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High resolution melting analysis as a tool to detect molecular markers of antiviral resistance in influenza A viruses

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A real-time PCR followed by high resolution melting analysis (HRMA) was developed, for rapid detection of antiviral resistance markers in influenza A viruses, of both H1N1 and H3N2 subtypes. The targets of these assays were the nucleotide substitution G806A (S31N mutation) in the M gene as marker of resistance to adamatanes in influenza viruses A(H3N2), the substitution A356T (E119V mutation) in the N2 gene of influenza viruses A(H3N2) and the substitution C823T (H274Y mutation) in the N1 gene of the pandemic A(H1N1) 2009 virus as markers of oseltamivir resistance. First, the designed primers and the overall protocol of the HRMA were validated using already characterized viral isolates either containing or lacking changes at the tested codons. Then, HRMA was used to search for the marker of oseltamivir resistance in 75 clinical samples, H1N1 2009 positives, analyzed previously by pyrosequencing and Sanger sequencing, and of both adamantane-derivatives and oseltamivir resistance in 57 H3N2 positive clinical samples. The results of HRMA of the H1N1 2009 isolates were in agreement with those obtained by sequencing. As regards the H3N2 isolates, HRMA revealed a widespread resistance to adamantanes with 89.5% nucleotide substitution G806A, while 3% were resistant to oseltamivir (A356T change).

HRMA, applied to the detection of markers of resistance to antiviral drugs against influenza A viruses, confirmed to be a procedure flexible, low cost and time-saving, suitable for application to epidemiological surveys and in clinical settings for diagnostic purposes.

1. Introduction

Two classes of antiviral drugs are currently approved for the prophylaxis and treatment of influenza A viruses: adamantane derivatives (amantadine and rimantadine) and neuraminidase inhibitors (NAIs) (zanamivir and oseltamivir). Adamantanes inhibit viral replication by blocking the ionic channel formed by the M2 protein. The resistance to this class of antivirals emerged soon and is now widespread among influenza A(H3N2) viruses and among seasonal type A (H1N1) viruses (Bright et al., 2005; Deyde et al., 2007). The pandemic (H1N1) 2009 virus was naturally resistant to these drugs (Garten et al., 2009). The most common mutation conferring resistance to adamantanes is due to the nucleotide substitution G806A with the consequent amino acid change from Ser to Asn at residue 31 (S31N) in the transmembrane domain of the M2 protein. This mutation can interfere with the drug's ability to block M2 ion channel activity and viral replication without affecting virus transmissibility or viral replication (Bautista et al., 2010; Ison, 2009; Puzelli et al., 2011). All A(H3N2) isolates examined in 2008–2009 were resistant to the adamantanes, because of S31N mutation (CDC, 2010; Puzelli et al., 2011; Weinstock and Zuccotti, 2009). The NAIs, zanamivir (Relenza, GlaxoSmithKline) and oseltamivir (Tamiflu, Roche Pharmaceuticals), were introduced into clinical practice in 1999. These compounds were specifically designed to bind the conserved neuraminidase (NA) enzymatic site of both influenza A and B viruses, preventing virus release from the host cell following replication. The frequency of resistance to oseltamivir, most widely used (Colman, 2005), remained low until 2007 (Lackenby et al., 2008). During the 2007–2008 and 2008–2009 seasons the majority of the H1N1 strains isolated were resistant to oseltamivir (but sensitive to zanamivir), independently of patient exposure to the drug (Renaud et al., 2011; Yang et al., 2011). Interestingly, the resistant viruses identified before 2007, had compromised growth and infection ability, while these resistant H1N1 strains behave, with regard to these properties as wild-type viruses (Dharan et al., 2009; Hauge et al., 2009). The pandemic H1N1 2009 virus was sensitive to both NAIs (Renaud et al., 2011). However, the widespread use predominantly of oseltamivir, for pandemic control in economically developed countries, was followed by great concern. Sporadic cases of oseltamivir resistance were observed, among pandemic 2009 strains, This was either the result of the antiviral treatment or

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was not the consequence of the exposure to the drug, suggesting, that the resistant strains may be transmitted (Arvia et al., 2012; Baz et al., 2009; Lackenby et al., 2011; Weinstock and Zuccotti, 2009). Resistance to NAIs results from changes in the NA active site which impairs its affinity for NAIs. Two types of mutations may be involved in this phenomenon, at the catalytic site (i.e., R292K in N2) that interacts with directly the substrate, or at framework sites (i.e., E119V in N2 protein, H274Y in N1, N294S in both N1 and N2) which support the catalytic residues (Ferraris and Lina, 2008; Kiso et al., 2004; Yen et al., 2006). This last kind of mutations induces resistance without much impairing the substrate binding NA activity, virus replicative capacity in vitro, infectivity and transmissibility in vivo (Bouvier et al., 2008; Herlocher et al., 2002; Richard et al., 2008; Yen et al., 2005). Mutations of NA, related to NAIs resistance, are type and subtype specific (Ferraris and Lina, 2008; Deyde et al., 2009). The most common mutation associated with oseltamivir resistance in influenza viruses, N1 subtype, is the aminoacid change from tyrosine to histidine at position 274 (H274Y) in NA, due to the nucleotide C/T transition at 823 position (Gubareva et al., 2001; Richard et al., 2011; CDC, 2010). In N2 subtype viruses, mutation at catalytic (R292K) and framework (E119V and N294S) NA residues have been detected in oseltamivir treated patients (Aoki et al., 2007; Richard et al., 2011). Replicative studies and transmissibility experiments in ferret model have shown that influenza A(H3N2) virus with R292K mutation was not transmitted as efficiently as the wild-type virus, unlike the virus with E119V mutation (Herlocher et al., 2004; Okomo-Adhiambo et al., 2010). In accordance with these experimental observations, the E119V substitution is the most common mutation associated with high levels of oseltamivir resistance in A(H3N2) viruses (Carr et al., 2011; Okomo-Adhiambo et al., 2010; Sheu et al., 2008).

