Development of a multiplex real-time RT-PCR that allows universal detection of influenza A viruses and simultaneous typing of influenza A/H1N1/2009 virus

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ABSTRACT

On June 11, 2009, the World Health Organization declared that the influenza A/H1N1/2009 virus had become the first influenza pandemic of the 21st century. Rapid detection and differentiation from seasonal and avian influenza would be beneficial for patient management and infection control. It was the aim of this study to develop a real-time RT-PCR that can detect all influenza A viruses and offer simultaneous typing for influenza A/H1N1/2009.

This would be a useful addition to existing diagnostic protocols for influenza A. Its routine use would allow laboratories to screen out influenza A/H1N1/2009 positive samples rapidly and would reduce overall testing costs.

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1. Introduction

On April 15 and 17, 2009, a novel swine-lineage influenza A (influenza A/H1N1/2009) infection was reported to the World Health Organisation (WHO) by the Centers for Disease Control and Prevention (CDC) in Atlanta in two children presenting with febrile respiratory illness from adjacent counties in southern California (CDC, 2009; Novel Swine-Origin influenza A (H1N1) Virus Investigation Team, 2009). These cases were not epidemiologically linked and neither child had exposure to swine.

Since the original identification of influenza A/H1N1/2009 in the United States and Mexico, sustained human-to-human transmission has been seen in other countries and on June 11, 2009, the World Health Organization declared that the virus had become the first influenza pandemic of the 21st century.

Although the influenza A/H1N1/2009 virus is likely to become the predominant influenza A type encountered in most countries, seasonal influenza A types may also co-circulate (Kelly et al., 2009) and in some countries sporadic H5N1 infections may still occur (WHO report of Avian influenza, 2009).

Determining the subtype of influenza virus is important as it has implications for patient management and infection control (Meijer et al., 2009; Beigel and Bray, 2008; Hall et al., 2009). For example, seasonal H1N1 viruses are often found to be resistant to oseltamivir and studies on H5N1 viruses have suggested that higher treatment doses of oseltamivir are necessary for successful treatment of infection (Lackenby et al., 2009; White et al., 2009).

In order to detect and then type influenza A viruses most laboratories use a two tier testing system comprising of a universal influenza A screening assay complemented with a suite of subtyping assays that determine whether the sample is seasonal influenza A (human H1N1 and H3N2), avian H5N1 or the influenza A/H1N1/2009 virus. Although useful for the reasons outlined above, the testing structure prolongs the time it takes to complete testing and is costly as most influenza A positive samples will have to be tested using the individual typing assays.

This article describes the development of a multiplex real-time reverse transcription polymerase chain reaction (rtPCR) that allows universal detection of all influenza A viruses and simultaneously subtypes all that are influenza A/H1N1/2009. An internal control rtPCR assay was also incorporated in order to detect PCR inhibition, failed extraction/PCR and technical error. Use of this assay will allow laboratories to screen respiratory samples for influenza A/H1N1/2009 virus in a rapid and cost effective format, ensuring that typing methods for seasonal and avian viruses are used on a smaller subset of samples.

2. Methods

2.1. Real-time RT-PCR method

The influenza A/H1N1/2009 rtPCR assay was designed to target segment 6 of the NA using published sequence data

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Table 1
Primer and probes sequences for the generic influenza A and the H1N1 swl rtPCR assays.

Virus target	Target gene	Forward primer	Reverse primer	Probe
H1N1 swl	N1	GTT AAC ATC AGC AAC ACC AAC TTT G	GAG AGG AAT TGC CCG CTA ATT	YAK-TGC TGG ACA GTC AGT GGT TTC CGT G-**BBQ**
Influenza A	Matrix	AAGACAAGACCAATYCTGTCACCTCT	TCTACGYTGCAGTCCYCGCT	**FAM**-TYACGCTCACCGTGCCCAGTG-**BBQ**
Internal control	EAV	CAT CTC TTG CTT TGC TCC TTA G	AGC CGC ACC TTC ACA TTG	**Cy5**-CGC TGT CAG AAC AAC ATT ATT GCC CAC-**BBQ**

A/California/04/2009 (Genbank Accession number: FJ969517) and sequences obtained from influenza A/H1N1/2009 detected in Scotland, which have not yet been submitted to Genbank. Based on the influenza A/H1N1/2009 sequences a conserved sequence was submitted to Primer Express Version 3.0 (Applied Biosystems). The chosen primers and probe (see Table 1) amplify a 73-bp amplicon and bioinformatics analysis using BLAST and Clustal alignments showed no significant homology to human or other known influenza A gene sequences (Fig. 1). The highest homology is 91% to classical swine flu, e.g. A/swine/England/WVL15/1997(H1N1). With this strain there were 4 mismatches in the probe sequence, which would be expected to prevent hybridisation although this was not experimentally tested. In addition we would not expect to be screening humans infected with classical swine flu strains. The chosen primers and probe do not span any of the variable antigenic sites minimising the possibility of point mutations occurring (Abed et al., 2002).

