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Original Article

Using the full spectral capacity (six channels) of a real-time PCR instrument can simplify diagnostic laboratory screening and typing protocols for pandemic H1N1 influenza

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Background Timely reporting of influenza A virus subtype affects patient management. Real-time PCR is a rapid and sensitive method routinely used to characterise viral nucleic acid, but the full spectral capability of the instruments is not employed.

Objectives To evaluate a hexaplex real-time PCR assay (Flu-6plx assay) capable of detecting influenza A and B, hMPV, respiratory syncytial virus (RSV) and distinguishing 2008 'human' influenza A/H1 from 2009 pandemic A/H1 subtypes.

Methods Respiratory specimens (n = 213) were tested using the Flu-6plx assay and a further four monoplex PCRs targeting hMPV, RSV, influenza A and B. The FDA-approved ProFlu ST test was used to validate the Flu-6plx PCR influenza A/H1 subtyping components. Discrepant 2009 pandemic A/H1 results were further tested using the CDC swine H1 assay.

Results The Flu-6plx assay had excellent sensitivity identifying 106/106 influenza A RNA–positive samples. The ProFlu ST test was a less sensitive subtyping test, and discrepant analysis could not confirm A/H1 status for four samples resulting in Flu-6plx PCR specificities of 98% and 95% for human A/H1 and 2009 pandemic A/H1, respectively. Co-infection affected the sensitivity of the Flu-6plx PCR hMPV component whereby low-level hMPV RNA could be masked by much higher concentrations of influenza A virus RNA.

Conclusions The Flu-6plx assay is a sensitive and specific test for the universal detection of influenza A infection and determination of A/H1 subtype. Concomitant detection of influenza B, hMPV and RSV demonstrates the utility of hexaplex real-time PCRs in viral diagnostics.

Keywords Hexaplex, influenza, metapneumovirus, real-time PCR, respiratory syncytial virus.

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Background

The 2009 influenza pandemic led to a massive increase in laboratory testing for the viral causes of acute respiratory illness.[1] Distinction of influenza infection from other respiratory viral aetiologies and identification of influenza A virus subtype aided patient management decisions. During an influenza outbreak in our hospital in December 2008, both influenza A/H1 and A/H3 strains were circulating.[2] Ninety nine per cent of all influenza A/H1 isolates characterised by the UK Health Protection Agency at this time were resistant to oseltamivir, whilst A/H3 strains remained sensitive to the drug.[3] As a result, first-line therapy for high-risk patients was switched from oseltamivir to zanamivir until the viral subtype was identified. The 2009 pandemic A/H1 strain has since become the dominant circulating subtype, and timely determination of this viral subtype has become important for epidemiology and infection control purposes.[4]

Real-time PCR offers a rapid and reliable methodology to detect and characterise viral nucleic acid, and numerous assays are available to help laboratories implement effective diagnostic services.[5–11] Many protocols are multiplexed to simplify workflow and reduce costs. However, no assay currently employs the full six fluorescent channel capacity of some real-time PCR instruments.

The aim of this study was to evaluate a six-target (hexaplex) real-time RT-PCR assay capable of detecting human metapneumovirus (hMPV), respiratory syncytial virus (RSV), influenza A/B and distinguishing seasonal human influenza A/H1 from the 2009 pandemic A/H1 subtype (Flu-6plx assay). The study retrospectively compared the hexaplex PCR results with four sensitive monoplex PCR protocols and validated subtyping results against the Prodesse ProFlu ST subtyping kit (Prodesse Inc., Waukasha, USA).

Methods

Patient samples and controls

The Flu-6plx assay was assessed with a panel of 213 samples comprising: 21 external quality assurance samples from Quality Control for Molecular Diagnostics (QCMD, Glasgow, UK), 154 nose and throat swabs submitted to the Royal Liverpool University Hospital Virology laboratory for investigation into respiratory illness between December 2008 and November 2009 and a further 38 paediatric nasopharyngeal aspirates collected at Instituto de Medicina Integral Professor Fernando Figueira Hospital, Recife, Brazil, between April 2008 and March 2009.

RNA Isolation

MS2 phage internal control (10 000 copies) was added to each 200 μl sample prior to nucleic acid extraction using the Qiagen Minelute Virus Spin kit or the Qiasymphony Virus/Bacteria Mini kit (Qiagen, Crawley, UK).

