

Detection of Influenza Virus Types A and B and Type A Subtypes (H1, H3, and H5) by Multiplex Polymerase Chain Reaction

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Infections with influenza virus type A and B present serious public health problems on a global scale. However, only influenza A virus has been reported to cause fatal pandemic in many species. To provide suitable clinical management and prevent further virus transmission, efficient and effective clinical diagnosis is essential. Therefore, we developed multiplex PCR assays for detecting influenza types A and B and the subtypes of influenza A virus (H1, H3 and H5). Upon performing multiplex PCR assays with type-specific primer sets, the clearly distinguishable products representing influenza A and B virus were separated by agarose gel electrophoresis. In addition, the subtypes of influenza A virus (H1, H3 and H5), which are most common in humans, can be readily distinguished by PCR with subtype-specific primer sets, yielding PCR products of different sizes depending on which subtype has been amplified. This method was tested on 46 influenza virus positive specimens of avian and mammalian (dog and human) origins collected between 2006 and 2008. The sensitivity of this method, tested against known concentrations of each type and subtype specific plasmid, was established to detect 10^3 copies/ μ l. The method's specificity was determined by testing against other subtypes of influenza A virus (H2, H4 and H6-H15) and respiratory pathogens commonly found in humans. None of them could be amplified, thus excluding cross reactivity. In conclusion, the multiplex PCR assays developed are advantageous as to rapidity, specificity, and cost effectiveness. ——— influenza A virus; H1N1; H3N2; H5N1; multiplex PCR

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Influenza virus infection has remained a major public health concern as it causes yearly epidemics mostly during the winter months. Influenza virus infection is associated with a diverse clinical spectrum, ranging from asymptomatic infection over respiratory symptoms such as high fever ($> 38^{\circ}\text{C}$) and either cough or sore throat (influenza-like illness) to severe complications due to secondary bacterial infections (Nicholson et al. 2003). Moreover, influenza virus infection can occasionally incur the severe and fatal complication of acute pulmonary microthromboembolism (Ohri et al. 2000).

Influenza viruses are members of the *Orthomyxoviridae* family and can be classified into types A, B and C, according to antigen variations in their nucleoprotein (NP) and matrix (M) protein. Most influenza pandemics are associated with type A. Influenza A viruses can be further classified into subtypes based on the antigen differences between their hemagglutinin (HA) and neuraminidase (NA) surface glycoprotein. Up to now, 16 HA and 9 NA subtypes of influenza A viruses have been identified (Fouchier et al. 2005).

At present, seasonally circulating influenza viruses in humans are influenza A virus subtypes H1N1 and H3N2 as well as influenza B virus. Since late 2003, outbreaks of highly pathogenic avian influenza A virus (HPAI) subtype H5N1 have been reported to infect poultry in Asia and subsequently in Europe and Africa. Moreover, human infections with this subtype have increasingly been diagnosed. WHO has documented 348 confirmed human cases infected with this virus with a mortality rate exceeding 60% (WHO 2007). However, most patients infected with the highly pathogenic H5N1 subtype initially display the typical influenza symptoms of high fever and an influenza-like illness (Beigel et al. 2005). Due to continuous H5N1 outbreaks in Asia and great genetic variability of this virus, a rapid and sensitive diagnostic assay is crucial for suitable clinical management and prevention of further virus transmission.

Hence, it is essential to test specimens from influenza-like illness patients, in cases where

there is clinical or epidemiological evidence of influenza A virus infection. Although virus isolation is a standard method for influenza virus diagnosis, reverse transcription-polymerase chain reaction (RT-PCR) offers an alternative method which can be used for rapid detection of the influenza virus genome. With multiplex polymerase chain reaction, more than one target sequence can be amplified by including more than one pair of primers in the same reaction (Elnifro et al. 2000). Therefore, multiplex PCR represents a method which is less laborious and time consuming as several influenza virus genes can be amplified simultaneously.

In this study, two multiplex PCR assays were developed for, firstly, typing influenza A and B virus with a set of three primer pairs specific for the M gene of influenza A and influenza B virus, and the glyceraldehyde 3-phosphate dehydrogenase (GAPDH) gene (a house-keeping gene of host cells) serving as an internal control. Secondly, for subtyping influenza A viruses a set of specific primers was designed to amplify the HA subtypes H1, H3, and H5 of currently circulating influenza A viruses.

