Improved detection of genetic markers of antimicrobial resistance by hybridization probe-based melting curve analysis using primers to mask proximal mutations: examples include the influenza H275Y substitution

David M. Whiley1,2*, Kevin Jacob1,2, Jennifer Nakos3, Cheryl Bletchly3, Graeme R. Nimmo3,4, Michael D. Nissen1–3 and Theo P. Sloots1–3

1Queensland Paediatric Infectious Diseases Laboratory, Queensland Children's Medical Research Institute, Children's Health Service District, Brisbane, Queensland, Australia; 2Clinical Medical Virology Centre, Sir Albert Sakzewski Virus Research Centre, The University of Queensland, Brisbane, Queensland, Australia; 3Microbiology Division, Pathology Queensland Central Laboratory, Herston, Queensland, Australia; 4Griffith University School of Medicine, Gold Coast, Queensland, Australia

*Corresponding author. Queensland Paediatric Infectious Diseases Laboratory, Sir Albert Sakzewski Virus Research Centre, Royal Children's Hospital & Health Service District, Herston Road, Herston, Queensland, Australia 4029. Tel: +61-7-3636 1623; Fax: +61-7-3636 1401; E-mail: d.whiley@uq.edu.au

Received 27 November 2011; returned 9 January 2012; revised 23 January 2012; accepted 24 January 2012

Objectives: Numerous real-time PCR assays have been described for detection of the influenza A H275Y alteration. However, the performance of these methods can be undermined by sequence variation in the regions flanking the codon of interest. This is a problem encountered more broadly in microbial diagnostics.

Methods: In this study, we developed a modification of hybridization probe-based melting curve analysis, whereby primers are used to mask proximal mutations in the sequence targets of hybridization probes, so as to limit the potential for sequence variation to interfere with typing. The approach was applied to the H275Y alteration of the influenza A (H1N1) 2009 strain, as well as a Neisseria gonorrhoeae mutation associated with antimicrobial resistance. Assay performances were assessed using influenza A and N. gonorrhoeae strains characterized by DNA sequencing.

Results: The modified hybridization probe-based approach proved successful in limiting the effects of proximal mutations, with the results of melting curve analyses being 100% consistent with the results of DNA sequencing for all influenza A and N. gonorrhoeae strains tested. Notably, these included influenza A and N. gonorrhoeae strains exhibiting additional mutations in hybridization probe targets. Of particular interest was that the H275Y assay correctly typed influenza A strains harbouring a T822C nucleotide substitution, previously shown to interfere with H275Y typing methods.

Conclusions: Overall our modified hybridization probe-based approach provides a simple means of circumventing problems caused by sequence variation, and offers improved detection of the influenza A H275Y alteration and potentially other resistance mechanisms.

Keywords: PCR, influenza, variation

Introduction

The H275Y (N1 numbering) mutation in the influenza A neuraminidase protein is associated with oseltamivir resistance, and identification of this alteration is of importance for both public health surveillance and informing patient treatment.1,2 The H275Y mutation is caused by a single-nucleotide polymorphism (SNP) at position 823 (cytosine to thymine) of the influenza A neuraminidase gene. Numerous real-time PCR assays, using technologies such as allelic discrimination and high-resolution melting curve analysis, have been described to detect this mutation for the H1N1 (2009) strain.3–6 However, ongoing sequence variation in the regions flanking the 275 codon have proven to impede assay performance. A recent study by Trevino et al.5 observed that the presence of a nucleotide substitution at position 822 (thymine to cytosine), directly flanking the codon of interest, interfered with the performance of several H275Y real-time PCR methods, particularly allelic
discrimination-based methods using allele-specific Taqman probes. Trevino et al.\textsuperscript{5} showed that the impact of the T822C mutation could be overcome through incorporation of degenerate bases into each probe at this position. Nevertheless, the solution was specific to this particular SNP and, as highlighted in the study, ongoing monitoring of target sequences is warranted.

Melting curve analysis using hybridization probes\textsuperscript{7} is a particularly useful method for characterization of SNPs, as even a single SNP in the sensor probe target can confer a melting temperature shift of several degrees. While this makes the technique highly suitable for defining SNPs of interest, it is also this attribute, combined with the large area of conserved sequence required to accommodate both the sensor and anchor probes, that makes the technique particularly vulnerable to the presence of additional SNPs in the probe targets.\textsuperscript{8,9} It is also for this reason that the technique would otherwise not be a feasible approach for detection of influenza A H275Y alteration, given that mutation rates for influenza viruses are high and that proximal SNPs such as T822C are common. However, in this study we explored a modification of the hybridization probe approach, one that uses primers to effectively mask proximal SNPs in the sensor probe target, thereby limiting the potential for sequence variation to interfere with typing by melting curve analysis.

