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Detection of influenza A H1N1 and H3N2 mutations conferring resistance to oseltamivir using rolling circle amplification

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ABSTRACT

In the event of an influenza pandemic, the use of oseltamivir (OTV) will undoubtedly increase and therefore it is more likely that OTV-resistant influenza strains will also arise. OTV-resistance genotyping using sequence-based testing on viruses isolated in cell culture is time consuming and less likely to detect the low-level presence of drug-resistant virus populations. We have developed a novel rolling circle amplification (RCA) method to achieve the sensitive detection of OTV-resistant viruses from clinical specimens. Using artificially created templates, RCA could detect the presence of OTV-resistant mutations (N2: 119V, 292K, N1: 274Y) even if the population carrying the mutations was <1% of the total. By applying RCA to clinical samples, we identified the emergence of the 274Y mutation in one OTV-treated patient, as well as in seven individuals who were treatment-naïve (confirming community transmission of 274Y-containing resistant influenza A H1N1). These results were further confirmed by neuraminidase region sequencing. In conclusion, RCA technology can provide rapid (<24 h), high-throughput diagnosis of OTV resistance mutations with a high specificity and sensitivity.

1. Introduction

Influenza pandemics occur occasionally when antigenically novel influenza A subtypes are introduced into the human population. Recently, the worldwide spread of highly pathogenic avian influenza A (H5N1) with associated human infections, and the onset of the novel swine-origin influenza A (H1N1) pandemic have ignited concerns about the global impact of influenza. With the availability of prophylaxis and treatment with neuraminidase (NA) inhibitors such as zanamivir (ZMV) and oseltamivir (OTV) (Kim et al., 1997; Hayden et al., 1999), the response to influenza pandemics will include the extensive use of antiviral drugs (mainly OTV), combined with other transmission-reducing measures and vaccines.

Although the prophylactic and therapeutic efficacy of these drugs is well established (Kim et al., 1997; Hayden et al., 1999), the use of antiviral agents on the scale required for pandemic control may create an unprecedented selective pressure for the emergence and spread of drug-resistant strains. Since the introduction of NA inhibitors, mutations associated with drug resistance have been reported in the active site of NA or in the haemagglutinin region (HA). Their prevalence has been low among field isolates (Barnett et al., 1999; Stilianakis et al., 2002; McKimm-Breschkin et al., 2003), but increasing prevalence was observed in children, with one study showing that 18% of OTV-treated children developed resistance (Kiso et al., 2004).

There are nine neuraminidase influenza A subtypes, forming two phylogenetically distinct groups. Structure-based neuraminidase inhibitors were designed to target conserved structure of the NA active site in all subtypes. However, differences in the drug-resistant mutation profiles between N1 and N2, together with crystal structure studies suggest that there is some variation between the active sites of each subtype (Russell et al., 2006). In N1 strains, a single amino acid change, H274Y, within the NA gene of influenza A H1N1 and H5N1 strains is linked to OTV resistance (Gubareva, 2004; Le et al., 2005). In N2 strains, two NA mutations, E119V and R292K, have been more frequently detected and shown to independently cause OTV resistance (Ives et al., 2000; Carr et al., 2002). Although earlier studies suggest that some of these OTV-resistant mutants may suffer from a loss of fitness and not be easily transmissible (Herlocher et al., 2002; Ives et al., 2002), it is possible, with increased OTV usage, that resistant strains will emerge with compensatory mutations to regain fitness and therefore allow ready transmission. In fact, the influenza seasons of 2007

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and 2008 were marked with the spread of OTV-resistant influenza A H1N1 strains carrying the H274Y mutation (Besselaar et al., 2008; Sheu et al., 2008). Of considerable concern was that these strains were isolated from individuals that had no recorded history of OTV treatment, indicating that these resistant viruses appeared to be sufficiently fit to be transmitted between individuals (Lackenby et al., 2008b; Rameix-Welti et al., 2008).

In influenza, resistance detection has required cell-culture methods to isolate viral strains, followed by genotypic or phenotypic analyses (McKimm-Breschkin et al., 2003). However, drawbacks to sequencing include reduced sensitivity for low levels of resistant viral quasispecies present in clinical samples, relatively high cost, and unsuitability for high throughput and rapid processing (Günthard et al., 1998; Palmer et al., 2006). Recently, the development of pyrosequencing approaches for the detection of the most common NA inhibitor resistance mutations allow improved sensitivity (detection level at 10%), are less time consuming and have high throughput for direct clinical samples detection (Deyde et al., 2009; Lackenby et al., 2008a). However, the technique remains very much machine- and software-dependent and the sensitivity needs further improvement. Therefore, more sensitive and simpler methods are needed to characterize and identify drug-resistant influenza viral strains, particularly in young and immunocompromised individuals where resistance is more likely to emerge (Ison et al., 2006; Boivin et al., 2002), or in influenza pandemics when the use of OTV prophylaxis creates a further selection pressure.

During the past few years, significant breakthroughs have been achieved in genetic analyses through the application of technologies based on analytical DNA-circularization reactions. Padlock probes have enabled parallel, high-throughput single nucleotide polymorphism (SNP) genotyping at increased scales (Faruqi et al., 2001; Tong et al., 2007; Kaocharoen et al., 2008). Padlock probes are linear oligonucleotides that comprise two target-specific end-sequences and a linking segment, typically carrying sequences used for identification and detection. The end-sequences hybridize head-to-tail to the target DNA, forming a nick between the ends which can further be recognized and sealed by a high fidelity DNA ligase (Nilsson et al., 1994). The subsequent detection by rolling circle amplification (RCA) provides a high-throughput, isothermal method of target signal amplification and rapid detection (Lizardi et al., 1998; Zhang et al., 1998).

