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Short communication

Rapid molecular detection of the H275Y oseltamivir resistance gene mutation in circulating influenza A (H1N1) viruses

Michael J. Carr [1], Naomi Sayre [1], Margaret Duffy, Jeff Connell, William W. Hall *

National Virus Reference Laboratory, University College Dublin, Belfield, Dublin 4, Ireland

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In early 2008, drug susceptibility surveillance of influenza viruses in Europe revealed that some influenza A viruses (subtype H1N1) circulating during the winter season of 2007 and 2008 were resistant to the neuraminidase inhibitor, oseltamivir. This resistance arises due to a histidine to tyrosine substitution in the neuraminidase active site (H275Y in N1 nomenclature). Current methods to detect this mutation involve an end-point reverse transcription polymerase chain reaction followed by nucleotide sequencing. While accurate, this approach has the limitation of being time-consuming, labour-intensive and expensive. Herein we describe a one-step allelic discrimination assay which rapidly (2 h) detects this resistance mutation. The sensitivity of the assay was as low as 10 copies per reaction and is capable of detecting the antiviral resistance mutation in a mixture of wild type H275 and mutant H275Y targets.

Influenza A virus is a member of the genus of *Orthomyxoviridae* RNA viruses. It is an important human pathogen and a cause of significant annual global morbidity and mortality. There is increasing and widespread concern on the possibility of the emergence of a pandemic strain of influenza (Lipatov et al., 2004). The M2 inhibitors were the first drugs to be used specifically against influenza A, in the mid-seventies. However, resistance to this drug emerges rapidly *in vitro* and *in vivo*, and the resistant strains appear to be fully pathogenic and transmissible (Hayden and Hay, 1992). Oseltamivir and zanamivir are neuraminidase inhibitors (NAIs), representing a more specific family of antiviral drugs, which are now licensed for prevention and treatment of influenza A and B (Gubareva et al., 2000; Hayden et al., 1997; Treanor et al., 2000). It was considered that this class of drug would be far less likely to promote the development of drug resistance because they interact with highly conserved residues in the viral neuraminidase (Burmeister et al., 1991; Taylor and von Itzstein, 1994). The global NAI Susceptibility Network, in collaboration with the World Health Organisation (WHO), was created to monitor the emergence of NAI-resistant viral mutants and has not found until recently any spontaneous resistance to NAIs, and very little emergence of resistance in treated patients (Zambon and Hayden, 2001). Surveillance in previous years have found <1% of circulating viruses to be resistant to oseltamivir and none to zanamivir (Monto et al., 2006). They usually occurred following treatment, but had not been shown to be readily transmissible and this was considered to be due possibly to decreased virus fitness (Carr et al., 2002; Colacino et al., 1997; Gubareva et al., 1997; Herlocher et al., 2004; Ives et al., 2002; Tai et al., 1998; Yen et al., 2005).

The predominant influenza A viruses that have being circulating in Europe during the 2007–2008 season are subtype H1N1 viruses, antigenically similar to the A/Solomon Islands/3/2006 virus which was included in the 2007–2008 northern hemisphere vaccine. However recently, surveillance studies have shown that a significant proportion of circulating viruses are presenting resistance to oseltamivir (Euro Surveill, 2008). Analysis of 2813 influenza A/H1N1 viruses from 24 European countries isolated between November 2007 and June 2008 showed that 701 were resistant to oseltamivir (mean 25%), but of those tested all samples analysed retained sensitivity to the alternative NAI, zanamivir (the E119 residue in NA1) and the M2 proton pump blocker amantadine (S31 in the M2 protein) (Euro Surveill, 2008). The studies to date have also shown considerable differences in the patterns of resistance in different geographical regions. There is no evidence that these influenza A/H1N1 viruses have arisen directly as a result of treatment by oseltamivir, which is currently not prescribed widely in most European countries. Global surveillance coordinated by the WHO has also subsequently found evidence of resistant viruses in North America and in the Far East. Clinical experience in Norway has suggested that individuals who become ill with an oseltamivir resistant strain of influenza A/H1N1 have a similar spectrum of illness to those infected with the wild-type seasonal influenza A (Euro Surveill, 2008).