Real-time PCR

The Flu-6plx PCR assay utilised the Superscript III qRT-PCR kit (Invitrogen, Paisley, UK) in a 25 μl reaction containing 5 μl purified RNA and oligonucleotides at the concentrations given in Table 1. MS2 phage internal control RNA was detected in a second in-house respiratory hexaplex reaction (PIV-6plx) run under identical conditions. RNA was denatured at 95°C for 1 minute prior to master mix addition on ice. Amplification and detection were performed on a Lightcycler 480 real-time PCR machine (Roche Diagnostics, Burgess Hill, UK) with reverse transcription at 50°C for 20 minutes, denaturation at 95°C for 2 minutes and 50 amplification cycles of 95°C for 15 seconds, 58°C for 45 seconds and 72°C for 1 seconds.

All nucleic acid extracts were assayed using a further four monoplex PCRs targeting the influenza A matrix gene, influenza B nucleoprotein gene, RSV and hMPV fusion genes to designate sample status.[5,12,13]

A subset ($n = 95$) of nucleic acid extracts identified as influenza A positive were further tested using the Prodesse ProFlu ST subtyping kit on the Smartcycler II system according to the manufacturer's instructions. Discrepant 2009 pandemic A/H1 subtype results were resolved using CDC 2009 pandemic A/H1 PCR assay.[14]

Results

A PCR result was considered positive when there was evidence of efficient amplification with a distinct sigmoidal curve. All Cp values were ≤37 cycles in Flu-6plx assay and ≤42 cycles in monoplex PCRs. The latter assays detected influenza A, influenza B, hMPV and RSV RNA in 106, 11, 20 and 24 study specimens, respectively (Table 2). The panel contained 58 monoplex-negative specimens, and six samples were co-infected with influenza A and either hMPV ($n = 4$) or RSV ($n = 2$). The Flu-6plx assay detected influenza A RNA in all 106 influenza A–positive samples. Four of these samples, all of which had high Cp values (Cp range, 31–35) in the monoplex assay, were positive in either the 2009 pandemic H1 ($n = 2$) or human H1 ($n = 2$) Flu-6plx channels but negative in the matrix channel. These four samples were considered positive for the purposes of this evaluation, giving the hexaplex assay 100% sensitivity and specificity for influenza A. The Flu-6plx assay correctly detected influenza B RNA in 11 samples but identified only 22/24 RSV- and 16/20 hMPV-positive samples. The six samples with false-negative hexaplex results all contained low-level viral RNA by monoplex PCR (Cp range, 33–41) and 4/6 were also influenza A RNA positive. No inhibitory samples were detected.

The ProFlu ST test was unable to subtype 17/95 influenza A–positive samples, whereas the Flu-6plx assay identified four of these as human A/H1 and 11 as 2009 pandemic A/H1 (Table 3). Three of the four discrepant human A/H1 samples were known human A/H1 weak-positive QCMD specimens, and 8/11 discrepant 2009 pandemic A/H1 samples were positive by the CDC 2009 pandemic H1 PCR. The Flu-6plx assay does not type influenza A/H3: 36/37 samples untyped by this assay were typed as influenza A/H3 (ProFlu ST PCR or QCMD data). The Flu-6plx assay had sensitivities and specificities of 100% and 98% for human A/H1, and 100% and 95% for 2009 pandemic A/H1 components, respectively.

Discussion

The Flu-6plx assay showed excellent sensitivity as an influenza A screening assay in comparison with a monoplex influenza A assay that targets a similar region of the influenza A matrix gene with high sensitivity.[5] However, the multiplex format compromised the sensitivity of the assay to detect low-level hMPV RNA when high amounts of influenza A RNA were present in the same specimen. The hMPV component of the hexaplex assay was able to detect all but one of the hMPV positive samples when run as a

