Journal of Virological Methods 163 (2010) 109–115

Contents lists available at ScienceDirect

Journal of Virological Methods

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

Rapid quantification of single-nucleotide mutations in mixed influenza A viral populations using allele-specific mixture analysis

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Article history:
Received 30 May 2009
Received in revised form 31 August 2009
Accepted 7 September 2009
Available online 15 September 2009

Keywords:
Single-nucleotide polymorphism (SNP) analysis
Quantitative genotyping
Allele-specific PCR
Antiviral resistance
Mixture analysis
Influenza resistance

A B S T R A C T

Monitoring antiviral resistance in influenza is critical to public health epidemiology and pandemic preparedness activities. Effective monitoring requires methods to detect low-level resistance and to monitor the change in resistance as a function of time and drug treatment. Resistance-conferring single-nucleotide mutations in influenza virus are ideal targets for such methods. In the present study, fives sets of paired TaqMan® allele-specific PCR (ASPCR) assays were developed and validated for quantitative single-nucleotide polymorphism (SNP) analysis. This novel method using ΔCt is termed allele-specific mixture analysis (ASMA) or FluASMA. The FluASMA assays target L26F, V27A, A30T, and S31N mutations in the A/Albany/1/98 (H3N2) M2 gene and H275Y mutation in the A/New Caledonia/20/99 (H1N1) NA gene and have a limit of quantification of 0.25–0.50% mutant. The error for % mutant estimation was less than 10% in all FluASMA assays, with intra-run ΔCt coefficient of variance (CoV) at ≤2% and inter-run ΔCt CoV at ≤5%. Results from the current study demonstrate that FluASMA is a highly sensitive and quantitative SNP analysis method, even for minor mutant components (<1%).

1. Introduction

The rise in antiviral resistance in influenza poses a major challenge to influenza pandemic preparedness (Regoes and Bonhoeffer, 2006). Given the limited treatment options in influenza, with adamantanes and neuraminidase inhibitors (NAI) as the two FDA-approved drug classes, development of rapid, sensitive resistance monitoring methods becomes time-critical and relevant globally (Lipsitch et al., 2007). Single-nucleotide mutations that confer resistance via a single amino acid substitution in influenza are well-characterized and have been used effectively as markers in monitoring resistance (Sheu et al., 2008). Of particular interest are the mutations in the two genes that encode the matrix protein 2 (M2) and neuraminidase (NA) proteins. M2 is a transmembrane ion channel protein responsible for acidifying the interior of the virion, for viral uncoating, and is the target of adamantanes (amantadine and rimantadine). NA is a surface peptide that affects the release of the virus from the cell surface, thereby playing an important role in influenza virulence and infectivity, and is the target of the NAIs (oseltamivir and zanamivir) (Moscona, 2005).

In the M2 gene, L26F, V27A, A30T, S31N are among the most prevalent amino acid substitutions and their molecular effects have been described in detail (Schnell and Chou, 2008). Adamantane resistance caused by mutations in the M2 gene was relatively rare until the 2003–2004 season (Bright et al., 2005; Cox and Klimov, 2005) but by 2005–2006, 92.3% of H3N2 isolates in the US were found to be resistant (Bright et al., 2006). Due to the widespread resistance to adamantanes globally, the Centers for Disease Control no longer recommend the use of this class of drugs as monotherapy for the treatment of influenza. In the NA gene, H275Y (N1 numbering) is a common and well-characterized mutation in the N1 subtype (Sheu et al., 2008; Baz et al., 2007; Abed et al., 2006; Ives et al., 2002). Oseltamivir-resistance was reported at <1% during the first three seasons of its use, beginning in the winter of 1999 (Monto et al., 2006), but H275Y-mediated resistance in H1N1 during the 2007–2008 season saw a rapid increase, with 12.3% found in the US (Dharan et al., 2009) and approximately 14% in Europe (Lackenby et al., 2008a). In the 2008–2009 season in the United States, nearly 100% of the H1N1 strains tested have been found to be oseltamivir-resistant (Moscona, 2009). This dramatic increase in adamantane- and oseltamivir-resistance poses a great challenge in antiviral selection and further empha-

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doi:10.1016/j.jviromet.2009.09.007

sizes the importance of effective global influenza drug resistance monitoring.