* Corresponding author. Tel.: +353 1 7161236; fax: +353 1 7161239.
E-mail address: william.hall@ucd.ie (W.W. Hall).
[1] These authors contributed equally to this work.

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Although sporadic low level transmission of drug-resistant viruses may have occurred since 1999 when the NAI class of drugs were first licensed (Carr et al., 2002; Escuret et al., 2008; Gubareva et al., 1998, 2001; Ives et al., 2000; Kiso et al., 2004; McKimm-Breschkin et al., 2003; Whitley et al., 2001), the 2007/2008 winter season is the first time there has been widespread and sustained transmission of such viruses in the community. Current methods for monitoring drug resistance include phenotypic and genotypic analysis of neuraminidase gene (Meijer et al., 2007), pyrosequencing (Bright et al., 2006), measure of hemagglutination titers in presence of drug (Bright et al., 2006; Su et al., 2008), microarray for the detection of adamantine resistance (Townsend et al., 2007) or flow cytometric analysis of virus-infected cells (McSharry et al., 2004), all of which are time-consuming.

New molecular techniques are required urgently for the rapid detection of the influenza A/H1N1 H275Y oseltamivir resistance mutation to monitor its transmission in the community and begin clinical investigations of disease severity and response to antivirals following infection. A rapid assay (2 h) was developed using a one-step allelic discrimination reverse transcription real-time polymerase chain reaction (AD RT-PCR) to detect the oseltamivir resistance mutation (H275Y) in the influenza A/N1 gene.

Respiratory samples are sent to the National Virus Reference Laboratory (NVRL) for epidemiological investigations and are tested by molecular screening for influenza by real-time PCR using an internally controlled multiplex influenza A and B assay (Gunson et al., 2005). Oseltamivir is not prescribed routinely in Ireland, and all patients from whom respiratory specimens were obtained had not been treated. This season, following direct sequencing of the influenza A/N1 cDNA, six from 62 samples (9.7% prevalence) were found to have the H275Y oseltamivir resistance mutation.

A validation panel was assembled comprising respiratory samples from influenza seasons from 2006 to 2008 ($n = 88$). This included RNA extracted from throat swab specimens from the following: 27 positive for wild-type influenza A/H1N1 (H275), five influenza A/H1N1 H275Y positive mutants, 22 influenza A/H3N2 positives, 10 influenza B positives and nine samples negative for both influenza A and B. The specificity of the AD RT-PCR assay was tested for cross-reactions with potential co-infecting viral pathogens including respiratory syncytial virus, parainfluenza virus-1, parainfluenza virus-3, adenovirus-4, adenovirus-7, enterovirus-7, rhinovirus-16, rhinovirus-72, rhinovirus-90, human metapneumovirus, human coronavirus 229E, human coronavirus OC43, mumps virus, measles virus, coxsackie B4 virus and an adenovirus/bocavirus co-infection. Two types of negative controls were included in each AD RT-PCR assay run comprising RNA from influenza A and B negative samples and no template controls. All the other samples (listed above) had been identified previously as such by PCR-based typing and/or sequencing methods.

RNA for the validation panel was extracted from respiratory specimens using a modified version of the manufacturer's instructions for the QIAamp viral RNA minikit (Qiagen, Crawley, UK). 150 μl of respiratory specimen was added to an equal volume of kit lysis buffer (AVL with carrier RNA added to 1 μg μl^{-1}) and heated at 70 °C for 15 min. 300 μl of 100% ethanol was added and applied to columns and washed as per manufacturer's instructions with a final elution in 50 μl of the kit EB buffer pre-heated to 50 °C. A one-step allelic discrimination reverse transcription real-time polymerase chain reaction (AD RT-PCR) was carried out using 5 μl of RNA eluate in a 25 μl reaction volume with the SuperScript III Platinum One-Step qRT-PCR System (Invitrogen) on an Applied Biosystems 7300 SDS with the following cycling parameters: 50 °C for 15 min; 95 °C for 15 s and 60 °C for 2 min for 40 cycles. Data acquisition was performed in both FAM and VIC filters during the annealing/extension stage. Oligonucleotide primers (900 μM final concentration; Applied Biosystems) for the AD RT-PCR were as follows: FluAN1-H275F 5′-CCGCCTCGTACAAAATCTTCAAGA-3′ (sense); FluAN1-H275R 5′-CAGTGTCTGGGTAACAGGAACATT-3′ (anti-sense) and 200 μM final concentration of each of the allele specific probes with 5′ dyes and 3′ minor groove binding non-fluorescent quenchers (MGBNFQ): FluAN1-H275 VIC-5′-CTCATAAT**G**AAAATTG-MGBNFQ (oseltamivir sensitive probe) and FluAN1-H275Y FAM-5′-CCTCATAAT**A**AAAATTG-MGBNFQ (oseltamivir resistance probe). The five influenza A/H1N1 H275Y mutants described in this work have been submitted to the influenza database as ISDN299176-ISDN299180 at http://www.flu.lanl.gov/.