A widely used universal influenza A rtPCR was used as part of the multiplex rtPCR. The assay targets the matrix region of the virus. Participation in various EQA schemes has shown this assay to detect influenza A viruses from humans and animals with high sensitivity (Carr et al., 2009). The rtPCR assay used for the internal control targets equine arteritis virus (EAV) and was taken from an existing publication (Scheltinga et al., 2005).

The primer and probe concentrations for each assay were individually optimised using in house protocols. All assays, singleton or multiplex, used the primers at an optimised concentration of 1 μM and the probe at 0.2 μM in a 15 μl reaction volume (all primers and probes are shown in Table 1).

For singleton assays, one-step rtPCR was performed on 6 μl of RNA extract with the Platinum One-step qRT-PCR kit (Invitrogen) on an ABI Prism 7500 SDS real-time platform (Applied Biosystems). The following thermal profile was used: a single cycle of reverse transcription for 15 min at 50 °C, 2 min at 95 °C for reverse transcriptase inactivation and DNA polymerase activation followed by 40 amplification cycles of 15 s at 95 °C and 34 s at 60 °C each (annealing-extension step).

For the multiplex rtPCR, one-step rtPCR was performed on 6 μl of RNA extract with the Qiagen quantifast mulitiplex RT-PCR kit (Qiagen, Crawley, United Kingdom) on an ABI prism 7500 SDS real-time platform. The following thermal conditions were used: a single cycle of reverse transcription for 20 min at 50 °C, 5 min at 95 °C for reverse transcriptase inactivation and DNA polymerase activation followed by 40 amplification cycles of 15 s at 95 °C and 30 s at 60 °C each (annealing-extension step).

Data acquisition occurred at the annealing step of each cycle and the threshold cycle (Ct) for each sample was calculated by deter-
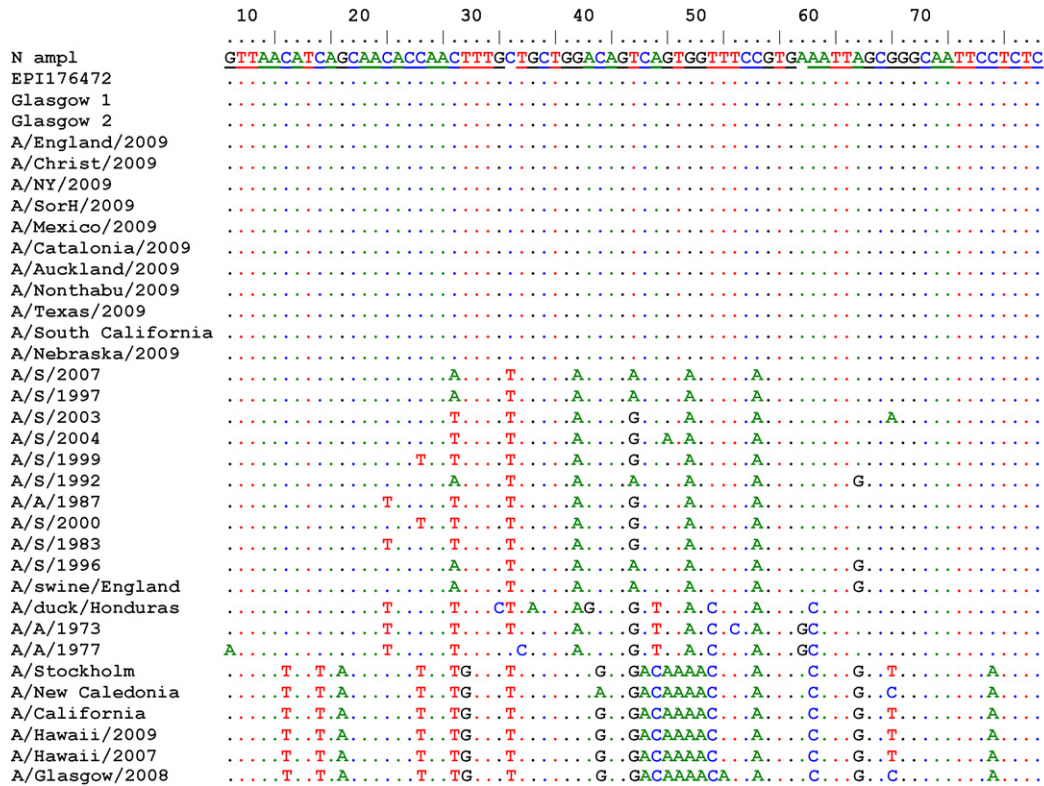