In this study, we designed NA inhibitor (NAI) resistance-specific padlock probes to target the resistant variants of influenza A H1N1/H5N1 (H274Y) and H3N2 (E119V and R292K). Using standard DNA templates, the presence of <1% resistant variants in NA was able to be detected. After demonstrating the highly specific and sensitive nature of padlock probes and RCA in detecting NA inhibitor resistance mutations, we screened 94 clinically confirmed influenza A infected patients to investigate the prevalence of NA resistance in both NAI-treated and untreated individuals.

2. Materials and methods

2.1. Patient information

Influenza A was detected in 94 samples collected from patients with influenza-like illness in 2006–2008, 74 from OTV treatment-naïve and 20 from treatment-experienced patients. In the treatment-experienced patients' group, one individual was an immunosuppressed bone marrow transplant recipient who had samples collected from five time-points (Day 12, 28, 42, 47 and 51) after receiving multiple courses of OTV. Of the 94 influenza A positive samples, 69 were subtyped by PCR amplification of the NA gene region as influenza A H3N2, and 25 were subtyped as influenza A H1N1.

2.2. RNA extraction, RT-PCR and sequencing

Viral RNA extraction from combined nasal and throat swabs was performed using the Qiagen Viral RNA extraction kit (Qiagen, Hilden, Germany) as per the manufacturer's instructions. In addition, RNA was extracted from an influenza A H5N1 isolate. RT-PCR amplifications were performed using the OneStep RT-PCR system (Qiagen, Hilden, Germany) to amplify the full NA gene and amplification was performed for 35 cycles. Amplicons were then purified using a Millipore vacuum apparatus (Millipore, Billerica, MA) and the full NA gene was sequenced. The chromatograms were examined at the sites where resistance is known to occur to look for previously described resistance mutations.

2.3. Design of padlock probes

Padlock probes, recognizing NAI resistance-specific SNPs (N2: 119Val and 292Arg, N1: 274Tyr and H5N1: 274Tyr) were designed as previously described (Wang et al., 2005). In addition, ambiguous positions were also introduced for broader resistance recognition during probe design according to the reported resistance mutation profile derived from Genbank. The genetic linker region was also carefully designed to minimize any similarity to potentially cross-reacting sequences after BLAST searches (http://www.ncbi.nlm.nih.gov/BLAST). Two primers used to amplify the specific circularized padlock probe signal during RCA were designed specifically to bind to the linker regions with a Tm of about 55 °C. The relevant primers and probes are listed in Table 1.

2.4. Novel design and production of artificial templates of wild-type and resistant variants

To validate the assay's sensitivity and specificity, artificial DNA templates containing wild-type (N2: 119Glu and 292Arg, N1: 274His, and H5N1: 274His) or resistant variants (N2: 119Val and 292Arg, N1: 274Tyr and H5N1: 274Tyr) were synthesized. Briefly, a 100 base pair oligonucleotide was designed based on the consensus sequence for the region and encompassing the base of interest. A second 100 bp oligonucleotide based on the complementary strand was designed, which shared a 20 bp region complementary to the first oligonucleotide at the 3′ end. The two oligonucleotides were manufactured and PAGE purified (Sigma–Aldrich, Sydney, NSW, Australia). They were then used in a PCR reaction by mixing 20 pmol of each paired long oligonucleotide with 2 U of *Taq* DNA polymerase (Promega, Madison, WI), 1× reaction buffer, 2.5 mM $MgCl_2$, and 0.4 mM of dNTPs. The reaction mix was incubated at 94 °C for 30 s followed by 55 °C for 30 s and 72 °C for 30 s for 5 cycles. After the reaction, the end product was run on a 2% agarose gel and visualized under UV light with ethidium bromide staining. The PCR products were cloned into the pGEM-T Easy Vector System II (Promega, Madison, WI) and transformed into competent *Escherichia coli* JM109 cells, according to the manufacturer's protocol. The plasmid DNA from positive clones was extracted using the QIAprep Spin Miniprep Kit according to the manufacturer's protocol (Qiagen, Hilden, Germany). The inserts were sequenced to confirm the presence of wild-type/resistance mutations. Liner templates containing wild-type and resistance changes were further generated by PCR amplification of plasmid DNA, followed by purification using the Millipore PCR purification plate (Millipore, Billerica, MA). The linear PCR products were quantified using a spectrophotometer and dsDNA DNA copy number were estimated using DNA calculator (http://www.uri.edu/research/gsc/resources/cndna.html). A total of 10^{11} copies of starting templates were used for testing the speci-

Table 1
Padlock probe sequence.

Probe name	Sequence
RCA-N2-119VAL	5′ APP-CTCTTGTCACCCAGATGTCCGATCATGCTTCTTCGGTGCCCATG AGGTGCGGATAGCTCGCGCAGACACGATAGTCTACATGACAC ATAAGGNA-3′
RCA-N2-292-LYS	5′ APP- TGCAGACACATCTGACACCAGATCATGCTTCTTCGGTGCCCAT GAGGTGCGGATAGCTCGCGCAGACACGATAGTCTACCTTTCCA GTTGTCYT-3′
RCA N1 274TYR:	5′ APP- RAAATTGGGTGCATTYAACTCGATCATGCTTCTTCGGTGCCCA TGAGGTGCGGATAGCTCGCGCAGACACGATAGTCTAGGAGCA TTCCTCATARTA-3′
RCA H5N1 274TYR	5′ APP- ATAATTAGGAGCATCCAATTCGACTGATCATGCTTCTTCGGTG CCCATGAGGTGCGGATAGCTCGCGCAGACACGATAGTCTAAG CATTCCTCATARTA-3′
Primers	
RCA primer 1	ATGGGCACCGAAGAAGCA
RCA primer 2	CGCGCAGACACGATA

A: the 5′-end of probe, P: indicates phosphorylation.

ficity of the padlock probe system. 10^{11} copies of templates were chosen because it provides sufficient template for padlock probe binding, it is an appropriate amount for the subsequent exonuclease treatment to remove the template, and is easily achievable from the amount of the PCR product.