In order to determine the limit of detection for the AD RT-PCR assay, a 108-bp cDNA fragment was amplified (using primers FluAN1-H275F and FluAN1-H275R) from a sequence confirmed influenza A/H1N1 H275 oseltamivir sensitive sample and a sequence confirmed influenza A/H1N1 H275Y oseltamivir resistant sample, with the Qiagen one-step RT-PCR kit (Qiagen, Crawley, UK) at the following reaction conditions: 50 °C for 30 min, 95 °C for 15 min, 30 cycles of 94 °C for 30 s, 58 °C for 30 s and 72 °C for 30 s and a final extension at 72 °C for 10 min. These influenza A/H1N1 H275 and A/H1N1 H275Y gene amplicons were purified using a PCR purification kit (Qiagen, Crawley, UK) and quantified on a Nanodrop ND-100 spectrophotometer. Standard curves were generated employing serial dilutions of these amplicons in nuclease free water. Linearity and assay sensitivity in copies per AD RT-PCR reaction were determined in triplicate employing the AD RT-PCR for each target.

The mutation conferring oseltamivir resistance arises through a cytosine to thymine substitution of the influenza A/N1 gene (nucleotide position 823 in the 2007/2008 influenza A/Solomon Islands/3/2006 (H1N1) vaccine strain) and was detected by an allelic discrimination strategy using two differently labelled probes across this single nucleotide polymorphism (SNP). The sensitivity of the AD RT-PCR for the influenza A/H1N1 H275 wild-type was 10 copies (3/3 reactions detected) whereas it is between 10 and 100 copies for the H275Y mutant target (the H275Y SNP detection probe detected 2/3 reactions at 10 copies of target and 3/3 reactions of 100 copies, see Fig. 1A and B). The assay was linear over the range 10^1 to 10^8 copies and the slopes were −3.5 with correlation coefficients (R^2-value) >0.99 for both targets demonstrating a broad dynamic detection range for the method and efficient amplification.

Analysis of the assay results showed that all the negative controls (no template control and influenza negative samples) emitted no detectable fluorescence in either FAM or VIC filters above background levels. The fluorescent signals emitted following AD RT-PCR from the validation panel can be seen in Fig. 2A and B. All influenza A A/H1N1 sequence confirmed oseltamivir sensitive specimens emit fluorescence detectable in the VIC filter (560 nm). However, all five influenza A/H1N1 H275Y oseltamivir resistant mutants containing the C>T SNP emit fluorescence with the FAM probe bound (530 nm). A 100% concordance was observed between the sequencing data and the new AD RT-PCR assay. The validation panel was run in duplicate and mean Ct values for replicates was in the range 26.5–37.8 for the wild-type A/H1N1 H275 and 30.4–37.2 for the oseltamivir resistant mutants A/H1N1 H275Y. In our experience the highest Ct values have been ~37 and were repeated in replicate samples. However, in routine diagnostic use, repeat extraction and/or sequencing could be recommended to confirm the results for these samples with high Ct values. There were no cross-reactions observed with the AD RT-PCR N1-specific primers and probes and the influenza A/H3N2 positive specimens or other common viral respiratory pathogens.

