

Validation of Multiplex Real Time RT-PCR Assay with an Internal Control for the Detection of influenza A/H1N1 2009

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Background

Molecular tests for rapid detection of influenza A/H1N1(2009) are necessary. In this regard, real-time polymerase chain reaction (PCR) based on TaqMan technology enables the accurate detection of viral genome over a broad range without the necessity for post-PCR handling. In this study, we developed a multiplex real-time RT-PCR assay (rRT-PCR) capable of simultaneously detecting influenza A, which includes novel influenza A/H1N1. The assay was validated clinically by direct comparison with a conventional PCR(cRT-PCR) and commercial PCR kit.

Methods

Primer and probe sets were used to target the HA gene of the pandemic influenza A/H1N1 2009, and the matrix gene of influenza A (Fig.1). We evaluated the analytical sensitivity, specificity, reproducibility, and reportable range of Influenza A virus and H1N1 RNA load using the rRT-PCR assay. The intra-assay variation was determined by using 2 plasmid DNA samples, one with low (5 X 10³ copies/mL) and the other with medium (10⁵ copies/mL) concentration. For greater accuracy, we also evaluated the relation between the multiplex rRT-PCR, cRT-PCR and commercial PCR kit by using 1087 respiratory samples obtained from July 2009 to January 2010.

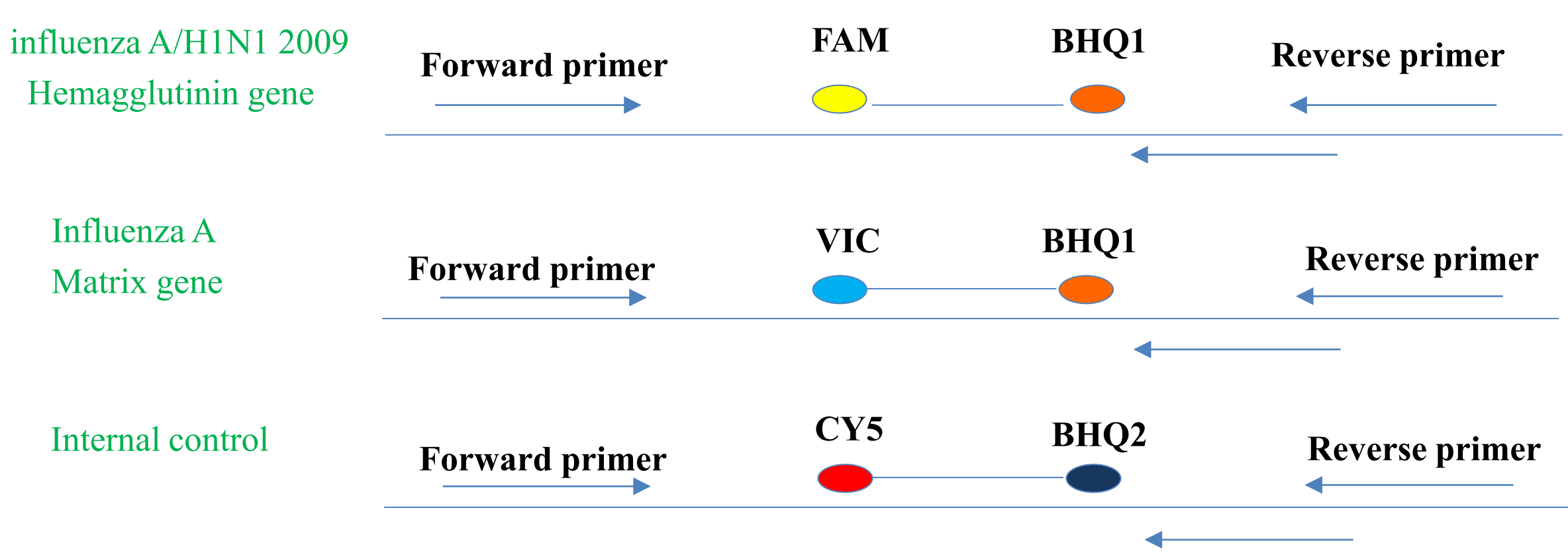


Figure 1. Location of amplification primers and TaqMan probe in multiplex real-time RT-PCR

Results

This assay detected influenza A/H1N1 RNA in a linear range from 500 to 10¹⁰ copies/mL (Fig. 2). The detection limit ranged between 1X10² and 2X10² copies/mL (Table 1). The percent coefficient of variation (%CV) value in the intra- and inter analysis was found to be almost under 5% (Table 2). None of the other viruses, showed cross-reactivity with influenza A (Table 3). The prevalence of influenza A/H1N1 2009 in the respiratory samples was 180 (16.5%) in the case of the multiplex rRT-PCR, 174 (16.0%) where the cRT-PCR was concerned, and 175 (16.1%) in the case of the commercial kit (Table 4).