### Materials and methods

Two different alterations associated with antimicrobial resistance from two different pathogens were investigated using the modified hybridization probe melting curve approach. These comprised the H275Y alteration in the influenza A (H1N1) 2009 neuraminidase protein associated with oseltamivir resistance and an adenine deletion in the Neisseria gonorrhoeae mtrR promoter region associated with resistance to a broad range of antimicrobials.\textsuperscript{10}

### Assay design

The H275Y-hybPCR assay was designed according to a standard hybridization probe-based format,\textsuperscript{7,10} in which two primers (H275Y-F and H275Y-R; Table 1) were used for amplification of the target region and two probes (H275Y-P1 and H275Y-P2; Table 1) were used for detection, with H275Y-P1 being the sensor probe and H275Y-P2 being the anchor probe. However, the difference in our approach lay in that the H275Y-F primer sequence was identical to the H275Y-P1 sensor probe, except that the probe had an additional three bases on the 3' end (Figure 1); it is these latter three bases on the 3' end of the sensor probe that target and enable typing of the codon at position 823–825 encoding amino acid 275. The benefit of our approach is that, upon PCR amplification, the H275Y-F primer sequence is incorporated into the PCR product, and hence any non-targeted SNPs (i.e. those not in the codon at position 823–825) are not represented in the resulting PCR product. More importantly, this means that the sensor probe will always match 100% with the resulting PCR product, unless any variations occur in the codon of interest. One limitation of having both primer and probe targeting the same sequence is that PCR amplification can be impeded by the probe blocking the primer (data not shown). To circumvent this problem, probes were added after PCR cycling as discussed below.

### Reaction mixture and cycling conditions

The H275Y-hybPCR assay was performed using the Qiagen One-Step RT–PCR Kit (Qiagen, Doncaster, Australia) with amplification and detection performed on a LightCycler 2.0 real-time PCR instrument (Roche Molecular Biochemicals, Mannheim, Germany). Briefly, an RT–PCR reaction comprising 0.8 µL of Qiagen One-Step enzyme mixture, 0.8 µL of Qiagen One-Step dNTP mixture, 4.0 µL of Qiagen One-Step 5 x RT–PCR Buffer, 4.0 pmol of H275Y-F primer (Table 1), 10.0 pmol of H275Y-R primer and 50–100 ng of the target DNA was performed. The primers and hybridization probes used in the assay are shown in Table 1.

### Influenza A H275Y hybridization probe PCR assay

#### Assay design

The Influenza A H275Y method (H275Y-hybPCR) was designed according to a standard hybridization probe-based format,\textsuperscript{7,10} in which two primers (H275Y-F and H275Y-R; Table 1) were used for amplification of the target region and two probes (H275Y-P1 and H275Y-P2; Table 1) were used for detection, with H275Y-P1 being the sensor probe and H275Y-P2 being the anchor probe. However, the difference in our approach lay in that the H275Y-F primer sequence was identical to the H275Y-P1 sensor probe, except that the probe had an additional three bases on the 3' end (Figure 1); it is these latter three bases on the 3' end of the sensor probe that target and enable typing of the codon at position 823–825 encoding amino acid 275. The benefit of our approach is that, upon PCR amplification, the H275Y-F primer sequence is incorporated into the PCR product, and hence any non-targeted SNPs (i.e. those not in the codon at position 823–825) are not represented in the resulting PCR product. More importantly, this means that the sensor probe will always match 100% with the resulting PCR product, unless any variations occur in the codon of interest. One limitation of having both primer and probe targeting the same sequence is that PCR amplification can be impeded by the probe blocking the primer (data not shown). To circumvent this problem, probes were added after PCR cycling as discussed below.