To ascertain the ability of the AD RT-PCR to amplify from mixed populations of wild-type influenza A/H1N1 H275 and H275Y

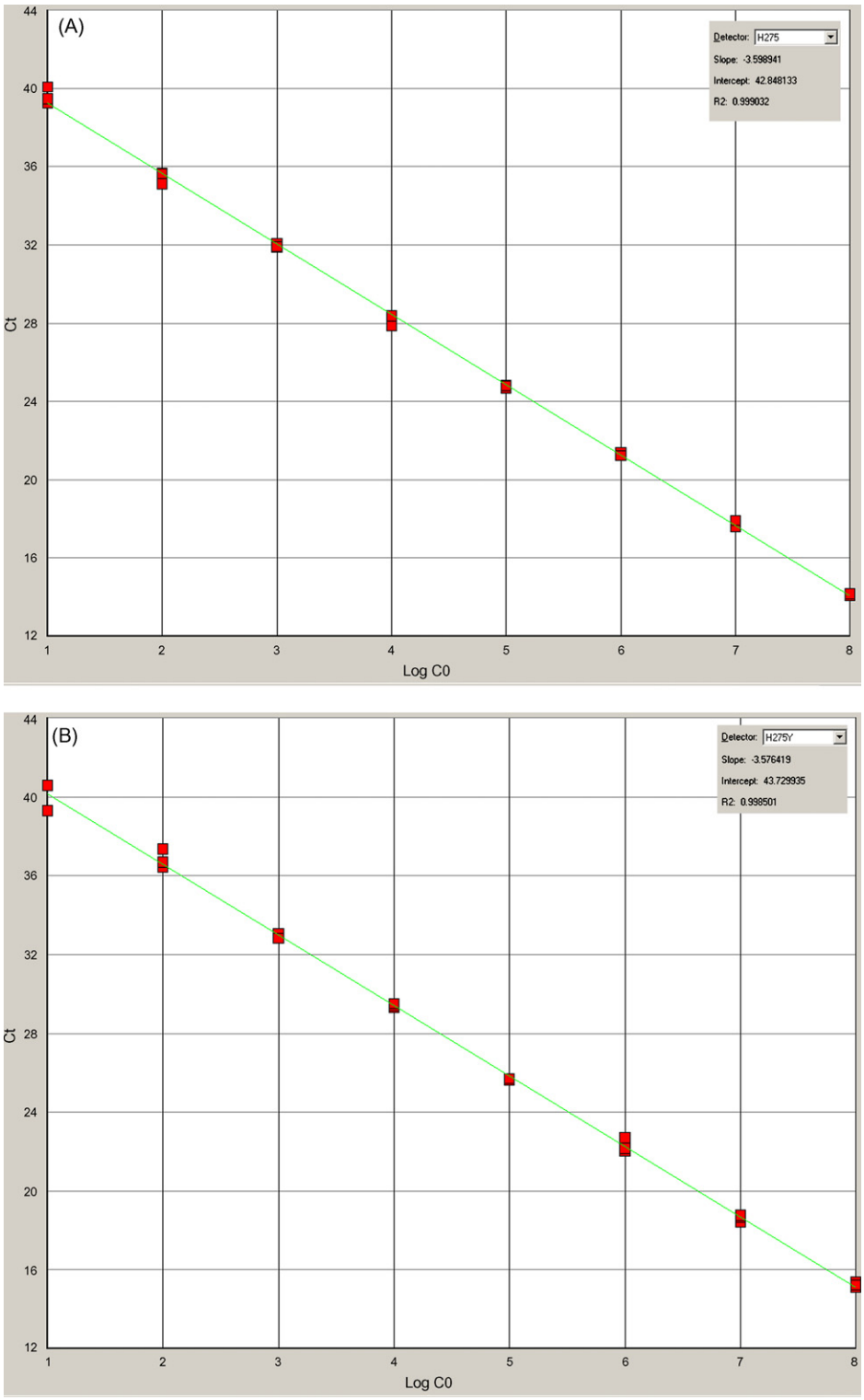

Fig. 1. Determination of the limit of detection of the AD RT-PCR assay for influenza A/N1 H275 (A) and H275Y (B). Quantified standards in the range 10^8 to 10^1 copies were run in triplicate for each target and plotted as Ct (crossing threshold) values versus the log of the copy number of the standard. PCR parameters are included for each assay.

