoms, before the start of oseltamivir administration, in 5 cases (4 children and 1 adult). In the other 5 cases (1 child and 4 adults), an oseltamivir sensitive strain was present in the specimen obtained at the onset of symptoms, while a resistant strain was

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