Table 1. Oligonucleotide primers and probes used in the Flu-6plx assay

Assay	Oligonucleotide	Sequence (5′–3′)	Concentration (μm)	Target gene	References
Flu-6plx	hMPV_383F	ACAAAGARGCAAGAAAAACAATGG	0·4		
	hMPV_451R	GGTGTGTCTGGTGCTGArGG	0·4	hMPV NP	Modified
	hMPV_424A	(FAM)– TCATCAGGyAATATy**C**CA**C**AAAATCAGAG –(BHQ1)	0·2		from 17
	hMPV_424B	(FAM)– TCATCAGGTAACATC**C**CA**C**AAAACCAGAG –(BHQ1)	0·1		
	RSV_F	GCAAATATGGAAACATACGTGAACA	0·4		
	RSV_R	GCACCCATATTGTWAGTGATGCA	0·4	RSV matrix	18
	RSV_LC610	(LC610)– CTTCACGAAGGCTCCACATACACAGCWG –(BHQ2)	0·1		
	IfB_F	AAATACGGTGGATTAAATAAAAGCAA	0·4		
	IfB_R	CCAGCAATAGCTCCGAAGAAA	0·4	Influenza B HA	18
	IfB_cyan500	(Cyan500)– CACCCATATTGGGCAATTTCCTATGGC –(BHQ1)	0·1		
	IfA_F	AAGACCAATCCTGTCACCTCTGA	0·4		
	IfA_R	CAAAGCGTCTACGCTGCAGTCC	0·4	Influenza A matrix	18
	IfA_VIC	(VIC)– TTTGTGTTCACGCTCACCGT –(MGB-NFQ)	0·1		
	H1_F	ATTGCCGGTTTCATTGAAGG	0·4		
	H1_R	ATGGCATTYTGTGTGCTYTT	0·4	Influenza A HA	19
	Swine_H1_LC640	(LC640)– ATGAGCAGGGGTCAGGATATGCAGCCGACC –(BHQ2)	0·1		
	Human_H1_LC670	(LC670)– ATGAGCAAGGATCTGGCTATGCTGCAGATC –(BHQ2)	0·1		
PIV-6plx	PIV1 HN525 F	GATTTCTGGAGATGTCCCGTAGG	0·4		
	PIV1 HN722 R	TGACTTCCCTATATCTGCACATCC	0·4	PIV type 1 HA-NM	
	PIV-1 HN556	(FAM)– TACTGAGCAACAACCC –(MGB-NFQ)	0·16		
	PIV-2 F	CCATTTACCTAAGTGATGGAA	0·4		
	PIV-2 R	CGTGGCATAATCTTCTTTT	0·4	PIV type 2 HA-NM	7
	PIV2 LC640	(LC640)– AATCGCAAAAGCTGTTCAGTCAC –(BHQ2)	0·16		
	PIV3 NP300 F	CTTTCAGACAAGATGGAACAGTGC	0·4		
	PIV3 NP800 R	AGTTACCAAGCTCTGTTGAGACC	0·4	PIV type 3 NP	
	PIV3 NP766	(LC610)– CCAATCTGATCCACTGTGTCACCGCTCA –(BHQ2)	0·16		
	PIV4 NP271 F	CAGGCCACATCAATGCAGAATC	0·4		
	PIV4 NP407 R	ATGTCATCCCAGCCAGATCTTG	0·4	PIV type 4 NP	
	PIV4 NP298	(LC670)– ATGATTGCTGCCAGAGCCCCAGATGC –(BHQ2)	0·16		
	hRV F	TGG ACA GGG TGT GAA GAG C	0·4		
	hRV R	CAA AGT AGT CGG TCC CAT CC	0·4	Rhinovirus 5′ UTR	7
	hRV HEX	(VIC)– TCC TCC GGC CCC TGA ATG –(BHQ1)	0·16		
	MS2 F1	TGG CAC TAC CCC TCT CCG TAT TCA CG	0·2		
	MS2 R1	GTA CGG GCG ACC CCA CGA TGA C	0·2	MS2 phage	20
	MS2 Cyan500	(Cyan500)– CACATCGATAGATCAAGGTGCCTACAAGC–(BHQ1)	0·08		

C denotes position of pdC nucleic acid bases. PIV; parainfluenza virus, NP; nucleoprotein, HA; haemagglutinin, NM; neuraminidase, UTR; untranslated region.
All oligonucleotides were purchased from Metabion Ltd, Martinsried, Germany, except MGB probes that were obtained from Applied Biosystems, Warrington, UK.

monoplex assay (data not shown). The remaining hMPV-positive sample had a Cp value of 39 by the alternative monoplex PCR.[13] Thus, the lower hMPV sensitivity of the Flu-6plx assay is a result of PCR competition in the multiplex format, and low-level hMPV co-infection cannot be excluded with this screening assay.

Multiple influenza A targets in a screening assay help overcome problems caused by sequence variation. However, samples with a positive influenza A/H1 PCR signal but negative influenza A matrix PCR may warrant further testing with a confirmatory monoplex PCR in the diagnostic setting. Similarly, the presumptive influenza A/H3 samples identified by the Flu-6plx assay (influenza A matrix PCR positive but both A/H1 PCRs negative) would require a further test to confirm subtype.