### Table 1. List of primers and probes used for the H275Y investigations

<table>
<thead>
<tr>
<th>Designation</th>
<th>Oligonucleotide sequence</th>
<th>GISAID accession number</th>
</tr>
</thead>
<tbody>
<tr>
<td>H275Y-hybPCR primers and probes</td>
<td></td>
<td></td>
</tr>
<tr>
<td>H275Y-F</td>
<td>AATCAGTGAAATGATGGCCCCTAATTAT</td>
<td>EPI301907</td>
</tr>
<tr>
<td>H275Y-R</td>
<td>CATGCCGTTACCTGGCACACA</td>
<td>EPI301907</td>
</tr>
<tr>
<td>H275Y-P1</td>
<td>AATCAGTGAAATGATGGCCCCTAATTAC-fluorescein</td>
<td>EPI301907</td>
</tr>
<tr>
<td>H275Y-P2</td>
<td>Quasar 670-ATGAGAAGCTCTGATCTCTAGATGAAATTC-phosphate</td>
<td>EPI301907</td>
</tr>
<tr>
<td>Modified reverse primers</td>
<td></td>
<td></td>
</tr>
<tr>
<td>mod-R1</td>
<td>GTGATTTCACTAGACCTAGGATAACAGGGACATTCCTCATA</td>
<td>EPI294206 (66.4°C, 66.7°C)</td>
</tr>
<tr>
<td>mod-R2</td>
<td>GTGATTTCACTAGAATCCGATAGACAGGGACATTCTCATA</td>
<td>EPI294380 (66.7°C, 66.9°C)</td>
</tr>
<tr>
<td>mod-R3</td>
<td>GTGATTTCACTAGAATCCGATAGACAGGGACATTCTCATA</td>
<td>EPI309672 (66.8°C, 66.8°C)</td>
</tr>
<tr>
<td>mod-R4</td>
<td>GTGATTTCACTAGAATCCGATAGACAGGGACATTCTCATA</td>
<td>EPI319536 (66.9°C, 67.0°C)</td>
</tr>
<tr>
<td>mod-R5</td>
<td>GTGATTTCACTAGAATCCGATAGACAGGGACATTCTCATA</td>
<td>EPI305478 (66.5°C, 66.8°C)</td>
</tr>
<tr>
<td>mod-R6</td>
<td>GTGATTTCACTAGAATCCGATAGACAGGGACATTCTCATA</td>
<td>EPI305475 (67.2°C, 67.3°C)</td>
</tr>
<tr>
<td>mod-R7</td>
<td>GTGATTTCACTAGAATCCGATAGACAGGGACATTCTCATA</td>
<td>EPI286671 (67.4°C, 67.5°C)</td>
</tr>
<tr>
<td>mod-R8</td>
<td>GTGATTTCACTAGAATCCGATAGACAGGGACATTCTCATA</td>
<td>EPI306145 (66.4°C, 66.6°C)</td>
</tr>
<tr>
<td>mod-R9</td>
<td>GTGATTTCACTAGAATCCGATAGACAGGGACATTCTCATA</td>
<td>EPI316414 (66.4°C, 66.7°C)</td>
</tr>
<tr>
<td>mod-R10</td>
<td>GTGATTTCACTAGAATCCGATAGACAGGGACATTCTCATA</td>
<td>EPI278865 (66.8°C, 67.0°C)</td>
</tr>
</tbody>
</table>

Bold, underlined bases in the modified reverse primers indicate mismatches with the H275Y-P2 probe sequence. Mismatches are based on SNPs documented in the GISAID database, and relevant accession numbers are provided. Melting temperatures obtained when using the modified primers in duplicate in the H275Y-hybPCR assay are provided in parentheses.
Improved antimicrobial resistance detection by hybridization probes

Clinical specimens
A total of 169 influenza A (H1N1) 2009 RT–PCR-positive respiratory specimens were included in the study. These were mainly nasopharyngeal swabs and aspirates collected in the period from June 2010 to September 2011, with the majority of samples (n=94) being successively collected between August and September 2011. Samples were extracted using the Corbett X-tratractor Gene (Qiagen, Australia; formerly Corbett Robotics, Australia) and the Corbett DX Xtraction kit. A further 34 samples collected over the same period and positive for the influenza A H3N2 type were also tested. Partial DNA sequencing of the neuraminidase gene
DNA sequencing was used as the reference standard for the H275Y-hybPCR assay evaluations. Briefly, samples were amplified using the Qiagen One-Step RT–PCR Kit (Qiagen, Doncaster, Australia), as described above, except that primers AGACAATGGGGCAGTGGCTGTGT and TAGGGCGTGGATTGTCCTCGAAA were used, designed to amplify a 392 bp fragment of the influenza A (H1N1) 2009 neuraminidase gene and encompassing the entire 99 base fragment of the H275Y-hybPCR. PCR products were then sent for automated fluorescent sequencing at the Australian Genome Research Facility (http://www.agrf.org.au/).