MATERIALS AND METHODS

Sources of clinical specimens

Of 46 specimens, 35 specimens were influenza A virus positive and 11 specimens were influenza B virus positive. The influenza A virus positive specimens comprised human H1N1 ($n = 8$), human H3N2 ($n = 10$), human H5N1 ($n = 1$), avian H5N1 ($n = 15$), and dog H5N1 ($n = 1$). The H5N1 subtype can be further divided into 2 clades, clade 1 ($n = 15$) and clade 2 subclade 3 ($n = 2$). Each sample's details are shown in Table 1.

Primer design

For the influenza virus typing assay, primers were selected from conserved nucleotide sequences specific for the M genes of influenza A and B viruses, as well as the house keeping gene, GAPDH, as an internal control. In order to design the influenza A virus M gene specific primer, the M gene nucleotide sequences from 15 subtypes of HA with a variable NA gene in each host were selected from the GenBank database. These sequences were aligned using the ClustalX (Thompson et al. 1997) program. The most conserved region was chosen to

TABLE 1. Lists of virus strains used in this study and the results obtained from typing and subtyping by multiplex PCR assays.

No.	Strain name	Multiplex-PCR		Direct sequencing	Accession no.		Source of specimen
		Typing	Subtyping		HA	NA	
1	A/Thailand/CU41/2006(H1N1)	A	H1	H1	EU021246	EU021247	NP
2	A/Thailand/CU53/2006(H1N1)	A	H1	H1	EU021248	EU021249	NP
3	A/Thailand/CU67/2006(H1N1)	A	H1	H1	EU021250	EU021251	NP
4	A/Thailand/CU51/2006(H1N1)	A	H1	H1	EU021254	EU021255	NP
5	A/Thailand/CU57/2006(H1N1)	A	H1	H1	EU021256	EU021257	NP
6	A/Thailand/CU44/2006(H1N1)	A	H1	H1	EU021258	EU021259	NP
7	A/Thailand/CU68/2006(H1N1)	A	H1	H1	EU021260	EU021261	NP
8	A/Thailand/CU32/2006(H1N1)	A	H1	H1	EU021264	EU021265	NP
9	A/Thailand/CU46/2006(H3N2)	A	H3	H3	EU021268	EU021269	NP
10	A/Thailand/CU280/2007(H3N2)	A	H3	H3	EU021272	EU021273	NP
11	A/Thailand/CU228/2006(H3N2)	A	H3	H3	EU021274	EU021275	NP
12	A/Thailand/CU282/2007(H3N2)	A	H3	H3	EU021276	EU021277	NP
13	A/Thailand/CU259/2006(H3N2)	A	H3	H3	EU021278	EU021279	NP
14	A/Thailand/CU260/2006(H3N2)	A	H3	H3	EU021280	EU021281	NP
15	A/Thailand/CU231/2006(H3N2)	A	H3	H3	EU021282	EU021283	NP
16	A/Thailand/CU-1101/2008(H3N2)	A	H3	H3	EU625363	EU625366	NP
17	A/Thailand/CU-1102/2008(H3N2)	A	H3	H3	EU625364	EU625367	NP
18	A/Thailand/CU-1103/2008(H3N2)	A	H3	H3	EU625365	EU625368	NP
19	A/Thailand/NK165/2005(H5N1)	A	H5	H5	DQ372591	DQ372593	Allantoic fluid
20	A/dog/Thailand-Suphanburi/KU-08/04(H5N1)	A	H5	H5	DQ530173	DQ530175	Allantoic fluid
21	A/Chicken/Thailand/PC-168/2006(H5N1)	A	H5	H5	DQ999880	DQ999881	Allantoic fluid
22	A/Chicken/Thailand/PC-170/2006(H5N1)	A	H5	H5	DQ999887	DQ999888	Allantoic fluid
23	A/Chicken/Thailand/NP-172/2006(H5N1)	A	H5	H5	DQ999872	DQ999873	Allantoic fluid
24	A/Chicken/Thailand/167/2006(H5N1)	A	H5	H5	-	-	Allantoic fluid
25	A/Chicken/Thailand/169/2006(H5N1)	A	H5	H5	-	-	Allantoic fluid
26	A/Chicken/Thailand/173/2006(H5N1)	A	H5	H5	-	-	Allantoic fluid
27	A/Chicken/Thailand/174/2006(H5N1)	A	H5	H5	-	-	Allantoic fluid
28	A/Chicken/Thailand/1947/2006(H5N1)	A	H5	H5	-	-	Allantoic fluid
29	A/Chicken/Thailand/3277/2006(H5N1)	A	H5	H5	-	-	Allantoic fluid
30	A/Chicken/Thailand/3348/2006(H5N1)	A	H5	H5	-	-	Allantoic fluid
31	A/Chicken/Thailand/3886/2006(H5N1)	A	H5	H5	-	-	Allantoic fluid
32	A/Chicken/Thailand/4802/2006(H5N1)	A	H5	H5	-	-	Allantoic fluid
33	A/duck/Nong-Khai/KU-56/2007(H5N1)	A	H5	H5	EU221249	EU221251	Allantoic fluid
34	A/chicken/Thailand/NS-339/2008(H5N1)	A	H5	H5	EU620652	EU620653	Allantoic fluid
35	A/chicken/Thailand/PC-340/2008(H5N1)	A	H5	H5	EU620660	EU620661	Allantoic fluid
36	B/Thailand/CU243/2006	B	ND	B	-	-	NP
37	B/Shanghai/MDH1/2007	B	ND	B	-	-	cDNA
38	B/Shanghai/MDH2/2007	B	ND	B	-	-	cDNA
39	B/Shanghai/MDH3/2007	B	ND	B	-	-	cDNA
40	B/Malaysia /MDH4/2007	B	ND	B	-	-	cDNA
41	B/Shanghai/MDH5/2007	B	ND	B	-	-	cDNA
42	B/Shanghai/MDH6/2007	B	ND	B	-	-	cDNA
43	B/Shanghai/MDH7/2007	B	ND	B	-	-	cDNA
44	B/Shanghai/MDH8/2007	B	ND	B	-	-	cDNA
45	B/Shanghai/MDH9/2007	B	ND	B	-	-	cDNA
46	B/Shanghai/MDH10/2007	B	ND	B	-	-	cDNA