Fig. 1. Alignment of the pandemic (H1N1) 2009 N1 amplicon with a representative selection of pandemic (H1N1) 2009 sequences, including 2 sequenced at RVL Glasgow (Glasgow 1 and 2), a selection of avian and swine N1 sequences and human H1N1 sequences, including 1 sequenced in Glasgow (A/Glasgow/2008) in the 2008–2009 influenza sequence. Homology with the amplicon is indicated by dots and differences with the bases showing. Primer and probe sequences are underlined. All pandemic (H1N1) 2009 sequences had 100% homology with the primers and probe, whereas there were a significant number of differences between both seasonal H1N1 and swine and avian N1 sequences. All reference sequences were obtained from the influenza sequence database (ISD) at Los Alamos. Sequences were aligned using Bioedit.

mining the point at which the fluorescence exceeded the threshold limit.

Please note that the Qiagen quantifast multiplex RT-PCR kit was used for the multiplex rtPCR only as, unlike the Invitrogen RT-PCR kit, it is specially designed to prevent competition from occurring between the PCR tests within a multiplex. Such competition often results in false negative reactions or strange traces which can impede result interpretation. Previous comparisons between this kit and the Invitrogen kit have shown it to be superior for multiplex rtPCR (Gunson et al., 2008).

2.2. Samples used for the assessment of the multiplex assay

The specificity of the multiplex assay was assessed using a recent World Health Organisation (WHO) panel. This panel contained examples of seasonal influenza A, H1N1 and H3N2, the influenza A/H1N1/2009 and numerous avian A/H5N1 viruses. A pool containing the following commonly encountered respiratory pathogens was also tested: influenza B, influenza C, parainfluenza 1–4, human metapneumovirus, respiratory syncytial virus, *Mycoplasma pneumoniae*, rhinovirus (untyped) and coronaviruses 229E, OC43 and NL63.

The end point detection limit of the new multiplex rtPCR was directly compared to each rtPCR in singleton format using a dilution series of an influenza A/H1N1/2009 clinical sample. This was carried out to ensure that multiplexing did not result in a reduction in end point detection limit.

The final assessment comprised of assessing the multiplex rtPCR on 92 clinical samples. Of the 92 samples, 40 were seasonal influenza A viruses (20 subtyped as H1N1 and 20 as H3N2) that had been submitted to the WoSSVC during previous respiratory seasons. The remaining 52 were samples that had been sent to the laboratory in 2009 and found to be influenza A/H1N1/2009 positive using an alternative rtPCR (Carr et al., 2009). All samples contained H1N1 at varying concentrations (Ct values range from 17 to 39).

Total nucleic acid was extracted from the aforementioned samples using QIAamp Viral RNA kit (Qiagen, Crawley, United Kingdom) on the Qiagen MDx according to the manufacturer's instructions. Please note that EAV was added to the lysis buffer prior to extraction in order to detect PCR inhibition, extraction failure or technical error ($300 \,\mu l \times 10^{-6}$ (Ct 23) of BHK tissue culture grown EAV was added to the lysis buffer to extract 96 samples). Internal control was also added to ensure that its presence did not compete with the diagnostic components of the assay and inhibit their performance.

3. Results

The multiplex rtPCR was evaluated using a recent panel distributed by the WHO comprising seasonal influenza A (H1N1 and H3N2), avian (H5N1) and influenza A/H1N1/2009 influenza A viruses (Table 2). The universal influenza A component detected all

Table 2
Evaluation of the specificity of the H1N1 swl N1 rtRT-PCR assay using the WHO panel.

