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Original Article

Comparison of Multiplex Nested RT-PCR with Virus Isolation for Detection of Influenza Viruses A and B

**T Mokhtari-Azad [1], F Rezaie-Khollari [1], AR Nadji [1], V Salimi [1], Z Noroozbabaie [1], M Naseri [1], AR Mirzaie [1], Z Saadatmand [1], MM Gouya [2], AR Esteghamati [2], R Hamkar [1]*

[1]Dept. of Pathobiology, School of Public Health and Institute of Health Research, Medical Sciences/University of Tehran, Iran
[2] Center for Diseases Control, Ferdosi Squar, Iranshar Street, No.68, Tehran-1581615117, Iran

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Abstract

Background: Influenza A viruses bearing all known HA and NA subtypes have been isolated from avian hosts: but only the A/H1N1,A/H2N2 and A/H3N2 influenza subtypes have been associated with widespread epidemics in humans until now .While influenza B detected only in humans. Subtype H5 infection in humans occurred for the first time during 1997-1998 in Hong Kong, then China in 2003, Vietnam and Thailand in 2004 and continued to 2005-2006 in Turkey and Egypt. In this study a Multiplex Nested RT-PCR was developed for simultaneous detection of influenza virus A and B in clinical samples.

Methods: Throat swab and throat wash was collected between October 2005 and September 2006 from 689 patients with a suspected influenza virus infection. Samples were transported to the National Influenza Center (Tehran University of Medical Science, School of Public Health). Each sample is divided into two parts in order to virus isolation and nested RT-PCR.

Results: The RT-PCR detected in 75 cases of influenza A and B, compared to 26 cases by virus isolation and 74 cases are positive by RT-PCR. In total, 50 out of 74 RT-PCR positive specimens were negative by virus isolation. The RT-PCR provides a sensitive and specific method for detecting and typing influenza viruses A and B.

Conclusion: The RT-PCR provides a sensitive and specific method for detecting and typing influenza viruses A and B. Multiplex RT-PCR can also be used to accurately detect more than one viral template in the same reaction mixture, allowing viral confections to be identified with the same respiratory specimen.

Key words: *Influenza viruses, RT-PCR, Isolation*

Introduction

Influenza viruses are enveloped, segmented negative-sense RNA viruses that belong to family orthomyxoviridae (1, 2).

In human, three types of influenza, types A, B and C, have been described on the basis of antigenic differences in matrix (M) protein and nucleoprotein (NP) (3, 4). Type influenza viruses are further subtyped on the basis of antigenic differences on the external glycoprotein's, the hemagglutinin (HA) and the neuraminidase (NA) Proteins. To date 15 distinct HA (H1 to H_{15}) and 9 NA (N_1 to N_9) subtypes have been identified (1, 2, 4, 5). Type A and B influenza viruses can cause a wide spectrum of illness, including lower respiratory tract disease, pneumonia, and even, in the case of type an influenza virus, encephalopathy and encephalitis (1, 3, 4, 6-8). Viruses bearing all known HA and NA subtypes have been isolated from avian hosts, but until recently only viruses of the, H_1N_1, H_2N_2 and H_3N_2 subtypes have been associated with widespread epidemics in humans (6-7). Subtype H_5 infection in humans occurred for the first time during 1997-1998 in Hong Kong, then China in 2003, Vietnam and Thailand in 2004 and continued to 2005-2006 in turkey and Egypt.H_5N_1 prompted us to highlight the need for a highly sensitive accurate and rapid diagnostic test for the infection (9). Such a test would be important, not only in infection control but also to facilitate early antiviral therapy (1, 9). Conventional diagnostic tools, cell culture, and serologic testing require from 2 d to weeks for results. On the other hand, commercially available rapid

***Corresponding author:** Tel: +98 21 88950595, Fax: + 98 21 88962343, E-mail: mokhtari@sina.tums.ac.ir

antigen test are rapid and simple, but subtyping of virus is not feasible and sensitivity and specificity is low**:** thus, they are less useful in making therapeutic and infection control decisions (1, 3-8, 10-12). Molecular diagnosis of influenza by reverse transcription polymerase chain reaction (RT-PCR) provides a sensitive and rapid means for detection and has facilitated the typing and subtyping of virus (3-6, 10, 13). In the so-called "multiplex" format PCR assays have been designed to amplify more than one respiratory viral target in the same PCR test (13).

With the strategy to detect and distinguish normally prevalent types of human influenza viruses, we have developed and evaluated an in-house multiplex reverse transcriptase-PCR assay for influenza A types H1, H3,H5 and for influenza virus B on 689 clinical samples including throat wash and swab and compared it to isolation with cell culture.

Materials and Methods

Clinical specimens Throat swab (virocult) and throat wash was collected between October 2005 and September 2006 from 689 patients with a suspected influenza virus infection. Samples were transported to the National influenza center. (Tehran University of Medical Science, school of public health).