2.5. Probe ligation, exonucleolysis and signal amplification by hyperbranch RCA

Ligation of the padlock probe to templates was carried out by mixing the 10^{11} copies of template with 1 pmol of individual padlock probes, 2 U of Pfu DNA ligase in 20 mM Tris–HCl (pH 7.5), 20 mM KCl, 10 mM $MgCl_2$, 0.1% Igepal, 0.01 mM rATP and 1 mM DTT in a total reaction volume of 10 μl. Multiple cycle ligation was conducted to validate the specificity of the probe in recognizing its corresponding template. The reaction conditions included 1 cycle of 5 min at 94 °C to denature the dsDNA followed by 1–15 cycles of 94 °C for 30 s and 4 min ligation at 65 °C. The ligation mixture was then subjected to exonucleolysis to remove any unreacted padlock probe and template PCR product in order to reduce subsequent ligation-independent amplification events (Tong et al., 2007). The exonuclease treatment was performed in a 20 μl volume by adding 10 U each of exonucleases I and III (New England Biolabs, Ipswich, MA) to the ligation mixture and incubating at 37 °C for 30 min followed by 94 °C for 30 s to inactivate the enzymes.

The amplification of circularized padlock probes was performed in a 50 μl volume by adding 8 U of Bst DNA polymerase (New England Biolabs, Ipswich, MA), 5 μl reaction buffer, 400 μM dNTP mix, 10 pmol of each RCA primer, 5% DMSO (v/v) and 1× Sybr Green I (Sigma–Aldrich, St Louis, MO) to the ligation mixture. The reaction was carried out at 65 °C for 30 min and the accumulation of dsDNA products was monitored on a Corbett RotorGene 3000 real-time PCR machine (Corbett, Mortlake, NSW, Australia). Alternatively, if a laboratory is not equipped with real-time instruments, the end product can be directly visualized by agarose gel electrophoresis, with the amplified probe signals showing the typical ladder-like pattern of dsDNA.

2.6. Determination of specificity and sensitivity of padlock probes and RCA system

Validation of the specificity of padlock probes was carried out by multiple cycle ligation of the probes with 10^{11} copies of corresponding resistance or wild-type template, or a mix of the two, to test the probes distinguishing ability. A total of 1–15 ligation cycles were tested followed by RCA of the probe signal according to the reaction conditions described above. Testing the sensitivity of the resistance-specific padlock was carried out using 10^{11} copies of standard templates containing 100%, 50%, 10%, 1%, 0.1% and 0% (wild-type only) resistance template in a background of wild-type template. The ligation reaction was performed for 15 cycles followed by RCA of the probe signal for detection.

2.7. Detection of NA inhibitor resistance mutation from patient samples using padlock probe and RCA

After conducting the sensitivity and specificity test of the padlock probe, 10^{11} copies of patient-derived NA gene PCR product were subjected to resistance-specific padlock probe ligation of 15 cycles in a similar fashion to the conditions described previously, followed by exonucleolysis and RCA of the probe signal. The signals were monitored on a Corbett RotorGene 3000 real-time PCR machine. All patient samples were run in parallel with standards containing resistance templates at various levels. The positive samples were recognized as having signals above the standard containing 1% of the resistance template and positive samples were tested again to confirm the results.

2.8. NA enzyme inhibition assay

Isolates that were found to contain the H274Y, E119V or R292K NA mutations were tested for OTV and ZMV susceptibility using a NA enzyme inhibition assay. The inhibition assay utilizes the fluorescent product 4-methylumbelliferone from the substrate 2-(4-methylumbelliferyl)-a-D-N-acetylneuraminic acid (MUNANA) (Sigma–Aldrich, St Louis, MO) as a measure of NA activity (Potier et al., 1979), following a previously published protocol (Hurt et al., 2004). Oseltamivir carboxylate, the active form of the ethyl ester prodrug oseltamivir phosphate, was kindly provided by Hoffman-La Roche, Basle, Switzerland. Zanamivir was used directly from the blister packaging of Relenza (5 mg zanamivir and 20 mg lactose) as distributed through pharmacies. IC_{50} values were calculated using a logistic curve fit program "Robosage" kindly provided by GlaxoSmithKline, UK.