Influenza NA and M2 sequencing analysis is used commonly for monitoring of resistance-conferring mutations in influenza, with Biotage®, a pyrosequencing platform emerging as the most frequently used method (Deyde et al., 2009; Duwe and Schweiger, 2008; Lackenby et al., 2008b). However, Sanger sequencing and Biotage® each have sensitivity limitations when analyzing mixed viral populations. Sanger sequencing has a detection threshold of 25% minor component in a mixed sample and relies on visual evaluation of the electropherogram for quantification (Larder et al., 1993). Biotage® is capable of quantitative sequencing but has a reported quantification threshold of >5% for H275Y mutant in H5N1 (Duwe and Schweiger, 2008).

Allele-specific real-time PCR (ASPCR) is rapid, sensitive, and highly quantitative. ASPCR is relatively unknown in influenza drug resistance monitoring but has had wide and successful usage in HIV, particularly in quantitative characterization of minor, emerging drug-resistant HIV populations (Metzner et al., 2009; Johnson et al., 2008; Louvel et al., 2008; Peuchant et al., 2008; Detsika et al., 2007; Metzner et al., 2005). ASPCR targets single-nucleotide polymorphisms (SNPs) by incorporating one or more mismatches in a selective primer either near or at the SNP. As a result, ASPCR assays can be designed to target different SNPs known to confer resistance with relative ease. Of the various approaches in ASPCR, HIV drug resistance monitoring has favored a combination of an ASPCR and a qPCR assay, where the ASPCR measures and amplifies preferentially the total mutant load and the "pan-HIV" qPCR measures the total viral load. Dividing the mutant load by the total viral load generates a ratio of mutant:total virus (Louvel et al., 2008). Using this approach, it is possible to characterize quantitatively minor resistant HIV populations for <0.1% mutant alleles to detect minor resistant virus (Metzner et al., 2005).

While this ASPCR approach is highly sensitive and quantitative, it has potential problems for influenza. As a segmented virus with M2 and NA genes on different gene segments, comparing the total influenza load generated from one segment with the mutant load generated from a separate segment may not be accurate. In addition, if the allele ratio at the SNP of interest is the desired genotyping output, it is more logical to quantify the wild-type viral load, not the total viral load. To do so, it is necessary to design ASPCR assays that quantify each allele state by generating separate threshold cycle values for the wild-type and the mutant allele. Ideally, this relative quantification method should be independent of an in-run standard curve, which is both time and resource consuming.

In the current study, we present the design and validation of five sets of allele-specific mixture analysis (ASMA) assays, referred to hereafter as "FluASMA". The FluASMA method is based on ASPCR and ΔCt (i.e., $Ct_{mutant} - Ct_{wild\text{-}type}$) and it quantitatively characterizes SNPs associated with amino acid changes known to confer antiviral resistance in influenza A (M2 gene: L26F, V27A, A30T, S31N; NA gene: H275Y).

2. Materials and methods

2.1. Generation of target cDNA mixtures for FluASMA validation

2.1.1. Plasmid construction using cDNA or synthetic plasmids

We generated plasmid standards containing the appropriate gene inserts using cDNA preparations as template in an initial PCR step, followed by insertion of the PCR amplicons into plasmid vectors for clonal propagation in *E. coli*. We employed sequence-confirmed cDNA (from A/Victoria/3/75) for the seasonal influenza (H3N2) M2 mutations: L26F, V27A, and S31N. For M2 mutation A30T and NA mutation H275Y, we generated synthetic DNA plasmid standards for use in assay validation (see below).