Monitoring of antiviral resistance is an essential component of influenza virus surveillance. Influenza infection in immune-compromised patients and in patients hospitalized in intensive care units with acute lung injury or acute respiratory distress syndrome, needs particularly fast assays for antiviral resistance detection.

High resolution melting analysis (HRMA) is applied increasingly in viral genomes characterization. Recently, Tong et al. (2011) and Lee et al. (2011) reported the use of two different HRM approaches for the detection and the quantification of the H274Y mutation as marker of oseltamivir resistance of pandemic virus A(H1N1) 2009 strains.

Since the last epidemic seasons were characterized by the circulation of both human influenza A subtypes, H1N1 2009 and H3N2, this study was aimed to develop a real-time PCR–HRMA, for rapid detection of antiviral resistance markers in these subtypes of influenza A viruses.

2. Materials and methods

2.1. Clinical samples

After Ethical Committee approval and patients' informed consent, a total of 136 respiratory samples from 119 patients were examined. Seventy-five samples from 62 patients, taken either during pandemic or during the influenza season 2010–2011, were positive for H1N1 2009 pandemic virus by real-time RT-PCR. Thirty patients (31 specimens) were children, 17 hospitalized in an onco-hematology unit (Meyer Children's Hospital, Florence, Italy) with severe influenza, and 13 outpatients with mild disease. Twenty of 32 adult patients (44 specimens) were hospitalized in Intensive Care Units (ICUs) (Careggi Hospital, Florence, Italy), and 12 outpatients had a mild disease. Other 61 samples from 57 patients, taken during the influenza seasons 2008–2009 and 2011–2012 were positive for H3N2 virus by real-time RT-PCR. Thirteen patients were children (two hospitalized in an onco-hematology unit with severe influenza syndrome and 11 outpatients with mild disease). Forty-four patients were adults, 27 hospitalized with severe influenza, and 17 outpatients with mild disease. All the samples tested had a threshold cycle (C_t) between 20 and 37 (median 32, mean 30.54) at the diagnostic real-time RT-PCR. This last real time RT-PCR was carried out, as a quantitative assay, according to the protocol for the M gene of influenza type A viruses (Galli et al., 2010), using serial dilutions of a standard RNA prepared to make a calibration curve. This calibrator consisted of the RNA sequence transcribed by the T7 RNA polymerase on the template of the product of the real time amplification of the M gene, cloned in the pGEM-T Easy Vector (Promega, Madison, WI, USA).

As controls for the real time PCR-HRM, for N1 and M genes, previously characterized isolates analyzed by pyrosequencing (Arvia et al., 2012) were employed. Instead, the control for the detection of subtype H3N2 resistance to oseltamivir was synthesized by PCR site specific mutagenesis.

2.2. Site-specific mutagenesis

The experimental pattern described by Erlich (1989) was used. A known sensitive isolate was employed as template for the site-specific mutagenesis PCR, in order to obtain a nucleotide sequence, including the site 356 of the N2 gene, with T instead of A in that position. At this purpose, the mutagenic primers 345F 5′-GGTGACAAGAGTACCTTATGTG-3′ and 366R 5′-CACATAAGGTACTCTTGTCACC-3′ were designed. Two one step RT-PCRs were performed using the QuantiTect virus kit, without ROX dye (Qiagen, Valencia, CA, USA), according to manufacturer's instructions, one with the primers 180F (Table 1) and 366R and another with the primers 345F and 471R (Table 1). Each reaction consisted of 30 cycles of amplification with the following thermal profile: 15 s at 95 °C, 30 s at 55 °C or 60 °C, depending on the primer sequences, 1 min at 72 °C. The amplification was followed by a final extension at 72 °C for 7 min. Similar amounts of the products of these two PCRs were mixed and 2 μl of this mixture was used as target for a further PCR with the primers 180F and 471R, in 20 μl reaction volume. After 35 cycles of amplification (95 °C for 30 s, 58 °C for 30 s, 72 °C for 1 min) a final extension at 72 °C for 7 min was performed. Applied Biosystems reagents were employed (2 μl of buffer 10×, 0.6 μl of dNTPs [10 μM], 1.6 μl of $MgCl_2$ [25 μM], 1 μl of each primer 180F and 471R [10 μM], 0.2 μl of TaqGold 250U). All amplification reactions were performed on 2720 Thermal Cycler (Applied Biosystems, Milan, Italy).

The product of this last PCR was cloned and analyzed by dideoxy Sanger sequencing. 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, WI, 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.

2.3. Viral RNAs extraction and one-step RT-PCR

Extraction of viral RNAs from clinical samples was carried out using a commercially available kit (E.Z.N.A. Viral RNA kit, Omega bio-tek, Norcross, GA, USA) according to manufacturer's instructions. QuantiTect virus kit, without ROX dye (Qiagen, Valencia, CA, USA) was used for each RT-PCR according to manufacturer's instructions, with the primers, designed using Primer3, listed in Table 1. In particular, after retro-transcription and denaturation, 30 cycles of amplification were performed (15 s at 95 °C, 30 s at temperature depending on the primer sequence (Table 1), 1 min at 72 °C), followed by a final extension at 72 °C for 7 min. The reaction