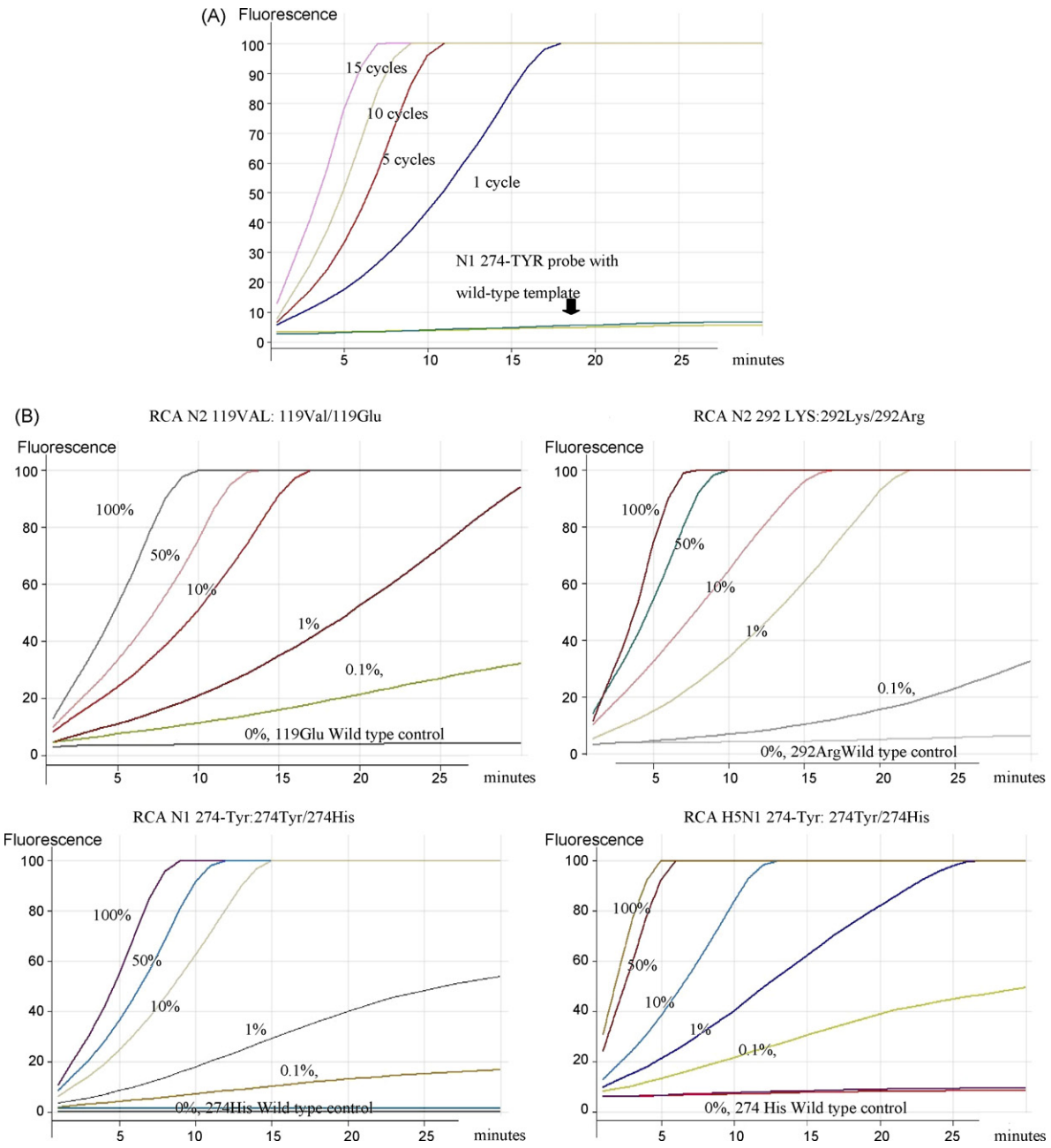

Fig. 1. (A) Specificity testing of the OTV resistance-specific probes targeting 10^{11} copies of standard templates. The ligation reaction was carried out in 1, 5, 10, and 15 cycles followed by RCA reaction to amplify circularized padlock probe. All four probes generated similar signal patterns and only the N1 274TYR probe was shown here as a representative. Specific probe signals were only detected with the standard N1-274Y template. No signal was detected in wild-type template. (B) Sensitivity testing of OTV resistance-specific probes targeting templates containing various percentages of the OTV resistance target. Significant elevation of signal was detected in all four probes even at the presence of 1% of the resistance template, while in wild-type control, no signal was detected.

3. Results

3.1. Generation of standard wild-type and resistance templates

After five PCR cycles using synthesized oligonucleotides, products from the paired synthesized long oligonucleotides were shown clearly on an agarose gel as ~180 bp dsDNA products. The PCR products were further cloned and sequencing of the clones proved the presence of either wild-type or resistant sequences. Linear templates from these clones were generated by PCR amplification and the concentration of standard template was adjusted to 2×10^{10} copies/μl. The sequence polymorphisms, which involved the 119V, 292K and 274Y resistance mutations, were all selected for testing the padlock probes specificity and sensitivity.

3.2. Specificity and sensitivity of padlock probes in the detection of N1 and N2 NA resistance mutations

All four probes were tested for their specificity in distinguishing resistance mutations from wild-type template. After 1–15 ligation cycles of the probe with either the resistance or wild-type template, RCA of the circularized probe showed that positive signals were only detected from reactions containing the resistance-specific padlock probe and resistance template; no significant elevation of signals were detected from wild-type templates despite only a

