Journal of Virological Methods 189 (2013) 265–270

Contents lists available at SciVerse ScienceDirect

Journal of Virological Methods

journal homepage: www.elsevier.com/locate/jviromet

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

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*Article history:*
Received 16 October 2012
Received in revised form 25 February 2013
Accepted 27 February 2013
Available online 7 March 2013

*Keywords:*
Amantadine
Neuraminidase inhibitors
Antiviral resistance
High resolution melting analysis
Influenza viruses

A B S T R A C T

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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http://dx.doi.org/10.1016/j.jviromet.2013.02.016

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

**Table 1**
Selected primers for each one-step RT-PCR, annealing temperature and amplicons length.

| One-step RT-PCR | Primers | Sequence | Annealing temperature | Length of the product |
|---|---|---|---|---|
| N1 | IC1 724F<br>IC2 905R | 5′-ATGACCGATGGACCAAG-3′<br>5′-GGTCGATTCGAGCCAT-3′ | 62 °C | 181 bp |
| N2 | 180F<br>471R | 5′-AAACATAACGAGATAGTGTAT-3′<br>5′-GGTCCGATAAGGGGTCCTAT-3′ | 58 °C | 291 bp |
| M2 | 681F<br>981R | 5′-ATTGGGACTCATCCTAGTTC-3′<br>5′-ACTGTCGTCAGCATCCACAG-3′ | 60 °C | 300 bp |

volume was 25 μl (5 μl of 5× QuantiTect virus master mix, 0.25 μl of 100× QuantiTect virus RT mix, 0.5 μl of each primer [10 μM], 5 μl of extracted RNA and $H_2O$ to reach the final volume). Several precautions were taken in order to avoid cross contamination, which is a potential drawback of the nested PCR: 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.

### 2.4. High resolution melting (HRM)

The primers (Table 2) were designed to amplify a conserved region, in order to avoid the emergence of substitutions in sites different from those of interest, and containing the nucleotide of interest in a central position with respect to the primers. The products of the one step RT-PCR, measured by NanoDrop 1000 Spectrophotometer (ThermoScientific, Wilmington, DE, USA), were adjusted in order to add 10 ng in 2 μl of each product to the successive real time PCR. 2× HRM PCR master mix of the Type-it HRM PCR kit (with EvaGreen dye) (Qiagen, Valencia, CA, USA) was used in a final reaction volume of 25 μl, with 1.75 μl of each 10 μM primer. After initial activation step, 30 cycles of amplification (95 °C for 10 s, 55 °C for 30 s, 72 °C for 10 s (acquiring Green)) were performed. For HRM analysis, ramp from 72 °C to 82 °C (N1 gene) or from 75 °C to 85 °C (N2 gene and M gene), were used, rising by 0.1 °C each step. The reaction was performed on Rotor Gene 6000 (Qiagen, Valencia, CA, USA).

The base change A/T (GAA/GTA) is the most difficult conversion to genotype, as the $T_m$ difference between A and T is very small (<0.2 °C). Thus, to detect the nucleotide change A356T in N2 gene a modification was made. Considering that the melting profile of heterologous products is better distinguishable from that of homologous products, to make more clear the difference due to A/T change, 5 μl of PCR products was mixed with a sensitive and with a resistant reference sample, separately. The mixed products were denatured at 98 °C for 1 min and renatured at 40 °C for 5 min before performing HRMA, as described previously.

### 2.5. Sanger sequencing

In order to validate the results of HRMA, the PCR products were analyzed by dideoxy Sanger sequencing. Sequencing was carried on an ABI Prism 377 automatic sequencer (Applied Biosystems, Milan, Italy), using the ABI Prism Dye Terminator cycle sequencing Ready Reaction kit.

## 3. Results

HRMA was used to determine the nucleotide sequence of three codons containing markers of adamantane derivatives and oseltamivir resistance in influenza A viruses of the H1N1 2009 subtype, as well as of the H3N2 subtype. The targets of these assays were the nucleotide substitution G806A in the M gene (S31N mutation) as a marker of adamantanes resistance of A(H3N2) viruses, and, as markers of oseltamivir resistance, A356T change in the N2 gene (E119V mutation) and C823T change (H274Y mutation) in the pandemic A(H1N1) 2009 virus. First, the designed primers and the overall protocol of the HRMA were validated using already characterized controls, either containing or lacking changes at the codons tested.