EQA sample	Expected result	Universal flu A result	H1N1 swl NA result
1	H5	20.82	Neg
2	H1N1 swl	21.51	21.51
3	H1 human	22.50	Neg
4	H5	20.35	Neg
5	H5	22.76	Neg
6	H1N1 swl	24.47	25.53
7	H5	24.48	Neg
8	H3 human	23.59	Neg
9	H5	20.56	Neg
10	H5	23.25	Neg

Table 3
Dilution series comparing the end point detection limit of the pandemic (H1N1) 2009 assay to the universal influenza A assay.

Sample	Single assays		Multiplex[a] assay	
	FluA	NA	FluA	NA
H1N1	27.10/27.08	27.02/26.77	28.45/27.93	28.20/27.62
-1	30.65/31.04	29.89/29.59	31.66/31.97	31.66/31.97
-2	35.06/34.43	37.65/34.16	34.18/36.13	34.18/36.13
-3	37.10/neg	Neg/neg	39.12/neg	Neg/neg
-4	Neg/neg	Neg/neg	Neg/neg	Neg/neg

[a] The triplex assay consists of the universal flu A assay, the pandemic (H1N1) 2009 NA assay and the EAV internal control.

samples as positive. The NA assay was positive in only two cases (samples 2 and 6) and did not detect the seasonal influenza A (H1N1 and H3N2) viruses or avian influenza viruses. The multiplex assay also did not detect any of the commonly encountered respiratory pathogens as positive.

The end point detection limit of both singleton rtPCR assays were compared using a dilution series of a influenza A/H1N1/2009 clinical sample. The universal influenza A test detected the 10-3 dilution in one out of two occasions (Table 3) whereas the NA assay was slightly less sensitive detecting the 10-2 dilution. Comparing the end point detection limit of each of the test components in singleton and multiplex form showed that multiplexing had no effect on test performance.

The final comparison assessed the multiplex assay on 92 clinical samples. The multiplex rtPCR detected all samples as influenza A positive. The NA assay detected all but 1 of the influenza A/H1N1/2009 samples as positive. The false negative sample had a Ct value >38 in the universal influenza and was confirmed as positive using an alternative influenza A/H1N1/2009 assay (Carr et al., 2009). None of the 40 samples containing seasonal influenza A were positive by NA assay.

4. Discussion

This article describes the development of a rapid, specific and sensitive multiplex rtPCR assay that detects all influenza A types and simultaneously identifies samples that contain the pandemic influenza A/H1N1/2009 virus. The assay also incorporates an internal control and thus is useful for detecting PCR inhibition or technical error. Multiplexing was shown to have no effect on the performance of the individual test components and, because the assay utilised the qiagen quantifast multiplex RT-PCR kit, the presence of internal control within the samples did not compete with the other diagnostic components.

The universal influenza A assay has been described elsewhere and the results shown here confirm it to be sensitive and able to detect a wide range of influenza A types. The NA assay was specific as it did not cross-react with seasonal influenza A strains currently circulating (subtypes H1N1 and H3N2) or other commonly encountered respiratory pathogens. In addition, the N1 assay did not detect as positive any of the influenza A, subtype H5N1 viruses. Close homology was observed between amplicon and classical swine influenza A (H1N1). The assay was not assessed against this virus and therefore we cannot be sure as to whether the test will detect this virus. However, four mismatches in the probe were observed which may reduce the likelihood of detecting this virus.

The sensitivity of the NA assay was slightly less than the frontline universal influenza A test. This was shown using the dilution series and was confirmed when it was assessed using the 52 influenza A/H1N1/2009 positive clinical samples where the NA assay failed to detect one sample which was positive using the universal influenza

A test and an alternative influenza A/H1N1/2009 assay. The Ct value of the sample was >38 in the universal influenza A assay which, based on the results from the dilution series, was likely to be beyond the detection limit of the H1N1 assay.

Based on the results shown here, the multiplex rtPCR is a useful addition to existing diagnostic protocols for influenza A. Its routine use would allow laboratories to screen out pandemic influenza A/H1N1/2009 positive samples rapidly. Such samples are likely to be in the majority and therefore only a small number of samples would require to be tested using seasonal or H5N1 specific assays. Use of this test would provide clinicians with complete results in rapid fashion and would reduce costs as far fewer samples will be sent for seasonal influenza or H5N1 typing.

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