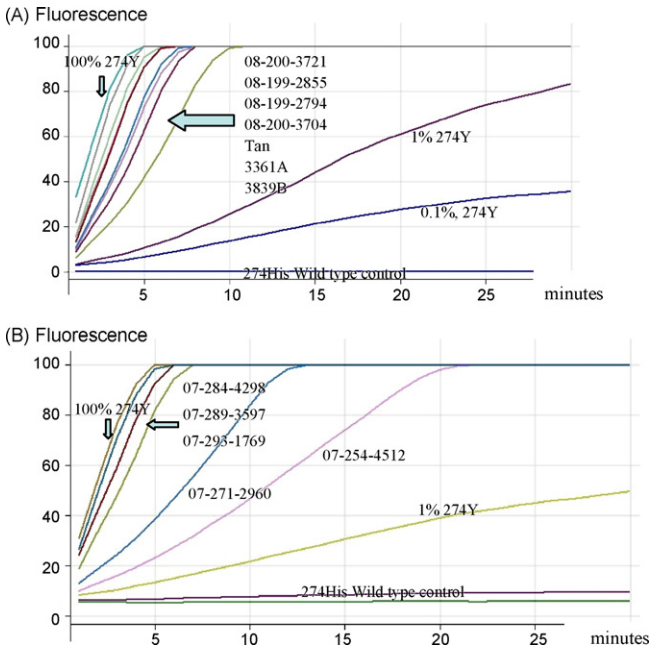

Fig. 2. (A) RCA testing of 24 H1N1 samples derived from OTV treatment-naïve individuals. Seven samples showed positive signal for N1 274TYR probe indicating the presence of N1-274Y mutation. (B) RCA testing of H1N1 samples derived from an immunosuppressed individual who received multiple course of OTV treatment. A total of five time-points were tested and all showed the positive signal for N1 274TYR probe. Two samples derived from early time points (07-254-4512, and 07-271-2960, Day 12 and 28 post-OTV treatment) showed the delayed signal detection indicating the lower copy numbers of the N1-274Y mutation.

single nucleotide difference from the resistance template (Fig. 1A). The earlier signal detection in response to the increased ligation cycles indicated that an increased amount of circular probes were generated after the probe recognition of its template. Also, we observed by increasing the ligation cycles to >20, that signals from wild-type templates could be detected but remained at very low levels (data not shown). Sensitivity tests were done using 10^{11} copies of standard template containing the resistance mutation at various percentages. It was clearly shown after 15 cycles of ligation followed by RCA signal amplification, that positive signals can be easily detected by all four probes, even when the resistance mutations consist of less than 1% of the total template tested, suggesting the RCA testing can detect the early emergence of resistance mutations (Fig. 1B). Repeat specificity and sensitivity testing revealed similar results.

3.3. Screening of patient samples using padlock probes and RCA

Two probes targeting resistance mutations at positions 119 and 292 of N2 strains failed to show any signal after examining PCR products from 69 samples from patients infected with influenza A H3N2 (data not shown), which is consistent with other surveillance data suggesting that the development of NAI resistance in H3N2 strains is rare (Hurt et al., 2009). Among 25 patients infected with influenza A H1N1 strains, NA gene PCR products from 17 patients were negative after RCA of the 274Y-specific padlock probe and eight patients showed positive signals, indicating the presence of NAI resistance mutations. Of the eight patients who tested positive for NAI resistance mutations, one patient (an immunosuppressed bone marrow transplant recipient) had received treatment with OTV, whereas the other seven did not. Among these seven patients, two patients (3361A and 3839B) were siblings who acquired virus from their mother who returned from a two-week visit to Khartoum, Sudan, to Australia (Fig. 2A). Interestingly, in the immunosuppressed individual who received multiple courses of OTV treatment, all five time-points showed a positive signal for 274Y (Fig. 2B). The signal from the first two time-points (07-254-4512 and 07-271-2960, Day 12 and 28 post-OTV treatment) were detected relatively late in comparison to three later time-points (Day 42, 47 and 51 post-OTV treatment respectively) despite the same input copy number of PCR template (Fig. 2B). Considering that the RCA is also a quantitative tool, the late detection of signal in the first two time-points indicates that the 274Y mutation was less frequent within the patient's viral quasispecies at these times.

3.4. Screening of patient samples by DNA sequencing

The NA gene of all 94 samples was also sequenced to validate the RCA data. For the 69 patient samples with influenza A H3N2 infection, all had the wild-type NA gene variant at positions 119 and 292, confirming the RCA data. Of the 25 patients infected with influenza A H1N1 strains, 17 were negative for the 274Y mutation and eight sequences showed the presence of the OTV resistance-associated 274Y mutations (Fig. 3A), again consistent with the RCA results. In the immunosuppressed bone marrow transplant recipient infected with influenza A H1N1 and treated with multiple courses of OTV, all five time-point samples showed the 274Y mutation. The sequence chromatograms indicated that the first two time-points harbored a mixture of both wild-type and resistance variants at position 274, while the resistance variants predominated at the latter three time-points (Fig. 3B). This data further confirm the RCA observation of a lower proportion of the 274Y mutation at the early two time points. Occasionally (~5%) the chromatograms had a relatively high level of background signal, which was most likely associated with PCR product purification.

3.5. NA enzyme inhibition assay

To further confirm the presence of H274Y in the correlation with NAI resistance, isolates from the eight cases detected by RCA containing the H274Y mutation were tested for susceptibility to both OTV and ZMV using a fluorescence-based NA inhibition assay. Oseltamivir IC_{50} values (the drug concentration required to inhibit 50% of the NA activity) for the H274Y mutants of the eight isolates ranged from 725 to 1586 nM, at least a 1500-fold increase compared to the mean OTV IC_{50} of normal fully sensitive influenza A H1N1 viruses (0.5 ± 0.4 nM; mean $\pm$ 1 SD; $n = 434$) isolated from the same time period in Australia. Although viruses with the H274Y mutation demonstrate significantly increased OTV IC_{50} values, ZMV sensitivity is not affected by this mutation. The ZMV IC_{50} values for the H274Y mutants of the eight isolates ranged from 0.3 to 2.0 nM, similar to the mean ZMV IC_{50} of normal fully sensitive influenza A H1N1 viruses (0.5 ± 0.6 nM; mean $\pm$ 1 SD; $n = 434$).

4. Discussion

The increased use of antiviral drugs often leads to the emergence of resistance strains, with implications for patient care and transmission to the community. The recent descriptions of the rapid spread of the H274Y mutation-containing influenza A H1N1 isolates in the 2007–2008 winter raise further concerns about widespread OTV resistance (Lackenby et al., 2008b; Rameix-Welti et al., 2008). Therefore, the monitoring of NA inhibitor resistance is important for the management of seasonal and pandemic influenza, especially as OTV use will undoubtedly increase during a pandemic.