TABLE 1. Continue.

No.	Strain name	Multiplex-PCR		Direct sequencing	Accession no.		Source of specimen
		Typing	Subtyping		HA	NA	
Specimens used for specificity test							
	A/Singapore/1/57 (H2N2)	A	-*	H2	L11142	AY209895	cDNA
	A/duck/Czeck/56 (H4N6)	A	-*	H4	-	-	cDNA
	A/turkey/Massachusetts/3740/65 (H6N2)	A	-*	H6	-	-	cDNA
	A/seal/Massachusetts/1/80 (H7N7)	A	-*	H7	AB269696	AB288844	cDNA
	A/turkey/Ontario/67 (H8N4)	A	-*	H8	-	-	cDNA
	A/turkey/Wisconsin/66 (H9N2)	A	-*	H9	DQ067444	DQ067439	cDNA
	A/chicken/Germany/N/49 (H10N7)	A	-*	H10	-	-	cDNA
	A/duck/England/1/56 (H11N6)	A	-*	H11	AB288845	AB288846	cDNA
	A/duck/Alberta/60/76 (H12N5)	A	-*	H12	AB288334	AB288335	cDNA
	A/gull/Maryland/704/77 (H13N6)	A	-*	H13	D90308	-	cDNA
	A/mall/Astrakhan/263/82 (H14N5)	A	-*	H14	AB289335	AB89336	cDNA
	A/duck/Australia/341/83 (H15N8)	A	-*	H15	L43916	-	cDNA

*; H1, H3 and H5 negative, ND, not detected; NP, Nasopharyngeal suction; Allantoic fluid, Allantoic fluid from virus infected embryonated chicken egg.

design the forward and reverse primers. For the assay aimed at determining subtypes H1, H3 and H5 of influenza A virus, primers were chosen specifically from the conserved regions of each HA gene's subtype. The primers were designed and analyzed by using the PCR primer software, FastPCR Version 4.0.27 by Ruslan Kalendar, (Institute of Biotechnology, University of Helsinki, Finland) to ascertain that they could be combined in a multiplex PCR format. All primer sequences are shown in Table 2.