Virus isolation Virus was isolated using MDCK cells, maintained in Dulbecco's Modified Eagle medium (supplemented with 10% fetal calf serum), 100 Iu/ml penicillin 100 mg/ml streptomycin and 2 mμl-glutamine. The cells were seeded onto tubes either 24 or 48 h prior to sample inoculation. The samples which were processed as fresh as possible, were inoculated (100 μL) induplicate on to mono layers of MDCK cells. Trypsin (0.25 ng/ml) was added to the medium for isolation in MDCK cells only. The cultures were maintained in serum-free medium at 34° C for 3-7 d (7).

RNA extraction and cDNA synthesis RNA extracted from 150μL of samples using the Neucleospin viral RNA (Neucleospin RNA extraction kit) according to the manufactures' instruction. cDNA synthesis was carried out in 30 μL reaction mixture containing 6μL of 5X RT Buffer, 2.5 μL of mixed dntps (2.5mμ each), 1μL of m-mulv enzyme (fermentase), 2.5 μL Random hexamer (Fermentase), 0.5μL Ransine (Fermentase) and 17.5μL RNA template, and Incubate 22° C for 10 min, 37° C for 30 min and 94° C for 5 min.

Primer design The oligonucleotide primers designed to amplify A/H_5 placed in HA Region of influenza virus. The G+C contents, melting temperature and lengths of primers were chosen and analyzed by suing oligo 5 primer design software (National Biosciences Inc.) to ensure that they not only met the essential criteria for optimal PCR primer but also could be used together in a multiplex PCR under similar conditions already determined to be effective for detection and subtyping of influenza A and B virus (Table: 1) (14).

PCR Each primer pair was used at 5 pmol in primary amplification and 25 pmol in the secondary amplification. For the primary PCR 10 μL of cDNA was added to 40μL of a reaction mixture containing 5μL of 10X PCR buffer (fermentase), 1.5μL of $Mgcl_2$ (50mm), 2μL of dntps Mixed (10mM) and 1.5u of tag polymerase. Amplification with a DNA Engine thermocycler (eppendorf) consisted of 1cycle at 94° C for 2 min, followed by 35 cycles of 94° C for 1 min, 50° C for 1 min, 72° C for 1. min 5 microliters of primary product was then transferred to 45 μL of the secondary amplification mixture as above, The samples were then incubated for 1 cycle at 94° C for 2 min and then 35 cycles of 94° C 1 min, 60° C min, and 72 °C 1 min. Ampilicons were visualized by ethidium bromide staining following electrophoresis on 1.5% agarose gels (14).

Table 1: Properties of primers used for influenza virus multiplex PCR

Amplification steps and primer[1]	Sequence(5′→3′)	Gene position	Melting temp (°C)	G+C Content (%)	Optimal annealing Temp (°C)	Maximum annealing Temp (°C)	Amplicon size (bp)	Sensitivity (PFU)
Primary								
AH IA(f)	CAGATGCAGACACA ATATGT	HA	55	40	52	63	1015	NA[2]
AH I FII(r)	AAACCGGCAATGGC TCCAAA	HA	72	50				
AH3A(f)	CAGATTGAAGTGAC TAATGC	HA	55	40	52	62	883	NA
AH3DII(r)	GTTTCTCTGGTACAT TCCGC	HA	62	50				
BHA A(f)	GTGACTGGTGTGAT ACCACT	HA	56	50	53	64	900	NA
BHA DII(r)	TGTTTTCACCCATAT TGGGC	HA	65	45				
H5F(f)	ACTCCAATGGGGGCGA TAAAC	HA		50	55	70	351	NA
H52R(r)	TCTGCATTGTAACGACC CATTG	HA		45				
Secondary								
AH IB(f)	ATAGGCTACCATGCG AACAA	HA	63	45	52	62	944	<1
AH I EII(r)	CTTAGTCCTGTAACC ATCCT	HA	55	45				
AH3B(f)	AGCAAAGCTTTCAG CAACTG	HA	63	45	54	69	591	<1
AH3CII(r)	GCTTCCATTTGGAGT GATGC	HA	65	50				
BHA B(f)	CATTTTGCAAATCTC AAAGC	HA	61	35	54	67	767	<1
BHA CII(r)	TGGAGGCAATCTGC TTCACC	HA	68	55				
H5R(f)	CAACGGCCTCAAACTG AGTGT	HA		50	56	72	298	10-15 copy/µl RNA
H5 inhouse(r)	CAT(A/C)CACCC(C/T)CT CACCATCGGGGAA	HA		55				

[1]F, forward; R, reveres
[2]NA: not applicable.