Fig. 1(A) shows the result of the HRMA of the N1 amplicon, 134 bp long, obtained by real time PCR, of a sensitive and a resistant H1N1 2009 isolate. An intermediate pattern was observed by HRMA of a mixed population, obtained experimentally, containing similar amounts of reference isolates both resistant and sensitive. Then, HRMA was used to analyze 75 clinical samples, H1N1 2009 positives, from 62 patients, analyzed previously by pyrosequencing (Arvia et al., 2012). The results of HRMA were in agreement with those already reported. In brief, the presence of resistant strains (with H274Y mutation) was demonstrated in 10 patients (16%), all hospitalized with severe influenza syndrome, seven (5 children and 2 adults) during the pandemic, and 3 (adults) during the season 2010/11. In 5 cases the resistant strain was already present in the first sample obtained, before antiviral treatment. In addition, 3 out of 75 samples (4%) had mixed populations with sensitive and resistant variants.

As it was known that the pandemic virus was resistant to M2 blockers, the susceptibility to adamantanes was tested only for A(H3N2) viruses. In Fig. 1(B) HRM curves of a sensitive and a resistant sequence are shown. Resistance to adamantanes among H3N2 isolates was widespread, as strains, carrying the S31N mutation due to nucleotide substitution G806A, were found in 51 out of 57 patients (89.5%). Only 6 isolates, all from adult patients, were sensitive, as inferred by the presence of G at position 806 in M gene. Two were collected during the season 2008/2009 and 4 during the season 2011/2012.

E119V due to nucleotide substitution A356T is the most common mutation associated with oseltamivir resistance in N2 gene. Using the HRM protocol described for the two other mutations studied, it was not possible to distinguish clearly (Fig. 2(A)) base

**Table 2**
Selected primers for real-time PCR-HRM and amplicons length.

| Real-time PCR-HRM | Primers | Sequence | Length of the product |
|---|---|---|---|
| N1 | 724F<br>858R | 5′-ATGACCGATGGACCAAGTRA-3′<br>5′-ACTAGAATCAGGATAACAGGAGC-3′ | 134 bp |
| N2 | 280F<br>408R | 5′-ATTACAGGATTTGCACCTTTT-3′<br>5′-CTGTCCAAGGGCAAATTGAT-3′ | 128 bp |
| M2 | 735F<br>853R | 5′-TTGCAGACCTATCAGAAACGA-3′<br>5′-AAAGACGATCAAGAATCCAC-3′ | 118 bp |