RNA Extraction and Reverse transcription

As for nasopharyngeal suction samples, RNA was extracted from 150 μ l of each sample using TRI REAGENT[®] LS (Molecular Research Center, Inc., Cincinnati, OH, USA) and dissolved in 12 μ l of DEPC treated water. As for embryonated chicken egg allantoic fluid, RNA was extracted from 140 μ l allantoic fluid of each sample using the QIAmp viral RNA mini kit (Qiagen, GmbH, Hilden, Germany) according to the manufacturer's specifications. The reverse transcription was performed using Moloney murine leukemia virus (MMLV) reverse transcriptase (Promega, Madison, WI, USA). The reaction mixture comprised 5 μ l of 5x MMLV reaction Buffer, 5 μ l of 10 mM dNTP, 40 units of RNasin[®] ribonuclease inhibitor, 200 units of M-MLV reverse transcriptase, 0.5 μ g of random hexamer, 12 μ l of RNA preheated to 70°C for 5 min then cooled on ice and

nuclease-free water to a final volume of 25 μ l. Subsequently, this reaction mixture was incubated at 37°C for 60 min. The resulting cDNAs were stored at -20°C until employed in the multiplex PCR reaction mixture.

Multiplex PCR and Thermal cycling conditions

Two separate reactions of multiplex PCR for typing and subtyping influenza virus were prepared. The reaction mixture of both reactions contained 10 μ l of 2.5X Eppendorf MasterMix (Eppendorf, Hamburg, Germany), 3 mM Mg²⁺, 200 μ M dNTPs, each set of primers for typing and subtyping at the final concentration of each primer equal to 0.4 μ M, 1 μ l of cDNA template and sterile water to a total volume of 25 μ l. Both typing and subtyping multiplex PCR assays were performed in a Mastercycler personal (Eppendorf) under the following conditions. Pre-denaturation at 94°C for 3 min, followed by 45 cycles at 94°C for 30 sec (denaturation), 58°C for 30 sec (primer annealing), 72°C for 1 min (extension) and concluded by a final extension step at 72°C for 7 min.

Agarose gel electrophoresis

A total of 10 μ l PCR products were mixed with loading buffer and run on a 1.5% agarose gel at 100 Volts for 60 min. After electrophoresis, the respective DNA bands were stained with ethidium bromide (FMC

TABLE 2. Primer set used for typing and subtyping of influenza virus.

	Target	Primer	Sequence (5' → 3')	Nucleotide position	Size (bp)
Influenza typing primer set	GAPDH	GAPDH-F85	GTGAAGGTCGGAGTCAACGG	85-104	107
		GAPDH-R191	TCAATGAAGGGGTCATTGATGG	191-170	
	Flu A (M gene)	FluA-M-F63	TCAGGCCCCCTCAAAGCCG	63-81	214
		FluA-M-R276	AGGGCATTTTGGACAAAKCGTCTA	276-253	
	Flu B* (M gene)	FluB-MF25	ATGTCGCTGTTTGGAGACACAAT	25-47	296
		FluB-MR320	TCAGCTAGAATCAGRCCYTTCTT	320-298	
Influenza subtyping primer set	H1	H1-F266	CTTAGGAAACCCAGAATGCG	266-285	362
		H1-R627	GCGGGTGATGAACACCCCA	627-609	
	H3	H3-F933	ATGGAAGCATTCCCAATGACAA	933-954	112
		H3-R1044	RTTYCGCATYCCTGTTGCCA	1044-1025	
	H5	H5-F886	ACTCCAATGGGGGCGATAAAC	886-906	188
		H5-R1073	CCCTCTATAAAACCTGCTATAG	1073-1052	

* Modified with degeneracy from Chi et al. 2002
K: (G/T); R: (A/G); Y: (C/T)

Bioproducts, Rockland, ME, USA) and then visualized on a UV transilluminator. The expected sizes of each PCR amplified product are summarized in Table 2.