For the initial PCR step, we used A/H3N2-specific M2 primers with forward primer 5′ CYA GCA CTA CAG CTA AGG CTA TGG AGC A 3′ and reverse primer 5′ CAT CCA CAG CAY TCT GCT GTT CCT 3′ to generate a 399 bp amplicon and seasonal A/H1N1-specific NA primers with forward primer 5′ CAA AGG AGA TGT TTT TGT CAT AAG AGA ACC 3′ and reverse primer 5′ CTC CAT CAA CAG TCA CTG GAT TAC AGC 3′ to generate a 698 bp amplicon. All PCR were performed using 1 μl of cDNA template in 14 μl of PCR master mix containing 200 nM primers, 300 μM dNTPs, 3 mM MgCl, 1 U Taq Polymerase per reaction (Invitrogen, Carlsbad, USA), and 1× Taq DNA Polymerase PCR buffer (Invitrogen) and the following thermocycling condition: 3 min at 95 °C for initial denaturation and 40 cycles of 30 s at 95 °C for denaturation, 30 s at 57.3 °C for annealing, and 1 min at 72 °C for extension, and final extension for 7 min at 72 °C. A TOPO TA Cloning Kit (Invitrogen) was used for the subsequent plasmid insertion and clonal propagation according to the kit instructions. Plasmid extractions were performed with QIAprep Spin Miniprep kit (Qiagen, Valencia, USA) and the plasmids preparations were sequence verified.

For the A30T and H275Y standards, plasmids with cloned synthetic gene inserts containing the targeted mutation were synthesized by Blue Heron Biotechnology GeneMaker® (Bothel, USA). Sequence of the synthetic A30T gene insert was generated by aligning common laboratory influenza A strains. Sequence of the H275Y gene insert was generated by aligning all available H275Y and select recent influenza A/H1N1 sequences from GenBank (GenBank accession nos. EU124179, CY030233, EU516196, EU566980, EU516267). An additional 100 bp of up- and down-stream sequences were also incorporated. Synthetic plasmid standards were supplied as pUC119 plasmid vectors with the appropriate gene inserts in *E. coli*. Clonal propagation of the plasmid and plasmid extraction were performed in an identical fashion as the plasmid standards generated from cDNA.

2.1.2. Generation of quantified plasmid mixtures

Quantified plasmid mixtures were generated at a concentration of 10^9 copies/μl using an in-house plasmid qPCR to first normalize the plasmid standards to 10^9 copies/μl. The normalized plasmids were then used to generate mutant:WT mixtures (50:50, 10:90, 5:95, 1:99, 0.5:99.5, 0.25:99.75) at 10^9 copies/μl.

2.2. FluASMA primer screening and assay validation

2.2.1. qPCR experimental procedure

All qPCR were performed in 10 μl reaction volumes in PRISM™ 384-well Clear Optical Reaction Plates (Applied Biosystems, Foster City, USA). In each 10 μl reaction, 1 μl of template was added to 9 μl of qPCR master mix containing 900 nM of each primer (Table 1), 225 nM of the appropriate TaqMan® MGB probe (Table 1), 1× TaqMan® Universal PCR Master Mix (Applied Biosystems), and molecular-grade water. The wild-type and the mutant assays were performed in separate reactions. All reactions, including the no-template controls (NTCs) were performed in triplicate. Amplification and real-time fluorescence detections were performed on the 7900HT Real Time PCR System (Applied Biosystems) using and the following PCR conditions: 3 min at 50 °C for UNG treatment, 10 min at 95 °C for Taq Polymerase activation, 40 cycles of 15 s at 95 °C for denaturation and 1 min at 60 °C for annealing and extension.

2.2.2. Primer screening and assay validation

For primer screening, all sets of candidate allele-specific PCR assays were screened using the appropriate mutant and WT cloned plasmid standards as templates across three 10-fold dilutions and a 50:50 mutant:WT mixture across two 10-fold dilutions. Assays meeting predetermined criteria (see Section 2.3.1) were moved