Further investigations of SNPs in the H275Y-P2 probe target
To minimize the potential for mismatches impacting upon the anchor probe, the H275Y-P2 probe was designed to have a melting temperature several degrees above that of the H275Y-P1 sensor probe (71.3°C compared with 66.9°C; Primer Express 2.0 software, Applied Biosystems Pty Ltd, Australia). The expectation was that the presence of a single mismatch in the anchor probe target would be insufficient to reduce the melting temperature of the anchor probe below that of the sensor probe such that viral typing would be affected. To test this, we introduced single mismatches into the H275Y-P2 anchor probe target of the PCR product by replacing the H275Y-R primer with modified primers (mod-R1 to mod-R10; Table 1). Briefly, these were designed to introduce recognized SNPs based on sequences from the Global Initiative on Sharing Avian Influenza Data (GISAID) public database (Table 1). Amplification and detection were performed using a wild-type (wt) influenza A (H1N1) 2009 strain in the H275Y-hybPCR assay as described above, except that the modified primers were used in place of the H275Y-R primer. Each primer was tested in duplicate and melting temperatures were then compared.

N. gonorrhoeae mtrR adenine deletion hybridization probe PCR assay
The approach used for the influenza A H275Y-hybPCR assay was replicated for an adenine deletion in the gonococcal mtrR promoter region. Briefly, the mtr-hybPCR assay was performed as previously described, except that the original mtr-R-F primer was replaced with a new forward primer (ACATAACCAGTGCACCGGATAAAA) designed to mask any potential SNPs in the sensor probe target (see Figure 2). Also, as per the H275Y-hybPCR assay protocol above, the probes were not added until after PCR. Evaluations of the mtr-hybPCR assay were conducted using 100 N. gonorrhoeae isolates for which the mtrR promoter had been previously characterized by DNA sequencing, comprising 75 isolates with the adenine deletion and 25 wt isolates. Of these, two wt isolates
Temperatures ranged from 66.4 to 67.5°C. The sequencing of the mtrR strains matched 100% with the results derived from DNA sequencing of the mtrR-P1 sensor probe target (wt/C6A and wt/C14G). Sequence numbering is based on the PCR product. Capitalized bases indicate mismatches with the primers or probes.

Results

**Influenza A H275Y-hybPCR assay**

Of the 169 influenza A (H1N1) 2009-positive clinical samples, 156 (92%) were typed by the H275Y-hybPCR assay and 13 (8%) samples were negative (no discernible melting curves). Of the typed samples, 151 were identified as wt strains (melting temperatures ranged from 66.6 to 67.9°C), and 5 strains were identified as harboring the H275Y alteration (melting temperatures ranged from 64.1 to 64.3°C, mean 64.2°C). DNA sequencing was performed on all 5 strains with the H275Y alteration and a random selection (n=98) of the strains identified as wt. The results of the DNA sequencing matched 100% with the results of the H275Y-hybPCR assay. The sequencing results also showed that four wt strains had the previously recognized T822C nucleotide substitution flanking the 275 codon and positioned in the H275Y-P1 sensor probe target (Figure 1). Notably, H275Y-hybPCR melting temperatures were not affected by this mutation; melting temperatures were consistent with other wt strains and ranged from 67.1 to 67.4°C. All 34 influenza A H3N2-positive samples were negative in the H275Y-hybPCR assay.

The impact of mismatches in the H275Y-P2 probe target was investigated by using modified primers (Table 1) to introduce SNPs into the PCR product of a wt influenza A (H1N1) 2009 strain. The results are provided in Table 1. Briefly, melting temperatures ranged from 66.4 to 67.5°C (mean 66.9°C, median 66.8°C). Notably, 17 of 20 melting temperatures fell within the range observed for the wt influenza A (H1N1) 2009-positive clinical samples. Also, the lowest observed melting temperature (66.4°C, one duplicate each for primers mod-R1 and mod-R8; Table 1) was only 0.2°C below the lowest melting temperature (66.6°C) observed for the wt influenza A (H1N1) 2009-positive clinical samples, yet was 2.1°C above the highest melting temperature (64.3°C) observed for H275Y-positive clinical samples.