mutant viruses, the quantified amplicons were mixed in the following copy number ratios (H275/H275Y): 100/900, 200/800, 300/700, 400/600, 500/500 for each target and assayed in triplicate (data not shown). The samples containing mixed populations of 500 copies each of H275 and H275Y, were found to emit fluorescence with both the FAM probe bound and the VIC probe bound (Fig. 3A) demonstrating that a specimen containing species of both the wild-type oseltamivir sensitive and the drug resistance mutation in the N1 gene can be distinguished employing the AD RT-PCR. The threshold concentration for detecting both wild-type and mutant was found to be at least 100 copies of H275Y mutant in a mixed population of 900 copies of wild-type H275 (Fig. 3B).

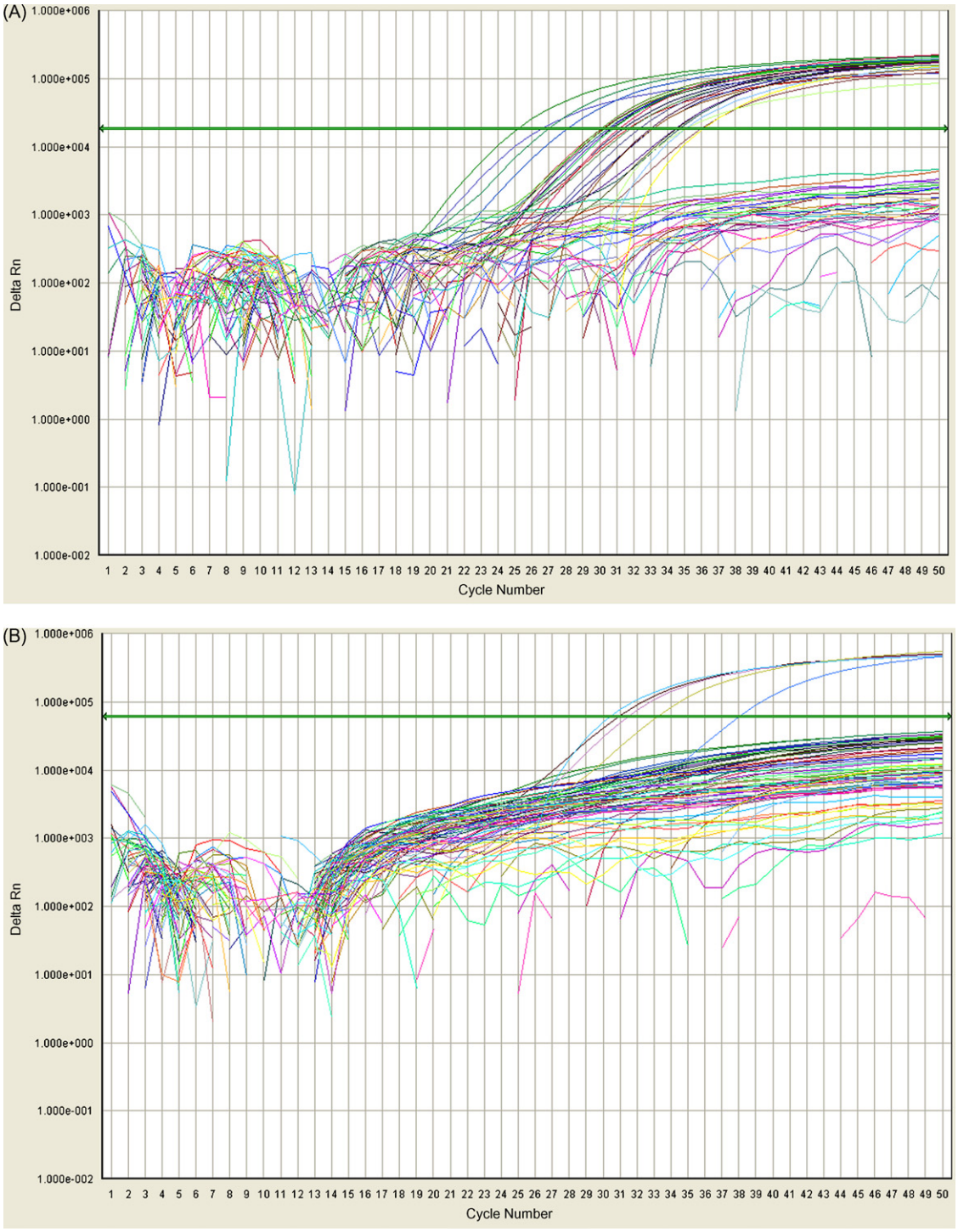

Fig. 2. Detection of oseltamivir resistant influenza A/H1N1 H275Y from clinical samples by allelic discrimination real-time RT-PCR. Delta Rn versus real-time cycle number plots showing fluorescent amplification curves from wild-type influenza A/H1N1 (H275, oseltamivir sensitive) with the VIC-TaqMan probe (A) and from A/H1N1 H275Y (B) oseltamivir resistant mutants with the FAM-TaqMan probe. Rn is the normalised signal reporter fluorescence divided by the passive internal reference dye, ROX.

There is considerable concern in the political, medical and scientific community of the likelihood of a future influenza pandemic, and for this reason many countries have decided to stockpile the NAI, oseltamivir. WHO has recommended that countries develop a strategic plan for pandemic preparedness and a crucial component of such plans involves the stockpiling of oseltamivir (WHO, 2005), that could be used to treat infected individuals in the initial stages of an outbreak to slow person-to-person spread of the virus and attempt to control its further propagation (Balicer et al., 2004; Ferguson et al., 2003, 2005, 2006; Gani et al., 2005; Germann et al., 2006; Hayden, 2004; Longini et al., 2004, 2005; Moghadas et al., 2008; Monto, 2003, 2005; Moscona, 2005, 2008).

The emergence of the influenza A/H1N1 H275Y antiviral resistance mutant across the Northern Hemisphere in 2007–2008 has

