Detection and discrimination of influenza A, B, and novel H1N1 influenza viruses using the 3M integrated cycler

T-19

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Abstract

The emergence of a novel H1N1 influenza virus, with concomitant circulation of other human origin influenza A and B strains, has demonstrated the need for a rapid and effective test that will detect and discriminate influenza viruses. Molecular techniques provide the ability to swiftly address this need, as they can be designed and developed immediately upon the availability of specific sequence targets. A multiplex assay was designed to detect influenza A viruses (of all hemagglutinin and neuraminadase types), influenza B viruses, and the novel swine-origin H1N1 influenza A virus. Specific regions of the hemagglutinin gene from the novel H1N1 virus were targeted to enable the detection of the new variant without cross reactivity or detection of previously circulating H1, H3, or H5 viruses. The assay was tested against a panel of known influenza strains and against a set of clinical specimens that were verified to be positive for the novel swine-origin H1N1 virus. The assay was able to detect the presence of all influenza A and influenza B viruses tested, and it was also able to detect the H1 gene target from the novel H1N1 influenza A virus; the results showed 100% specificity with respect to the discrimination of the novel H1N1 virus from other influenza A virus strains. Other respiratory pathogens including closely related parainfluenza viruses were not detected by the assay. The use of this assay on the 3M integrated cycler provides a sensitive method for the rapid detection and discrimination of influenza viruses, and it may prove useful in detecting outbreaks in a community, and in providing physicians with a tool to predict response to antiviral therapy.

Methods

Sample Preparation: Influenza clinical samples were obtained from the Focus Diagnostics Reference Laboratory. Pandemic H1N1 (A/California/7/2009), seasonal influenza virus (A/PR/8/34), and influenza B virus (B/GL/1739/54) were obtained from Virapur, Advanced Biotechnologies Inc, and ATCC, respectively. Clinical specimens (200 uL) and virus stocks were extracted using a Roche MagNA Pure LC instrument, and its corresponding Total Nucleic Acid Isolation Kit.

Multiplex Influenza RT-PCR: PCR primer sequences were designed to target influenza A and B (Matrix genes), influenza A H1N1 (2009) (HA gene), and an internal control. Probes specific for influenza A, B, H1N1, and the internal control were labeled with FAM, JOE, CAL Fluor Red 610, and Quasar 670, respectively. The final primer concentration for each reaction was 600 nM for each influenza target, and 150 nM for the internal control primers. RT-PCR was carried out using the 3M Integrated Cycler (Fig. 1) with the following cycling parameters: stage 1: 47˚C for 15 minutes, stage 2: 97˚C for 10 minutes, and stage 3: 40 cycles of 97˚C for 15 seconds and 60˚C for 30 seconds.

PCR Efficiency: The RT-PCR amplification efficiency was determined by amplifying serially diluted viral RNA. A curve slope was obtained after plotting Ct vs. log concentration values for influenza A, influenza B, and influenza A H1N1 (2009) targets.

Limit of Detection: Dilutions were made for each virus (seasonal influenza A and B, and influenza A H1N1 2009), and replicate specimens were run. The lowest concentration with >95% detection was considered to be the LOD.

Methods (Cont.)

Specificity: A panel of viral and bacterial pathogens was extracted using the Roche MagNA Pure, and was then amplified to determine if the assay would cross react with other respiratory pathogens.

Method Comparisons: Three different method comparisons were performed. Initially, a panel of clinical samples containing H1 and H3 subtypes, as confirmed by the California State Department of Public Health (CA DOH), was examined with the multiplex influenza RT-PCR assay. In addition, a panel of 6 influenza A H1N1 (2009) samples (confirmed using the CDC PCR protocol at CA DOH) was tested. Finally, a comparison was performed between the test method and the Emergency Use Authorized Influenza A H1N1 (2009) RT-PCR assay (Focus Diagnostics) using a panel of 60 characterized clinical samples.

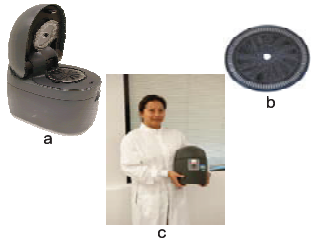

Figure 1. 3M Integrated Cycler (a) shown along with sample disc (b) and a demonstration of its compact nature (c).

Results

Detection of influenza viruses using the 3M Integrated Cycler: The 3M Integrated Cycler performance was demonstrated with the multiplex flu RT-PCR. As shown in Figure 2a-e, robust detection of influenza A (2a), B (2b), H1N1 (2c), and co-infection of influenza A and B (2d) as well as H1N1 and influenza B (2e) are achieved.

PCR Efficiency : PCR efficiencies were 95% or better for all targets, based on slopes of -3.34, -3.46, and -3.27 for the influenza A, influenza B, and influenza A H1N1 (2009) amplifications respectively (Table 1).