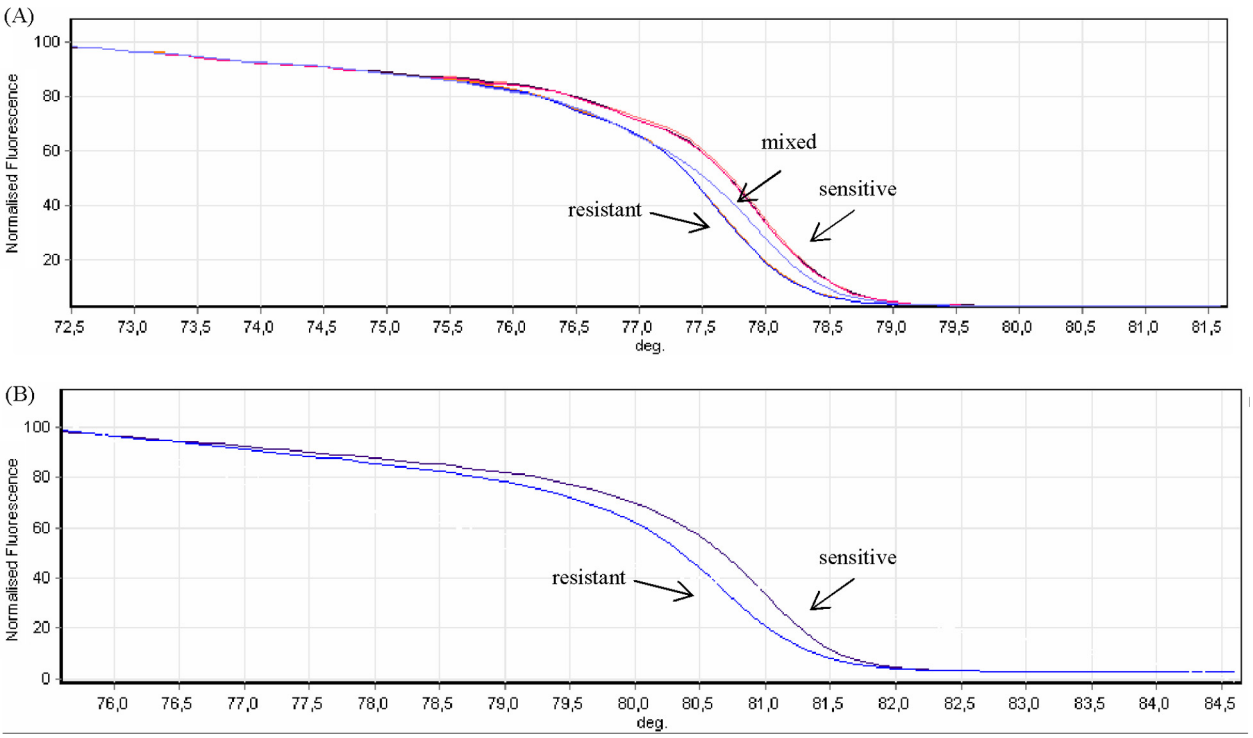


**Fig. 1.** High resolution melting analysis of N1 gene (A) and M gene (B) partial sequences encompassing the sites of interest: mutation C823T as marker of oseltamivir resistance in H1N1 subtype (A) and mutation G806A as marker of adamantane resistance in H3N2 subtype (B). (A) Arrows indicate melting profiles of N1 gene partial sequences of a wild type, sensitive, strain (C823), of a mutated, resistant, strain (T823) and of a mixed population (containing both wild type and mutated strains). (B) Melting profiles of M gene partial sequences of a wild type, sensitive, strain (G806) and of a mutated, resistant, strain (A806).

change A/T (GAA/GTA), because of the very small $T_m$ difference between A and T (<0.2 °C). Thus, to detect this mutation a modification was made. After real-time PCR, the PCR product was mixed with a same volume of the PCR product of a sensitive reference sample and, separately, of a resistant reference sample. After denaturation and renaturation of the mixed products, HRM was performed as described. If the sample analyzed contained a sensitive strain the resulting melting profile would be overlapping with that of the homologous sensitive reference sample, but, when mixed with the resistant reference sample, the profile of the heterologous product obtained would be clearly distinguishable (Fig. 2(B)). In two out of 61 isolates of H3N2 subtype from 57 patients (3%), the nucleotide T instead of A at position 356 was found. These resistant isolates were obtained during the influenza season 2011/2012 from two different hospitalized adult patients, before antiviral treatment. HRMA was able to distinguish the presence, in the same sample, of mixed populations containing both sensitive and resistant variants (Fig. 2(C)). Fig. 2(C), in fact, represents the HRMA of samples created experimentally in order to obtain mixed populations containing different percentages of sensitive and resistant reference sequences. The HRMA was able to distinguish clearly mixed population with similar amounts of sensitive and resistant variants and also 25% of resistant and 75% of sensitive variant. In all cases the results of Sanger sequencing analysis confirmed the HRMA results.

The sensitivity of this assay can be inferred by its ability to amplify and analyze clinical samples with high $C_t$ (up to 37, 20 copies/μl) at the diagnostic RT real time PCR. Each clinical sample was assayed in duplicate. In addition, in order to assess the intra-assay and inter-assay variability of HRMA, ten different samples for M and N1 genes (five wild-type and five with mutation) and seven different samples for N2 gene (five wild-type and two with the mutation E119V) were assayed three time in the same run as well as in four different runs with always reproducible results.

