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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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A B S T R A C T

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.

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 IC2 R 905	ATGACCGATGGACCAAG GGTCGATTCGAGCCAT	181 bp
Second round	801F 900R biotinylated	CGAAATGAATGCCCCTAAT ATTCGAGCCATGCCAGTTAT	99 bp

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

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 H_2O 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 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′-AAATGAATGCCCCTAAT-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 byotinilated for this use. The product of the PCR was purified using QIAquick Gel Extration 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

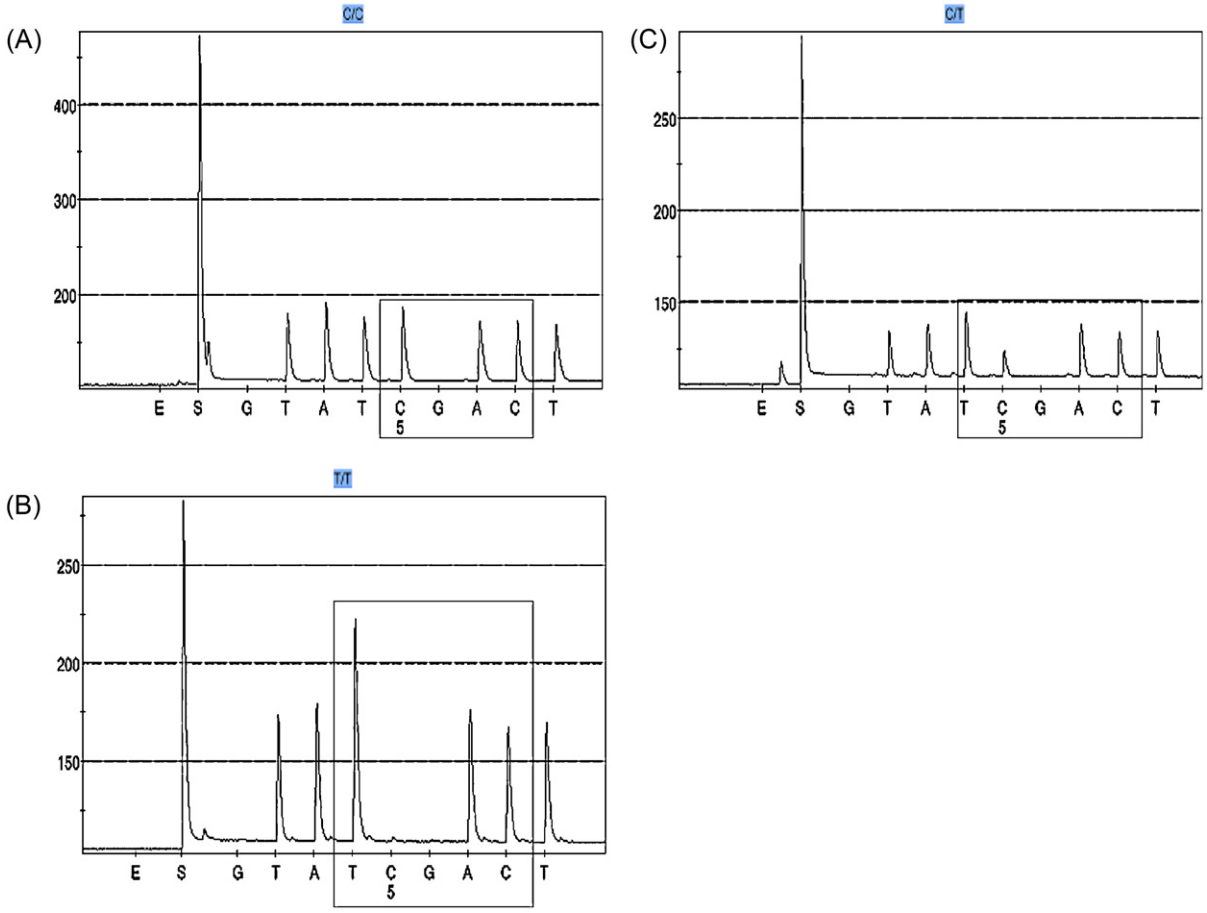

Fig. 1. Pyrograms of the sequence encompassing the target site C823T. Pyrograms representative of a sensitive strain (CAC), coding for histidine at position 275 (N1 numbering) (A), of a resistant strain (TAC), with tyrosine at position 275 (B) and of a mixed population consisting of sensitive and resistant variants (C).

demonstrated in a second specimen, taken 5–12 days later, during antiviral treatment (data not shown). Pyrosequencing proved to be a sensitive, specific and fast assay when applied to the detection of the H275Y mutation in the NA gene of the Influenza virus A(H1N1) 2009, as a marker of oseltamivir resistance. In fact, the use of a nested PCR targeting a very short sequence of the NA allowed us to detect the mutation H275Y by a pyrosequencing assay even in specimens with low viral load, inferred approximately by the Ct (>30) of the diagnostic real time (data not shown). The specificity of the pyrosenquencing, assessed by comparison with the classic Sanger method, was 96%, due to the presence of 3 samples with mixed variants. These samples, without cloning, were classified by the Sanger sequencing as sensitive or could not be accurately classified. Another advantage of pyrosequencing is its ability to detect mixed populations of resistant and sensitive variants, without the need for cloning. The presence of such mixed populations could be a warning to consider for a correct therapeutic approach. The data show that pyrosequencing can detect at least 10% of resistant (or sensitive) variants in a mixed population. These findings are in agreement with data from the literature reporting that pyrosequencing could detect as little as 5% mutants in a mixed viral population (Deng et al., 2011; Renaud et al., 2011; Yang et al., 2011). This pyrosequencing assay enables the analysis of approximately 20 samples at the same time, in about 5 h, assuring a fast turn-around time in comparison with Sanger sequencing.

The neuraminidase inhibitor oseltamivir was widely employed during pandemic and also in the following epidemic season in patients developing severe influenza. In this study resistance to oseltamivir seemed to be quite wide-spread, in contrast to other published data (Longtin et al., 2011; Hurt et al., 2011). However, the number of the patients included in this study is too small to draw statistical conclusions. Moreover, the hospitalized patients analyzed within this study were selected as at risk patients for the development of resistant virus due to immunodepression and/or prolonged antiviral treatment.

This study confirms that resistance to oseltamivir may develop mainly during antiviral treatment and that the resistant strains may be transmitted. The finding of strains with the H275Y mutation in the NA gene, at the hospital admission, before antiviral treatments, could suggest this conclusion (Baz et al., 2009; Lackenby et al., 2011). As regards the demonstration of resistant strains at the first day of disease in 4 children, we cannot ignore that three of the cases occurred within the same days in the same hospital. Therefore, the transmission could have taken place within the hospital. In 7 of the 10 cases, viral clearance occurred without turning to another antiviral drug. This could be documented by the availability of a successive, real time PCR negative, specimen taken 4–14 days after the emergence of the resistant strain. In only one patient the resistant strain persisted for at least 24 days. In this case, it was not possible to establish the time of viral clearance, because the patient recovered and was discharged from the hospital. The duration of the infection by resistant or sensitive strains in patients with severe influenza disease (data not shown) seems to indicate a trend towards a more prolonged infection by the resistant strains. Broader and more in-depth studies are needed to confirm this observation.

In conclusion, the development or the transmission of resistant H1N1 2009 strains is of concern and must be controlled, as a report

from Australia outlined (Hardie, 2011). The high sensitive nested-PCR amplicon pyrosequencing assay described in this paper may be a useful tool in achieving this purpose.

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