We have successfully designed padlock probes to target the most frequently observed mutations associated with OTV resistance. The technology used is rapid, simple, relatively inexpensive,

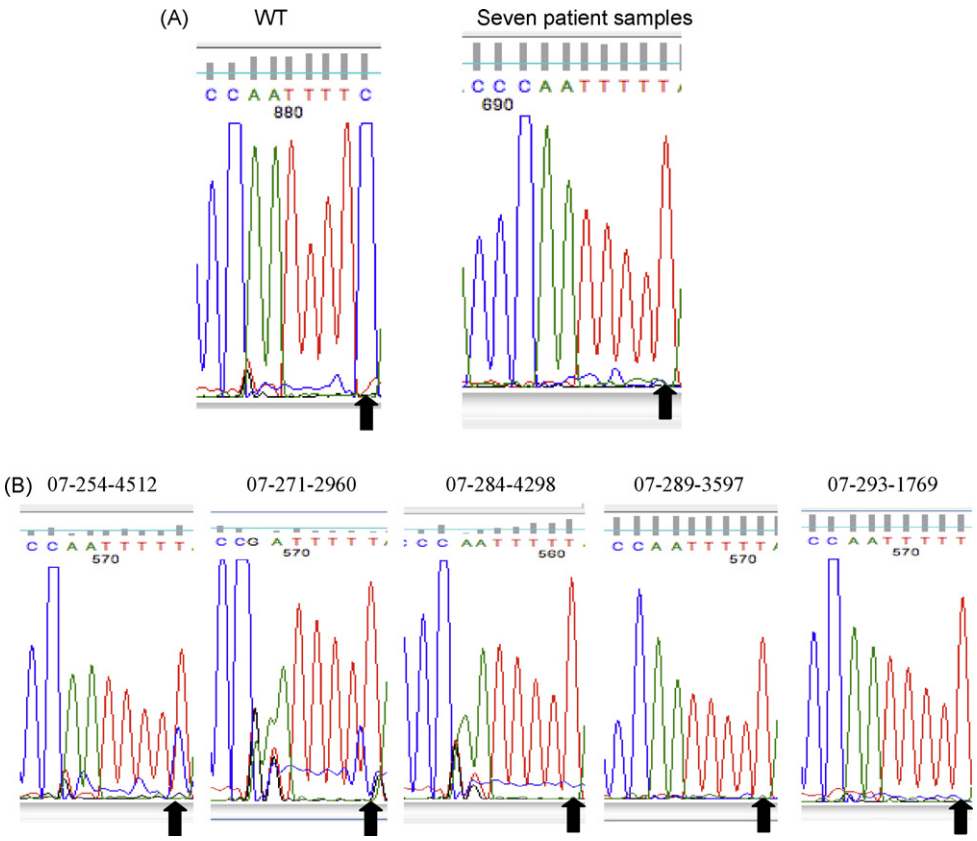

Fig. 3. (A) Sequence chromatogram of NA gene region at position 274 from seven individuals indicates the presence of T (right panel) in comparison to wild-type C (left panel). Only one representative sequence chromatogram is shown. (B) NA gene region chromatogram at position 274 from five time-points in an immunosuppressed bone marrow transplant recipient. The chromatograms of the two early time points (07-254-4512, and 07-271-2960, Day 12 and 28 post-OTV treatment), at position 274, showed the presence of both T and C (resistance mutation and wild-type virus), and the three late time point (07-284-4299, 07-289-3597, and 07-293-1769, Day 42, 47 and 51 post-OTV treatment respectively) showed the predominance of the resistance genotype.

and easily transferable to laboratories in resource-poor environments. The use of ligation-based allele discrimination rather than primer-based discrimination provided this type of assay with a high level of specificity and, in conjunction with RCA, sensitivity (Zhang et al., 1998). The concordance between RCA in the detection of H274Y mutation and sequencing result along with NA enzyme inhibition assay validation further confirmed the accuracy of such test in clinical diagnosis.

Currently the RCA technology is only suitable for screening previously known drug resistance mutations, although once new mutations are identified via the traditional methods of sequencing cell culture-derived isolates, new padlock probes for each mutation can be easily designed and rapidly implemented (Zhang et al., 1998; Schweitzer and Kingsmore, 2001). In addition, although the presence of genetic variations, other than those conferring resistance, may influence the detection sensitivity by interfering the probe–template hybridization, such mutations may only decrease the hybridization efficiency marginally and will not interfere with the formation of circular molecules—this is mediated by DNA ligase and only requires a perfect match between the template at the 5′ and 3′ end of the oligonucleotides. In this study, amplified PCR products were used from clinical specimens to look for the presence of resistance mutations, and therefore is limited by the sensitivity of the diagnostic PCR. However, the use of PCR products will ensure the input template quantity, which is important for the probe-based quantitative assays such as RCA.

Low levels of influenza A OTV resistance have been seen in treated populations, based on clinical trials prior to widespread drug use. Of note is the recent dramatic shift with high prevalence of H274Y mutants in untreated populations in 2007/2008, suggesting that virus is sufficiently fit to be easily transmitted between individuals. Our study confirms this observation with NAI resistance being detected by RCA and confirmed by sequencing in eight patients, seven of whom were treatment-naïve. This and other reports of these viruses circulating in the 2007–2008 influenza season (Lackenby et al., 2008b; Rameix-Welti et al., 2008) indicate community transmission of OTV-resistant viruses.