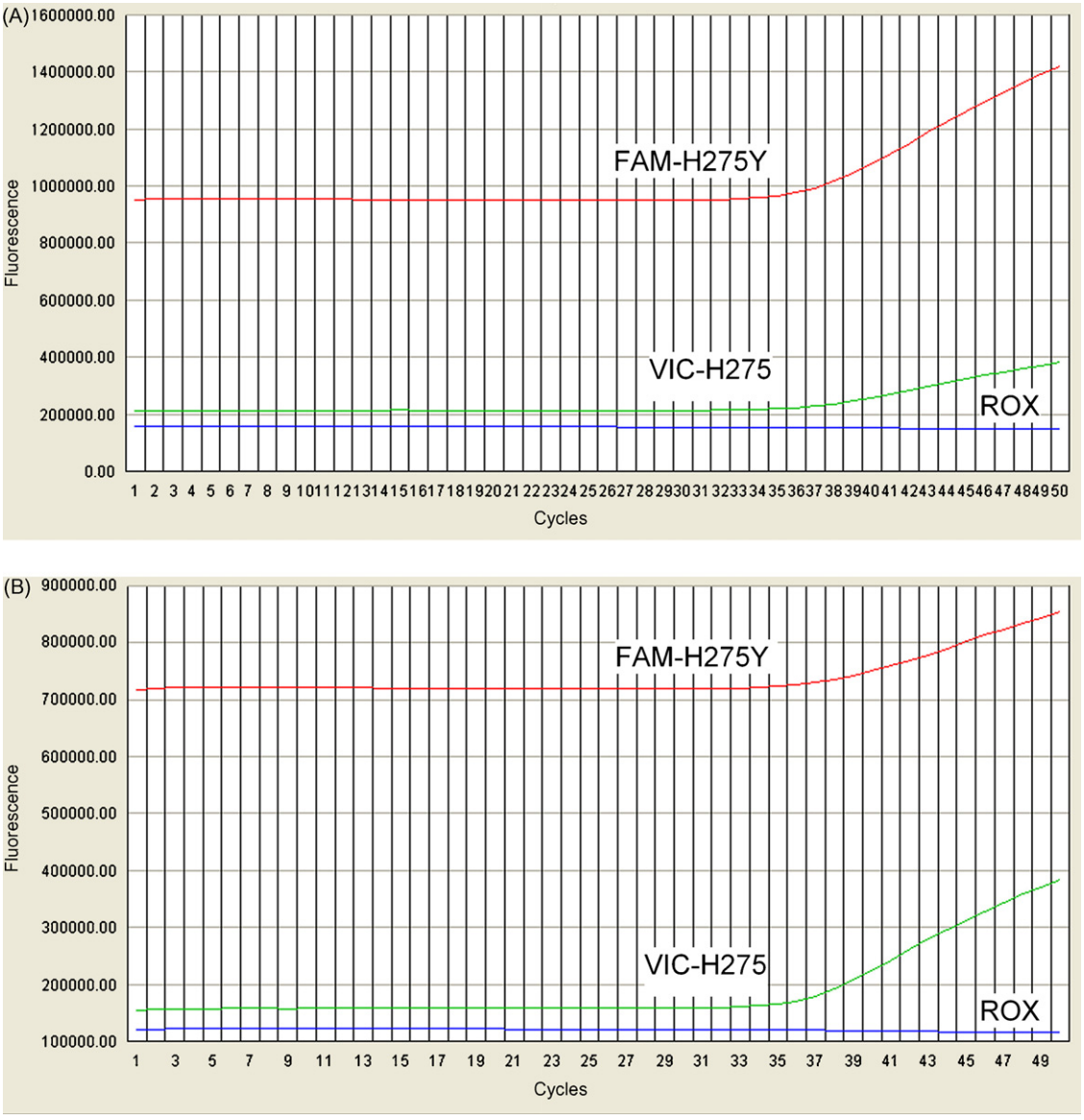

Fig. 3. Component Plots of fluorescence emission versus cycle number of mixed populations of influenza A/N1 H275 and H275Y amplicons. Detection of 500 copies of the influenza A/N1 H275 oseltamivir sensitive amplicon and 500 copies of an influenza A/N1 H275Y oseltamivir resistant amplicon by AD RT-PCR showing an increase in both FAM fluorescence and VIC fluorescence (A); 900 copies of an oseltamivir sensitive influenza A/N1 H275 amplicon mixed with 100 copies of an oseltamivir resistant influenza A/N1 H275Y amplicon was also detectable by the AD RT-PCR approach showing an increase in both FAM and VIC fluorescence (B).

forced a reappraisal of the transmissibility and fitness of these viruses. Therefore, improved and more rapid diagnostic methods are required to identify rapidly these new circulating resistant strains and also to rationalise the use of alternative therapeutic options early in infection. Chutinimitkul et al. (2007) reported a similar approach to detect the homologous H274Y mutation in the highly pathogenic avian influenza H5N1. However, little sequence homology exists between the avian and human N1 genes and the AD RT-PCR assay described above is absolutely specific to human influenza A/H1N1.

Genetic drift in viral RNA genomes lacking proof reading can cause changes in assay sensitivity over time due to mutation in the primer and/or probe binding sites. Design of degenerate oligonucleotide primer and probe sets can obviate decreases in assay sensitivity due to genetic drift in target sequences. If the AD RT-PCR assay is employed downstream of a primary screening approach to detect influenza positives then failure of the SNP assay may well be attributable to genetic drift however this is not something we have encountered to date.

The present assay will allow a more comprehensive screen of the influenza A/H1N1 viruses without the time-consuming and costly sequencing approaches. We envisage it could be employed as part of an influenza A molecular confirmatory strategy in conjunction with influenza A subtyping. We suggest it as a second line assay, following a first influenza diagnosis. It would then be an invaluable tool not only for surveillance and epidemiological purposes, but also as a diagnostic and therapeutic aid for physicians intending to treat patients with oseltamivir. The influenza A/H1N1 H275Y mutation is, at the moment, the only mutation conferring resistance to a NAI to be detected circulating from person to person, and appears therefore to be the only relevant mutation to screen in the general, non-treated population. It is important to point out that this is not the case concerning patients already undergoing treatment, for whom

a more extensive screening for resistance mutations would be required.

Finally, this new molecular detection assay could also be used in prospective clinical studies, to ascertain whether there are differential long term outcomes and treatment response between patients infected with either the wild-type influenza A/H1N1 H275 or viruses harbouring the antiviral resistance H275Y gene mutation.

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