Limit of Detection: The LOD, which was defined as the lowest dilution with >95% detection, was determined to be 3 TCID50/mL for both seasonal influenza A and influenza A H1N1 (2009), and 15 ECID50/ml for Influenza B.

Specificity Studies: As shown in Table 3, the assay does not detect any of the pathogens tested and was specific for the targeted viruses. In addition, database searches indicate that the targeted regions do not have significant homology with sequences from other pathogens.

Method Comparison Studies: As shown in Tables 4 and 5, the test method provided results that were 100% concordant with the results obtained from the CA state department of public health. Comparison with the EUA method also showed 100% concordance, with each method identifying 44 H1N1 positive and 16 H1N1 negative samples. (Table 6).

Results (Cont.)

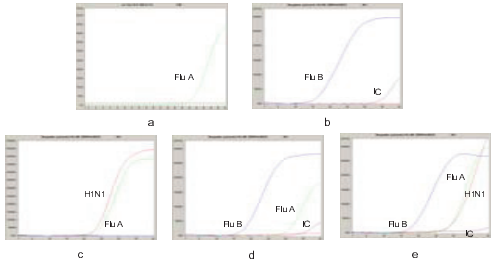

Figure 2. Amplification plots showing detection of influenza A (a), influenza B (b), influenza A H1N1(2009) (c), co-infection of influenza A and B (d), and co-infection of influenza B and H1N1(e) by the 3M Integrated Cycler

Table 1. Multiplex influenza RT-PCR amplification efficiency

Virus	Slope	Amplification Efficiency
Influenza A	-3.34	99%
Influenza B	-3.46	95%
H1N1(2009)	-3.27	~100%

Table 2. Limit of detection studies.

Flu A		Flu B		H1N1	
TCID	# Detected	ECID	# Detected	TCID	# Detected
3000	3/3	16000	3/3	3000	3/3
300	3/3	1600	3/3	300	3/3
30	3/3	160	3/3	30	3/3
3	3/3	16	3/3	3	3/3
0.3	1/3	1.6	0/3	0.3	0/3

Table 3. Specificity testing with different respiratory pathogens

Pathogen	Results	Pathogen	Results
Adenovirus 7	-	*Parainfluenza -1*	-
Bordetella pertussis	-	*Parainfluenza -2*	-
Bordetella parapertussis	-	*Parainfluenza -3*	-
Chlamydophila pneumoniae	-	*Rhinovirus-16*	-
Coronavirus 229E	-	*RSV A*	-
Coronavirus OC43	-	*RSV B*	-
Legionella pneumophila	-	*Mumps virus*	-
Mycobacterium tuberculosis	-	*H5N1**	-
Mycoplasma pneumoniae	-	*Human Metapneumovirus*	-

* positive with influenza A probe

Results (Cont.)

Table 4. Method comparison between test method (multiplex influenza RT-PCR) and expected results (influenza A typing)

Influenza A typing*		Multiplex influenza RT-PCR		
H1	H3	Flu A	H1N1	Flu B
+	-	+	-	-
+	-	+	-	-
+	-	+	-	-
+	-	+	-	-
-	+	+	-	-
-	+	+	-	-
-	+	+	-	-
-	+	+	-	-
-	+	+	-	-

* performed by California State VRDL lab

Table 5. Method comparison between test method (multiplex influenza RT-PCR) and expected results (CDC Developed H1N1 PCR assay)

H1N1 PCR assay*		Multiplex influenza assay		
Swine Flu A	Swine H1	Flu A	H1N1	Flu B
+	+	+	+	-
+	+	+	+	-
+	+	+	+	-
+	+	+	+	-
+	+	+	+	-
+	+	+	+	-

* performed by California State VRDL lab

Table 6. Concordance table for influenza A H1N1 (2009) detection comparing test method (multiplex influenza RT-PCR) and expected results (Focus Diagnostics H1N1 RT-PCR assay)

		Multiplex RT-PCR +	Multiplex RT-PCR -
EUA Assay	+	44	0
	-	0	16

Conclusions

- The LOD's for influenza virus A, B, and influenza A H1N1(2009) are 3 TCID, 15 ECID, and 3 TCID, respectively.
- Concordant results from parallel studies with other methods shows that the multiplex influenza RT-PCR assay effectively detects both seasonal influenza viruses (H1, and H3) and Influenza A H1N1 (2009).
- The multiplexed influenza RT-PCR using the 3M Integrated Cycler provides robust and sensitive detection for influenza A and B, and influenza A H1N1 (2009), and the convenience of the instrument (small footprint, rapid cycling, and high throughput) shows that it provides an effective option for clinical laboratory testing.

Acknowledgements

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