Nucleotide Sequencing

Type and subtype of samples whose sequence was lacking in the database were confirmed by sequencing. After PCR amplicons were analyzed by 1.5% agarose gel electrophoresis and purified using the Perfectprep Gel Cleanup Kit (Eppendorf), direct DNA sequencing was performed using the Big Dye Terminator V.3.0 Cycle Sequencing Reaction kit (ABI, Foster City, CA, USA). The reactions were carried out in an ABI-Prism 310 Genetic Analyzer (Perkin Elmer, Norwalk, CT, USA). The initial sequences were edited and analyzed by Chromas Lite version 2.01 (Technelysium Pty Ltd., Australia) and the Bioedit Sequence Alignment Editor V.7.0.5.3 (Hall 1999). Subtypes of influenza A viruses were determined by BLAST analysis.

Positive controls

To optimize the influenza virus typing (A/B) and subtyping (H1/H3 and H5) assays, a total of six plasmid DNAs were constructed by insertion of the M gene of influenza A virus (A/chicken/Nakorn-Patom/Thailand/

CU-K2/2004(H5N1); nt 1-1027), M gene of influenza B virus (B/Malaysia/2506/2004-like vaccine strain; nt 1-682), GAPDH genes (nt 85-191), H1 gene (A/Thailand/CU51/2006(H1N1); nt 266-1672), H3 gene (A/Thailand/CU46/2006(H3N2); nt 10-1771) and H5 gene (A/chicken/Nakorn-Patom/Thailand/CU-K2/2004(H5N1); nt 880-1694) into the pGEM-T Easy Vector (Promega) by TA-cloning strategy. Then the plasmids were extracted by FastPlasmid Mini kit (Eppendorf) and subsequently confirmed by nucleotide sequencing. The plasmid concentrations were determined in a UV spectrophotometer in order to calculate copies/ μ l.

Specificity test

The specificity of the multiplex PCR assays was evaluated by cross-reaction tests with cDNA obtained from other subtypes of influenza A viruses (H2-H4 and H6-H15) as shown in Table 2. These subtypes of influenza A virus specimens were kindly provided by the Department of Livestock Development, Bangkok, Thailand. The assays were also evaluated against other respiratory viruses including human bocavirus (HBoV) ($n = 9$), adenovirus ($n = 16$), parainfluenza (PIVs) ($n = 12$), respiratory syncytial virus (RSV) subgroups A & B ($n = 24$), human metapneumovirus (hMPV) ($n = 22$) first

described by Chieochansin et al. (2007) as well as respiratory bacteria including *Haemophilus influenzae* ($n = 1$), *Streptococcus pyogenes* ($n = 1$), *Streptococcus pneumoniae* ($n = 1$) and *Staphylococcus aureus* ($n = 1$).

RESULT

In this study, a multiplex RT-PCR assay for typing and subtyping influenza virus was developed by using two specific primer sets. To establish influenza virus type, the conserved regions in M genes of influenza A and B virus were amplified. The clearly distinguishable amplicons of influenza A and B virus are shown in Fig. 1A. The PCR product size after amplification of influenza A and B virus were 214 bp and 296 bp, respectively. In this typing assay, a primer specific for the GAPDH gene was added to ascertain that in the specimen collection and RNA extraction step we did obtain cells and RNA. Therefore, every positive result of influenza A or B virus typing must also display the GAPDH band at 107 bp which simultaneously rules out false positive results.

In order to subtype influenza A virus, the conserved and specific regions of the HA gene of H1, H3 and H5 subtype were amplified. This assay provided different product sizes represent-

ing each influenza A virus subtype. The product sizes of H1, H3 and H5 were 362, 112 and 188 bp, respectively. The results of the subtyping assay are shown in Fig. 1B. For all specimens tested, the typing and subtyping results obtained by multiplex PCR were in complete agreement with type or subtype determinations by direct nucleotide sequencing. The specimens were identified as either influenza A (H1N1, H3N2 and H5N1) or B as shown in Table 1.

The assay detection limit was established by using 10-fold serial dilutions (10^9 or 10^8 - 10 copies/ μ l) of standard plasmid DNA of each gene. Amplified products were visible at plasmid DNA concentrations as low as 10^3 copies/ μ l of the M gene of influenza A and B, GAPDH, H3 and H5 plasmid. To detect the H1 plasmid concentrations of 10^4 copies/ μ l were required (data not shown).