## 4. Discussion

The resistance to antivirals against influenza viruses, either adamantane derivatives or NAIs, may develop and spread in an unforeseeable manner. Amantadine resistance was rare among influenza viruses A(H3N2) until 2004, whereas in 2005 the incidence of resistant strains was increased and exceeded 90% in several countries (Deyde et al., 2007). NAIs resistance among influenza viruses A(H1N1) was low until 2007 while the seasons 2007–2008 and 2008–2009 were characterized by an unprecedented circulation of oseltamivir resistant H1N1 strains (Deyde et al., 2009). In patients at risk and in severe influenza syndromes antiviral drugs may be used for prophylaxis and treatment respectively. These considerations involve the need of suitable assays for antiviral resistance detection. Phenotypic assays give wide and accurate information concerning antiviral resistance of influenza virus strains. However, the procedure to perform such tests is complex and time consuming, whereas for diagnostic purpose rapid assays are required. Here, HRMA was applied to the detection of three nucleotide substitutions associated with the development of resistance to antiviral drugs available for influenza viruses of type A, either of the subtype H1N1 or of the subtype H3N2. This assay allowed us to identify the nucleotide substitutions in the target genes (N1, N2 and M) after a PCR amplification of the target sequence directly on the clinical sample, without need of viral amplification in cell cultures. To perform HRMA we used a commercial kit containing EvaGreen, the novel double-stranded DNA-binding fluorescent dye, which does not interact with single-stranded DNA. In contrast to the Sybr-Green, Eva-Green can be used in higher concentrations and shows equal binding affinity for GC-rich and AT-rich regions with no apparent sequence preference. HRMA enables to assess the sensitivity (or the resistance) of the influenza virus present in the sample in about 3 h. In fact, it requires about 20 min and about 2 h and a half are needed for