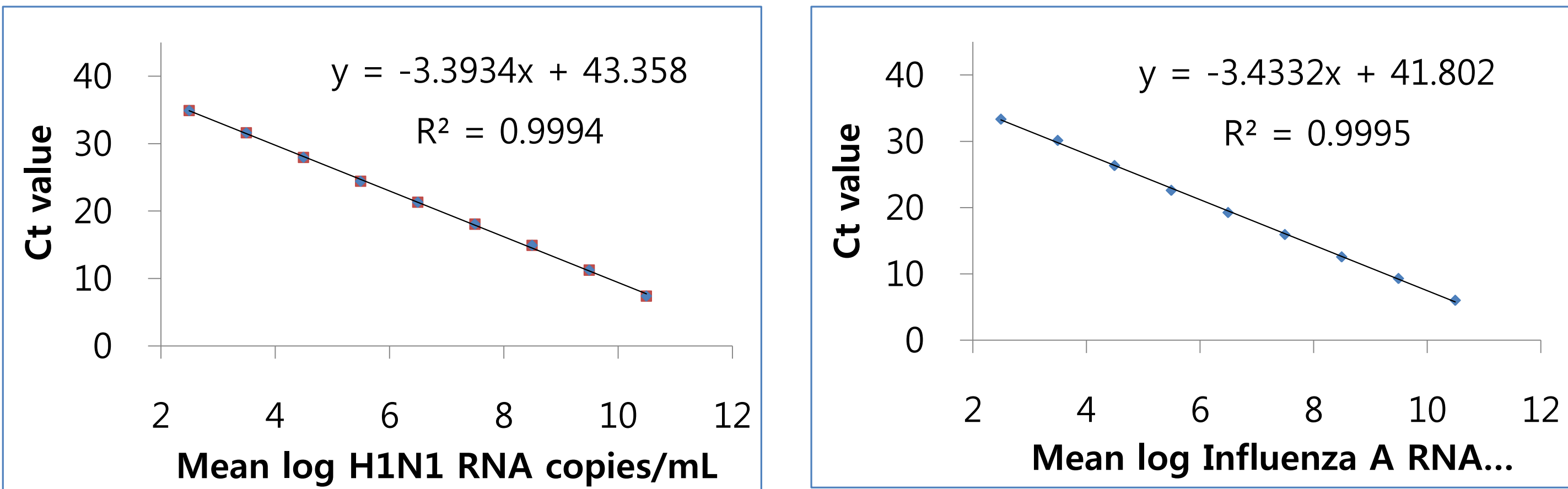


Fig. 2. Standard curves generated by multiplex real-time RT-PCR assay using serial dilutions of plasmid standard

Table 1. Detection limit of multiplex real-time RT-PCR assay

Input H1N1 (copies/mL)	Result		
	Replicates	Positive reaction	%
2000	18	18	100
500	18	18	100
100	18	15	83.3
20	18	6	33.3

Input Influenza A (copies/mL)	Result		
	Replicates	Positive reaction	%
2000	18	18	100
500	18	18	100
100	18	16	88.9
20	18	5	27.8

Table 2. Intra- and inter-assay variability of plasmid standard

Copy no. of plasmid standard	H1N1			Influenza A		
	Mean Ct	SD Ct	%CV	Mean Ct	SD Ct	%CV
Intra-assay (n=10)						
5 X 10 ⁵ copies/mL	24.33	0.64	2.63	22.71	0.45	1.98
5 X 10 ³ copies/mL	31.49	0.95	3.02	30.14	0.59	1.96
Inter-assay (n=20)						
5 X 10 ⁵ copies/mL	24.38	0.35	1.44	22.66	0.22	0.97
5 X 10 ³ copies/mL	31.55	0.99	2.83	30.17	0.79	2.62

Table 3. Analytical specificity of in-house real-time PCR assay

	SWH1	Influenza A	Internal control
Respiratory syncytial virus	-	-	+
Parainfluenza	-	-	+
Enterovirus	-	-	+
Adenovirus	-	-	+
Hepatitis B virus	-	-	+
Hepatitis C virus	-	-	+
Epstein-barr virus	-	-	+
Cytomegalovirus	-	-	+
BK virus	-	-	+

Table 4. Comparison with multiplex real-time RT-PCR, conventional PCR, commercial kit for detection of influenza A/H1N1 2009-positive sample

Detection methods	No.(%) of Influenza A/H1N1 2009 positive sample	
	Total (n=1087)	
multiplex rRT-PCR	180 (16.5)	
cRT-PCR	174 (16.0)	
commercial PCR kit	175 (16.1)	

Conclusion

The mutiplex real-time PCR assay designed to detect influenza A/H1N1 2009 and the influenza A group showed good analytical sensitivity, specificity, high reliability, and a broad reportable range.