Among a combination of all plasmids employed in the typing assay (M gene of Flu A and B and GAPDH; 1:1:1 ratio) the sensitivity of the assay was approximately 10^3 - 10^4 copies/ μ l (Fig. 2A), while the sensitivity of the subtyping (H1, H3 and H5; 1:1:1 ratio) assay was approximately 10^4 copies/ μ l (Fig. 2B).

For the specificity test, cDNA obtained from other subtypes of influenza A viruses including

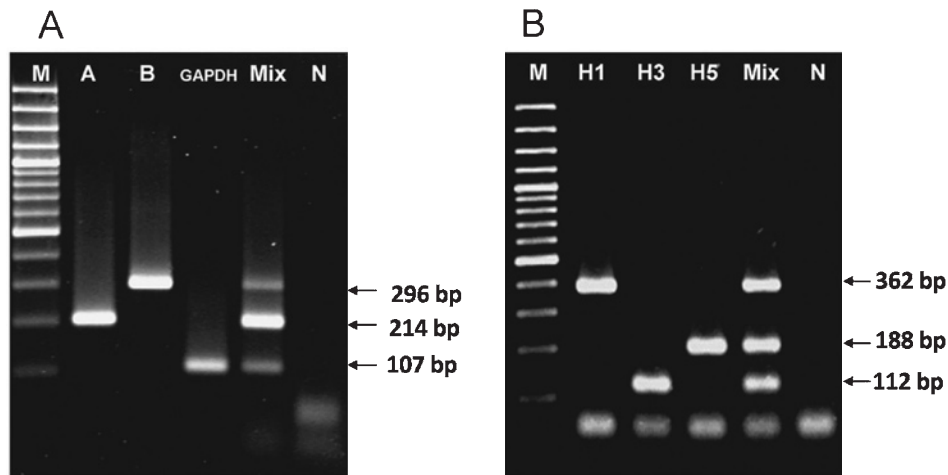


Fig. 1. Typing and subtyping multiplex PCR assay. The pattern of PCR product size from typing (A) (M; 100-bp marker, A; M gene of Influenza A plasmid, B; M gene of Influenza B plasmid, GAPDH; GAPDH plasmid, Mix; mixture of M gene of influenza A, M gene of influenza B and GAPDH plasmids, N; Negative control) and subtyping (B) of influenza virus (M, 100-bp marker; H1, H1 plasmid; H3, H3 plasmid; H5, H5 plasmid; Mix, mixture of H1, H3 and H5 plasmids; N, Negative control).

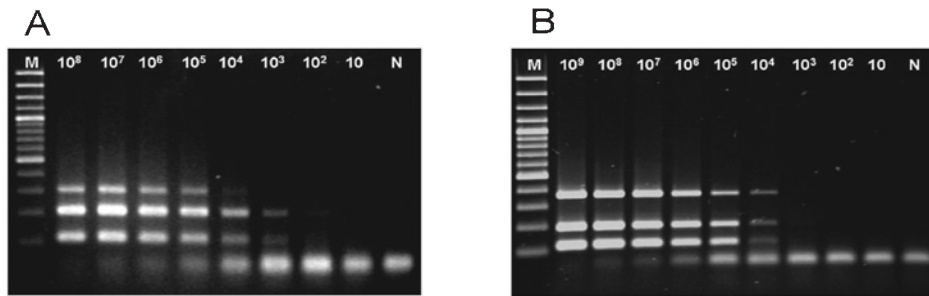


Fig. 2. The sensitivity test. The sensitivity of typing assay (A) and subtyping assay (B). DNA plasmid concentrations (copies/ μ l) are indicated on top of the lanes (M, 100-bp marker; N, Negative control).