The high prevalence of OTV resistance among recent circulating influenza A H1N1 strains together with the spread of pandemic (H1N1) 2009 (accompanied by extensive use of neuraminidase inhibitors for treatment and prophylaxis) may significantly increase the possibility of drug resistance development via genetic mutation, genome reassortment and recombination. Hence, continuous and ‘real-time’ surveillance for the emergence of NA inhibitor-resistant influenza virus variants (including avian origin influenza A H5N1 and pandemic (H1N1) 2009) is necessary. High-throughput RCA technology that selectively targets known resistance SNPs shows promise as a rapid, sensitive and specific detection platform, and has the potential to be performed on clinical specimens at large scale.

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References

Barnett, J.M., Cadman, A., Burrell, F.M., Madar, S.H., Lewis, A.P., Tisdale, M., Bethell, R., 1999. In vitro selection and characterisation of influenza B/Beijing/1/87 isolates with altered susceptibility to zanamivir. Virology 265, 286–295.

Besselaar, T.G., Naidoo, D., Buys, A., Gregory, V., McAnerney, J., Manamela, J.M., Blumberg, L., Schoub, B.D., 2008. Widespread oseltamivir resistance in influenza A viruses (H1N1), South Africa. Emerg. Infect. Dis. 14, 1809–1810.

Boivin, G., Goyette, N., Bernatchez, H., 2002. Prolonged excretion of amantadine-resistant influenza a virus quasi species after cessation of antiviral therapy in an immunocompromised patient. Clin. Infect. Dis. 34, 23–25.

Carr, J., Ives, J., Kelly, L., Lambkin, R., Oxford, J., Mendel, D., Tai, L., Roberts, N., 2002. Influenza virus carrying neuraminidase with reduced sensitivity to oseltamivir carboxylate has altered properties in vitro and is compromised for infectivity and replicative ability in vivo. Antivir. Res. 54, 79–88.

Deyde, V.M., Okomo-Adhiambo, M., Sheu, T.G., Wallis, T.R., Fry, A., Dharan, N., Klimov, A.I., Gubareva, L.V., 2009. Pyrosequencing as a tool to detect molecular markers of resistance to neuraminidase inhibitors in seasonal influenza A viruses. Antivir. Res. 81, 16–24.

Faruqi, A.F., Hosono, S., Driscoll, M.D., Dean, F.B., Alsmadi, O., Bandaru, R., Kumar, G., Grimwade, B., Zong, Q., Sun, Z., Du, Y., Kingsmore, S., Knott, T., Lasken, R.S., 2001. High-throughput genotyping of single nucleotide polymorphisms with rolling circle amplification. BMC Genomics 2, 4.

Gubareva, L.V., 2004. Molecular mechanisms of influenza virus resistance to neuraminidase inhibitors. Virus Res. 103, 199–203.

Günthard, H.F., Wong, J.K., Ignacio, C.C., Havlir, D.V., Richman, D.D., 1998. Comparative performance of high-density oligonucleotide sequencing and dideoxynucleotide sequencing of HIV type 1 pol from clinical samples. AIDS Res. Hum. Retroviruses 14, 869–876.

Hayden, F.G., Treanor, J.J., Fritz, R.S., Lobo, M., Betts, R.F., Miller, M., Kinnersley, N., Mills, R.G., Ward, P., Straus, S.E., 1999. Use of the oral neuraminidase inhibitor oseltamivir in experimental human influenza: randomized controlled trials for prevention and treatment. JAMA 282, 1240–1246.

Herlocher, M.L., Carr, J., Ives, J., Elias, S., Truscon, R., Roberts, N., Monto, A.S., 2002. Influenza virus carrying an R292K mutation in the neuraminidase gene is not transmitted in ferrets. Antivir. Res. 54, 99–111.

Hurt, A.C., Barr, I.G., Hartel, G., Hampson, A.W., 2004. Susceptibility of human influenza viruses from Australasia and South East Asia to the neuraminidase inhibitors zanamivir and oseltamivir. Antivir. Res. 62, 37–45.

Hurt, A.C., Ernest, J., Deng, Y.M., Iannello, P., Besselaar, T.G., Birch, C., Buchy, P., Chittaganpitch, M., Chiu, S.C., Dwyer, D., Guigon, A., Harrower, B., Kei, I.P., Kok, T., Lin, C., McPhie, K., Mohd, A., Olveda, R., Panayotou, T., Rawlinson, W., Scott, L., Smith, D., D'Souza, H., Komadina, N., Shaw, R., Kelso, A., Barr, I.G., 2009. Emergence and spread of oseltamivir-resistant A (H1N1) influenza viruses in Oceania, South East Asia and South Africa. Antivir. Res. 83, 90–93.

Ison, M.G., Gubareva, L.V., Atmar, R.L., Treanor, J., Hayden, F.G., 2006. Recovery of drug-resistant influenza virus from immunocompromised patients: a case series. J. Infect. Dis. 193, 760–764.

Ives, J.A., Carr, J.A., Mendel, D.B., Tai, C.Y., Lambkin, R., Kelly, L., Oxford, J.S., Hayden, F.G., Roberts, N.A., 2002. The H274Y mutation in the influenza A/H1N1 neuraminidase active site following oseltamivir phosphate treatment leave virus severely compromised both in vitro and in vivo. Antivir. Res. 55, 307–317.

Ives, J., Carr, J., Roberts, N.A., Tai, C.Y., Mendel, D.B., Kelly, L., Lambkin, R., Oxford, J., 2000. An oseltamivir treatment-selected influenza A/N2 virus with a R292K mutation in the neuraminidase gene has reduced infectivity in vivo. J. Clin. Virol. 18, 251–269.