**N. gonorrhoeae mtr-hybPCR assay**

The results of mtr-hybPCR assay for the 100 *N. gonorrhoeae* strains matched 100% with the results derived from DNA sequencing of the mtrR promoter region; 75 were identified as harbouring the adenine deletion (melting temperatures ranged from 60.0 to 64.6°C, mean 64.4°C) and 25 strains were identified as wt (melting temperatures ranged from 66.5 to 67.0°C, mean 66.8°C, median 66.8°C). Notably, both isolates with additional mutations in the sensor probe target (wt/C6A and wt/C14G; Figure 2) provided melting temperatures consistent with that of wt: 66.9°C and 66.8°C, respectively. When tested using the original method (i.e. with the original forward primer) these isolates provided melting temperatures of 62.4 and 59.9°C, respectively.

Discussion

Sequence variation is a common problem affecting the success of molecular assays, particularly those used to characterize infectious agents, such as influenza A, that exhibit high mutation rates. Melting curve analysis using hybridization probes is one such method that, in our opinion, is particularly susceptible to problems caused by non-targeted mutations and it is because of such issues that our interest in this technique had previously waned. However, in this study we present a means of limiting the potential for non-targeted SNPs to interfere with hybridization probe-based typing. The system exploits the fact that primers can still readily attach to mismatching targets, particularly single mismatches, and that upon PCR application it is the sequence of the primers, not the target, which is incorporated into the PCR product. Overall, use of a primer to ‘mask’ proximal SNPs in sensor probe targets was highly successful in this study, with the results of the PCR typing being 100% consistent with the results of DNA sequencing for both the influenza A H275Y-hybPCR and N. gonorrhoeae mtr-hybPCR assays. Of particular interest was that the H275Y-hybPCR assay correctly typed influenza A strains harbouring the T822C nucleotide substitution, previously shown to interfere with other H275Y typing methods.

It should be noted that SNPs in anchor probe targets may still have the potential to impact upon melting curve analysis for these assays, particularly if multiple mismatches are present. For the H275Y-hybPCR assay it was our original intention to design a reverse primer to mask potential variations in the H275Y-P2 anchor probe target. We did trial such a primer, which was similar in design to the modified primers (Table 1) but without the mismatching SNPs. However, the assay had severely compromised sensitivity, failing to type many samples with low viral load (data not shown). The problem appeared to be due to a previously observed primer dimer. Nevertheless, ‘masking’ of anchor probe targets does not appear to be needed for the influenza A and *N. gonorrhoeae* mechanisms investigated in this study. Our sequencing data showed that the anchor probe targets for both the H275Y-hybPCR and mtr-hybPCR assays were 100% conserved for all influenza A
and N. gonorrhoeae strains sequenced (data not shown). In addition, we investigated sequence data on the GISAID database and found that of 693 influenza A (H1N1) 2009 strains collected in the period January to October 2011, 58 (8.4%) had at least one (non-targeted) mismatch with the H275Y-P1 sensor probe whereas only 24 (3.5%) had a mismatch with the H275Y-P2 anchor probe. Notably, all of these 24 mismatches in the anchor probe target were single-nucleotide substitutions, and data from our modified primer experiments showed that single mismatches with the H275Y-P2 anchor probe were insufficient to impede typing.

While primer-masking was successful in this study, the technique potentially involves a sacrifice in sensitivity and may restrict PCR primer design. These limitations therefore need to be weighed against the potential benefits of masking unwanted mutations, and as such the utility of the technique may be limited to problematic targets such as the ones described above, rather than being adopted as standard practice in mutational analysis. Consideration also needs to be given to the fact that there are only so many mismatches that a primer can accommodate before amplification will fail or sensitivity will be compromised. Thus, the system may not work for targets exhibiting numerous SNPs. In fact, sequence variation in primer targets may account for the negative results returned by 13 samples in the H275Y-hybPCR assay. A further limitation of the primer-masking approach is that amplification curve data cannot be obtained during the PCR amplification process, given that the probes are not present in the mixture at that stage. However, we believe that this limitation is outweighed by the benefits of the improved melting curve analysis, particularly for assays such as those investigated here, where typing is the sole purpose of the method. Also, novel mutations in the targeted codon, such as a C825T in the neuraminidase 275 codon, could still impact upon melting curve analysis. This nevertheless highlights the need for ongoing monitoring of target sequences.

In summary, the results show that primers can be used to mask proximal SNPs in hybridization probe targets. This is a simple solution to the problem of sequence variation affecting hybridization probe-based melting curve analysis.

### Transparency declarations
None to declare.

### References

### Funding
This work was supported by core funding of the Queensland Paediatric Infectious Diseases Laboratory provided by the Queensland Children’s Medical Research Institute and Royal Children’s Hospital Foundation.