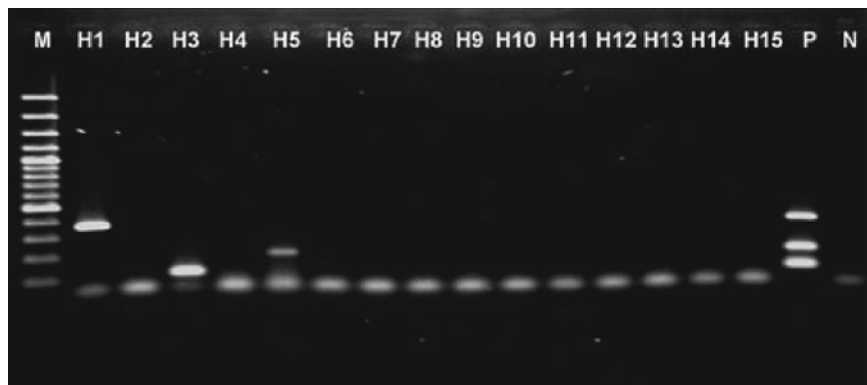


Fig. 3. The specificity test. Specificity test for H1, H3 and H5 subtyping assay with 15 influenza A virus reference subtypes (H1-H15). Virus subtypes are indicated on top of the lanes (M, 100-bp marker; P, H1, H3 and H5 mixed plasmids; N, Negative control).

H2N2, H3N8, H4N6, H6N2, H7N7, H8N4, H9N2, H10N7, H11N6, H12N5, H13N6, H14N5 and H15N8 were used as templates. The results showed that all subtypes of influenza A viruses (H1-H15) could be amplified with the primers specific for the M gene of influenza A virus, indicating the broad detection range of the primers in the typing assay. The subtyping assay produced the specific bands corresponding to H1, H3 and H5 subtypes only, demonstrating the high specificity of the primers as shown in Fig. 3. These results proved the suitability of primer sets and multiplex PCR conditions for successful influenza virus typing and subtyping.

DISCUSSION

The rapid and accurate diagnosis of respiratory viruses is essential to improve patient management and provide information as to which

preventive measures may be taken. This is particularly important with H5N1 avian influenza infection whose initial clinical symptoms are indistinguishable from seasonal influenza or other respiratory virus infections. In addition, the subtyping assay can detect co-infections of H1, H3 and/or H5 viruses which may result in genetic reassortment between avian and human influenza viruses. Identification of the pathogen will limit unnecessary administration of antibiotics and increase effectiveness of treatment.

Methods appropriate for influenza diagnosis ought to be highly accurate, sensitive, specific and rapid. Virus culture, the most accurate method, is still considered the gold standard. Yet, its limitation lies in the time required to achieve results, which especially with H5N1 infected patients might negatively impact the outcome.

Previous studies suggest that multiplex PCR

could resolve the limitations associated with traditional diagnostic techniques by combining increased sensitivity, specificity, and rapid achievement of results and thus, effectively detect multiple respiratory infections (Bellau-Pujol et al. 2005). Live virus is not necessary for the multiplex PCR assay to detect the viral genome and thus, it is more flexible for specimen collection and transportation than virus isolation. Several studies have reported simultaneous influenza type and subtype detection by multiplex RT-PCR as for example, a multiplex RT-PCR for H1N1, H3N2, H5N1 and type B detection (Poddar 2002), for detection of H5 and H7 avian influenza viruses (Thontiravong et al. 2007) and a single step reaction for H5N1 detection (Payungporn et al. 2004). Hence, multiplex PCR can be widely used for influenza virus detection because the reagents and equipment required for the assays are accessible in many countries. According to the high degree of mutations within the HA and NA genes, it may be necessary to update the primer sequences for detection of particular subtypes. Since our assays used primer sequences that were based on circulating strains of influenza viruses, this represented an improvement over other assays.

In the present study, two multiplex PCR assays were developed to simultaneously detect influenza viruses A and B, as well as discriminate between the subtypes capable of infecting humans, H1, H3 and H5. Especially with respect to the H5 subtype, our primer was specifically designed in relation to the recent outbreaks of H5N1 which can be divided into 2 clades. The result showed that the H5 subtyping primer can amplify both clades. Both reactions could be accomplished under simple conditions without the necessity for nested PCR. Moreover, the PCR products could be clearly separated by agarose gel electrophoresis. Applying these assays, the seasonally circulating human influenza viruses (H1N1, H3N2 and type B) including the highly pathogenic subtype H5N1 can reliably be diagnosed.

In conclusion, the multiplex RT-PCR assays described here provide a simple, rapid and cost effective method for influenza virus type and sub-

type detection from avian, mammalian and also human clinical specimens and hence, ought to be implemented for large-scale detection aimed at controlling influenza virus outbreaks.

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