Kaocharoen, S., Wang, B., Tsui, K.M., Trilles, L., Kong, F., Meyer, W., 2008. Hyperbranched rolling circle amplification as a rapid and sensitive method for species identification within the *Cryptococcus* species complex. Electrophoresis 29, 3183–3191.

Kim, C.U., Lew, W., Williams, M.A., Lui, H., Zhang, L., Swaminathan, S., Bischofberger, N., Chen, M.S., Mendel, D.B., Tai, C.Y., Laver, W.G., Stevens, R.C., 1997. Influenza neuraminidase inhibitors possessing a novel hydrophobic interaction in the enzyme active site: design, synthesis and structural analysis of carbocyclic sialic acid analogues with potent anti-influenza activity. J. Am. Chem. Soc. 119, 681–690.

Kiso, M., Mitamura, K., Sakai-Tagawa, Y., Shiraishi, K., Kawakami, C., Kimura, K., Hayden, F.G., Sugaya, N., Kawaoka, Y., 2004. Resistant influenza A viruses in children treated with oseltamivir: descriptive study. Lancet 364, 759–765.

Lackenby, A., Democratis, J., Siqueira, M.M., Zambon, M.C., 2008a. Rapid quantitation of neuraminidase inhibitor drug resistance in influenza virus quasispecies. Antivir. Ther. 13, 809–820.

Lackenby, A., Hungnes, O., Dudman, S.G., Meijer, A., Paget, W.J., Hay, A.J., Zambon, M.C., 2008b. Emergence of resistance to oseltamivir among influenza A (H1N1) viruses in Europe. Euro. Surveill. 13, pii: 8026.

Le, Q.M., Kiso, M., Someya, K., Sakai, Y.T., Nguyen, T.H., Nguyen, K.H., Pham, N.D., Ngyen, H.H., Yamada, S., Muramoto, Y., Horimoto, T., Takada, A., Goto, H., Suzuki, T., Suzuki, Y., Kawaoka, Y., 2005. Avian flu: isolation of drug-resistant H5N1 virus. Nature 437, 1108.

Lizardi, P.M., Huang, X., Zhu, Z., Bray-Ward, P., Thomas, D.C., Ward, D.C., 1998. Mutation detection and single-molecule counting using isothermal rolling-circle amplification. Nat. Genet. 19, 225–232.

McKimm-Breschkin, J., Trivedi, T., Hampson, A., Hay, A., Klimov, A., Tashiro, M., Hayden, F., Zambon, M., 2003. Neuraminidase sequence analysis and susceptibilities of influenza virus clinical isolates to zanamivir and oseltamivir. Antimicrob. Agents Chemother. 47, 2264–2272.

Nilsson, M., Malmgren, H., Samiotaki, M., Kwiatkowski, M., Chowdhary, B.P., Landegren, U., 1994. Padlock probes: circularizing oligonucleotides for localized DNA detection. Science 265, 2085–2088.

Palmer, S., Boltz, V., Maldarelli, F., Kearney, M., Halvas, E.K., Rock, D., Falloon, J., Davey Jr., R.T., Dewar, R.L., Metcalf, J.A., Mellors, J.W., Coffin, J.M., 2006. Selection and persistence of non-nucleoside reverse transcriptase inhibitor-resistant HIV-1 in patients starting and stopping non-nucleoside therapy. AIDS 20, 701–710.

Potier, M., Mameli, L., Bélisle, M., Dallaire, L., Melançon, S.B., 1979. Fluorometric assay of neuraminidase with a sodium (4-methylumbelliferyl-alpha-D-N-acetylneuraminate) substrate. Anal. Biochem. 94, 287–296.

Rameix-Welti, M.A., Enouf, V., Cuvelier, F., Jeannin, P., van der Werf, S., 2008. Enzymatic properties of the neuraminidase of seasonal H1N1 influenza viruses provide insights for the emergence of natural resistance to oseltamivir. PLoS Pathog. 2008 4, e1000103.

Russell, R.J., Haire,.L.F., Stevens, D.J., Collins, P.J., Lin, Y.P., Blackburn, G.M., Hay, A.J., Gamblin, S.J., Skehel, J.J., 2006. The structure of H5N1 avian influenza neuraminidase suggests new opportunities for drug design. Nature 443, 45–49.

Schweitzer, B., Kingsmore, S., 2001. Combining nucleic acid amplification and detection. Curr. Opin. Biotechnol. 12, 21–27.

Sheu, T.G., Deyde, V.M., Okomo-Adhiambo, M., Garten, R.J., Xu, X., Bright, R.A., Butler, E.N., Wallis, T.R., Klimov, A.I., Gubareva, L.V., 2008. Surveillance for neuraminidase inhibitor resistance among human influenza A and B viruses circulating worldwide from 2004 to 2008. Antimicrob. Agents Chemother. 52, 3284–3292.

Stilianakis, N.I., Perelson, A.S., Hayden, F.G., 2002. Drug resistance and influenza pandemics. Lancet 359, 1862–1863.

Tong, Z., Kong, F., Wang, B., Zeng, X., Gilbert, G.L., 2007. A practical method for subtyping of *Streptococcus agalactiae* serotype III, of human origin, using rolling circle amplification. J. Microbiol. Methods 70, 39–44.

Wang, B., Potter, S.J., Lin, Y., Cunningham, A.L., Dwyer, D.E., Su, Y., Ma, X., Hou, Y., Saksena, N.K., 2005. Rapid and sensitive detection of severe acute respiratory syndrome coronavirus by rolling circle amplification. J. Clin. Microbiol. 43, 2339–2344.

Zhang, D.Y., Brandwein, M., Hsuih, T.C., Li, H., 1998. Amplification of target-specific, ligation-dependent circular probe. Gene 211, 277–285.