The influenza A/H1 typing components in the Flu-6plx assay showed excellent sensitivity in comparison with the FDA-approved ProFlu ST test. However, the discrepant analysis model used in this study meant that the specificity calculations for the hexaplex assay were adversely affected by the four samples in which the Flu-6plx identified as A/H1, but this status could not be confirmed with any of

Table 2. Flu-6plx assay results in comparison with resolved data from monoplex PCRs for 213 respiratory samples

	Monoplex result	Flu-6plx result	Flu-6plx sensitivity	Flu-6plx specificity
Influenza A positive	106	106*	100	100
Influenza B positive	11	11	100	100
hMPV positive	20**	16	80	100
RSV positive	24***	22	92	100
Negative	58	60		
Total tested	213	213		
Single infections	149	151		
Co-infections	6	2		

*Includes four influenza A/H1 component positive, matrix component negative Flu-6plx results. The Flu-6plx assay identified subtype in these samples as 23 human H1, 42 2009 pandemic H1 and 41 presumptive H3.
**Low-level hMPV RNA detected in four additional samples by monoplex PCR (Cp range, 35–41). Three were co-infections with influenza A.
***Low-level respiratory syncytial virus (RSV) RNA detected in two additional samples by monoplex PCR (Cp range, 33–37). One was a co-infection with influenza A.

Table 3. Flu-6plx assay influenza A subtyping results in comparison with ProFlu ST subtyping assay for 95 influenza A–PCR positive respiratory samples

	ProFlu ST result	Flu-6plx result	Resolved subtype*	Flu-6plx sensitivity	Flu-6plx specificity
Influenza A subtype					
Human A/H1	18	22	21	100	98
2009 pandemic A/H1	25	36	33	100	95
Human A/H3	35	ND	36		
Untyped	17	37**	5***		

*Influenza A subtype resolved using ProFlu ST test, CDC 2009 pandemic H1 PCR and QCMD data.
**Flu-6plx assay does not identify influenza A/H3 subtype. These samples correspond to the 36 resolved influenza A/H3 samples plus another untyped low-level influenza A RNA matrix PCR (Cp = 37).
***Five samples with unresolved subtypes all contained low-level influenza A RNA (matrix PCR Cp > 34).
ND, not determined.

the other assays used in this evaluation. Sensitivity of the Flu-6plx assay to detect mixed influenza A/H1 infections was not tested.

Crosstalk between fluorescent channels was minimised by the application of an assay-specific colour compensation file. These files allow the user to balance assay sensitivity against elimination of crosstalk. The file used in this study allowed the sensitive detection of low signal, although weak bleed-through was occasionally detected in an adjacent channel. This crosstalk was recognised by identical crossing points in the two channels and was often more evident with the far red dyes. Signal strength should be altered in the colour compensation file dependant on the needs of the laboratory.

Identification of human influenza A/H1 subtype was important for patient management during the 2008/9 winter season as this virus was oseltamivir resistant but susceptible to zanamivir.[3] Emergence of the 2009 pandemic H1 virus as the dominant circulating strain compelled laboratories to adopt new subtyping protocols for infection control and patient management. The Flu-6plx assay reported here combines both A/H1 subtyping functions in a sensitive and specific assay for influenza and also facilitates the detection of influenza B, hMPV and RSV RNA in the same reaction. Multiplex real-time PCR has simplified the molecular diagnosis of viral respiratory disease, but the majority of these PCRs contain only three or four targets in each reaction.[6,8–10] Several real-time PCR platforms have six detection channels, but these instruments have not been fully exploited in routine diagnostic virology laboratories. A hexaplex real-time PCR assay has been reported for screening genetically modified organisms in food, but the protocol used a five-channel instrument with two components detected in the same channel.[15] Real-time six-channel technology has been used by commercial tests to determine antibacterial resistance but, to the best of our knowledge, this is the first report validating a laboratory-developed assay capable of distinguishing six individual viral targets.[16] Utilising real-time PCR platforms to their full spectral capacity can reduce the number of reactions required for a respiratory virus screening panel, thereby saving both time and resources.

Funding

None.

Conflicts of interest

None declared.

Ethical approval

Analysis of respiratory samples from Instituto de Medicina Integral Professor Fernando Figueira Hospital (IMIP), Brazil, was approved by the Human Subjects Office at IMIP and the National Research Ethics Office of Brazil (protocol number 896-06).