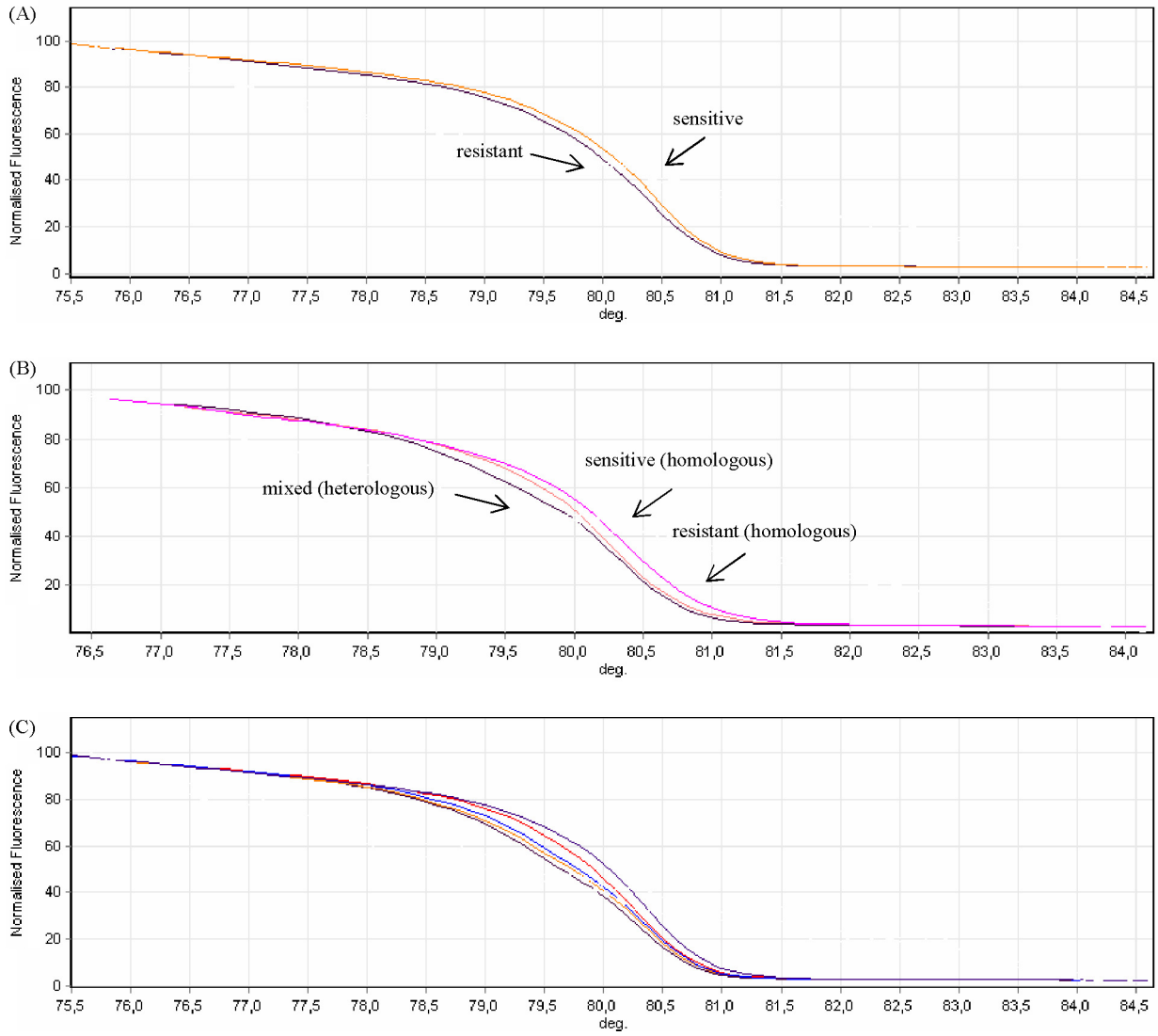


**Fig. 2.** High resolution melting analysis of N2 gene partial sequence encompassing the site 356. (A) Arrows indicate melting profiles of the sequence with A356 (GAA → glutamic acid at position 119) and of sequence with A356G (GTA → valine at position 119). (B) Arrows indicate melting profiles of mixed sensitive sequences (GAA) (homologous), of mixed resistant sequences (GTA) (homologous) and mixed sequences of sensitive and resistant variants (heterologous). (C) Melting profiles of mixed populations containing different percentages of sensitive and resistant sequences (50% of sensitive and resistant, 25% resistant and 75% sensitive, 75% resistant and 25% sensitive).

the amplification reactions. It must be stressed however that this assay, as other genotypic assays, at variance with the phenotypic assays, is able to detect only known specific markers of resistance.

HRMA is a high-throughput assay, that can allow diagnostic laboratories to analyze from 30 to more than 100 samples in the same run (depending on the type of instrument).

In addition, HRMA does not require fluorescent-probes or specific and expensive instruments. In fact, it can be performed using a number of commercial instruments for real time PCR, provided with the HRMA software.

One-step RT-PCR coupled to the real time PCR–HRMA was able to analyze in a reproducible manner also clinical samples with low viral load (20 copies/μl).

With regard to the diffusion of the resistance to oseltamivir among the H1N1 2009 pandemic strains, the results of this study confirm those obtained in a previous analysis on the same samples performed by pyrosequencing (Arvia et al., 2012).

With regard to the resistance to adamantanes and to oseltamivir among influenza virus strains of subtype A(H3N2), the results reported herewith confirm that the resistance to adamantanes is largely spread. In fact, only a small percentage (10.5%) showed sensitive to these drugs, while only 3% of the isolates of subtypes H3N2 were resistant to oseltamivir. In these cases the resistant strain was present before the begin of the antiviral drug treatment. This study confirms that resistance to oseltamivir may develop during antiviral treatment and also that resistant strains of both subtypes may be transmissible.

In conclusion, HRMA applied to the detection of markers of resistance to antiviral drugs against influenza A viruses showed to be specific, sensitive and reproducible. However, the analysis of a larger number of clinical samples is required to fully validate the specificity of the proposed assays. HRMA confirmed, also in this application, to be a low cost, time-saving, and flexible procedure, suitable for epidemiological surveys and for diagnostic purposes in clinical settings.

## Acknowledgments

This study was supported in part by grants from Istituto Superiore Sanità, Progetto finalizzato 2010 “Studio della

farmaco-resistenza nei virus influenzali pandemici ed epidemici" and from the Foundation "Istituto di ricerche virologiche Oretta Bartolomei Corsi" (Florence, Italy).

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