References

1 Scriven J, Mcewen R, Mistry S *et al.* Swine flu: a Birmingham experience. Clin Med 2009; 9: 534–538.

2 Health Protection Agency. Outbreak of influenza at an acute hospital in the north-west of England. Health Protection Agency Weekly Report 2009; 3: 4–5. Available at http://www.hpa.org.uk/hpr/archives/2009/hpr0509.pdf (Accessed 18 May 2010).

3 McLean E, Murdoch H, Reynolds A *et al.* Surveillance of influenza and other respiratory viruses in the United Kingdom: October 2008 to April 2009. Health Protection Agency Weekly Report 2009; 3: 7–24 Available at http://www.hpa.org.uk/hpr/archives/2009/hpr3909.pdf (Accessed 31 March 2010).

4 Health Protection Agency. Weekly pandemic flu media update, 14 January 2010. Health Protection Agency 2010: Available at http://www.hpa.org.uk/NewsCentre/NationalPressReleases/2010PressReleases/100114Weeklypandemicflumediaupdate/ (Accessed 31 March 2010).

5 Carr MJ, Gunson R, Maclean A *et al.* Development of a real-time RT-PCR for the detection of swine-lineage influenza A (H1N1) virus infections. J Clin Virol 2009; 45:196–199.

6 Gunson R, Maclean A, Davies E, Bennett S, Miller R, Carman WF. 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. J Virol Methods 2010; 163:258–261.

7 Gunson RN, Collins TC, Carman WF. Real-time RT-PCR detection of 12 respiratory viral infections in four triplex reactions. J Clin Virol 2005; 33:341–344.

8 Hymas WC, Hillyard DR. Evaluation of Nanogen MGB Alert Detection Reagents in a multiplex real-time PCR for influenza virus types A and B and respiratory syncytial virus. J Virol Methods 2009; 2:124–128.

9 Ieven M. Currently used nucleic acid amplification tests for the detection of viruses and atypicals in acute respiratory infections. J Clin Virol 2007; 40:259–276.

10 Lassauniere R, Kresfelder T, Venter M. A novel multiplex real-time RT-PCR assay with FRET hybridization probes for the detection and quantitation of 13 respiratory viruses. J Virol Methods 2010; 165:254–260.

11 Templeton KE, Forde CB, Loon AM *et al.* A multi-centre pilot proficiency programme to assess the quality of molecular detection of respiratory viruses. J Clin Virol 2006; 35:51–58.

12 Curran MD, Ellis J, Zambon M. Real-time quadruplex for the detection of influenza. Health Protection Agency 2006: Available at http://www.hpa-standardmethods.org.uk/documents/vsop/pdf/vsop25.pdf (Accessed 18 May 2010).

13 Kuypers J, Wright N, Corey L, Morrow R. Detection and quantification of human metapneumovirus in pediatric specimens by real-time RT-PCR. J Clin Virol 2005; 33:299–305.

14 Centers for Disease Control. CDC Protocol of Realtime RTPCR for Influenza A (H1N1). World Health Organisation 2009: Available at http://www.who.int/csr/resources/publications/swineflu/CDCRealtimeRTPCR_SwineH1Assay-2009_20090430.pdf (Accessed 31 March 2010).

15 Bahrdt C, Krech AB, Wurz A, Wulff D. Validation of a newly developed hexaplex real-time PCR assay for screening for presence of GMOs in food, feed and seed. Anal Bioanal Chem 2010; 396:2103–2112.

16 Helb D, Jones M, Story E *et al.* Rapid detection of Mycobacterium tuberculosis and rifampin resistance by use of on-demand, near-patient technology. J Clin Microbiol 2010; 48:229–237.

17 Hopkins MJ, Redmond C, Shaw JM *et al.* Detection and characterisation of human metapneumovirus from children with acute respiratory symptoms in north-west England, UK. J Clin Virol 2008; 42:273–279.

18 Brittain-Long R, Nord S, Olofsson S, Westin J, Anderson LM, Lindh M. Multiplex real-time PCR for detection of respiratory tract infections. J Clin Virol 2008; 41:53–56.

19 Wang R, Sheng ZM, Taubenberger JK. Detection of novel (swine origin) H1N1 influenza A virus by quantitative real-time reverse transcription-PCR. J Clin Microbiol 2009; 47:2675–2677.

20 Rolfe KJ, Parmar S, Mururi D *et al.* An internally controlled, one-step, real-time RT-PCR assay for norovirus detection and genogrouping. J Clin Virol 2007; 39:318–321.