Results

Sensitivity The sensitivity of detection of influenza virus with nested primer sets used individually and in a multiplex reaction was determined. Serial 10-fold dilution of stock of influenza A/H_5 (A/Chicken/combodia/7/04, Containing 10^8 copy /ml) was prepared. The end point of detection of viral RNA by primer sets use individually and in a multiplex RT-PCR. The obtained sensitivity for RT-PCR assay ranged between 10-15 copies RNA per reaction after the second amplification step. In both eases, the end point of multiplex RT-PCR detection was unaltered by the presence of all the primer sets in a multiplex reaction (Fig. 1).

Specificity The multiplex RT-PCR was tested for its specificity for all of the viral targets by first using the H_5 primer and then add-

ing each of the influenza H_1, H_3 and B primer pairs sequentially to simulated clinical specimens. No mispriming was observed when all of primer sets were present with either influenza H_1, H_3 or B virus or influenza H_5 template, a product of the expected size was obtained for each viral template by the multiplex RT-PCR with all of the primer sets present. There was no detectable PCR product following nucleic acid extraction and multiplex RT-PCR amplification from 40 clinical samples (Throat swab and wash throat) continuing adenovirus (n= 16), Para influenza (n= 20) and measles virus (n= 4) which were positive by immunofluorescence (Data not shown).

Dual infection The ability of the multiplex reaction to detect presence of more than one viral template in the clinical samples. The multiplex reaction was capable of detecting all four templates simultaneously. (Fig. 2). This indicates that coinfection could be detected by multiplex RT-PCR.

Evaluation of RT-PCR on Clinical Specimens The diagnostic outcome of RT-PCR was compared to virus isolation. 19 out of 20 (95%) influenza a culture- positive were detected by RT-PCR, and 6 out of 6 (100%) influenza B were detected by RT-PCR assay. Discrepant test result of culture and RT-PCR assay are shown in Table 2.

Noteworthy were 52 samples positive only in RT-PCR. The cases of influenza a virus detected by RT-PCR were of H_1 and H_3 and no H_5 subtype was detected (Data shown in Table: 3).

Evaluation of RT-PCR and Cell Culture on Clinical Specimens Cell culture and RT-PCR were evaluated on 698 clinical specimens, of which 527 were throat wash and 161 were throat swab (Virocult) RT-PCR detected 43 influenza A and B in throat swab and 35 in wash throat specimens. In contrast cell culture detected 21, 4 Influenza A, B viruses in throat swab and wash throat respectively. Cell culture and RT-PCR overall detected 43 influenza viruses in throat wash and 35 in throat swab (Table 4).

Sensitivity and specificity of RT-PCR VS cell culture Sensitivity and specificity of RT-PCR versus cell culture in detection of influenza virus were 95.8% and 92.16%, respectively (Table 2).

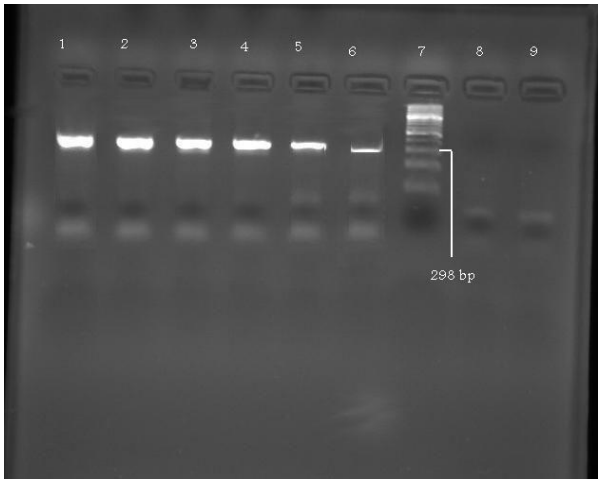

Fig. 1: Serial 10-fold dilutions of A/H5 influenza RNA was prepared (A/Chicken/Cambodia/7/04) Each dilution was subjected to Multiplex RT-PCR Primer sets of A/H5. Lanes 1 to 6 and 8 (10^{-2} to 10^{-8}). Lane 7, size marker (100 bp) and Lane 9, water control.

Fig. 2: typing and subtyping of influenza viruses A&B by multiplex RT-PCR .Lane 1, mixture of H1, H3, H5 B influenza. Lanes 2 to 5(H5, B, H1 and H3). Lane 6, Negative control. (Length of feragmets, H1; 944, H3; 591, B; 767, H5; 298 bp).M, size marker (100bp).

Table 2: Comparision of cell culture and RT-PCR in detection of influenza A

RT- PCR	Positive	Negative	Sensitivity & specificity of RT-PCR VS cell culture		PPV &NPV (95% confidential interval)	
			Sensitivity	specificity	PPV	*NPV*
Positive (%)	23(2.6)	52(7.8)				
Negative (%)	1 (0.2)	612(92.2)	95.83	92.16	30.66	99.83

Table 3: Typing and subtyping of influenza viruses

Detection	A(%)	B(%)	Neg(%)	H_1(%)	H_3(%)	H_5(%)	Neg(%)
Culture (%)	20 (2.9)	6 (0.9)	663 (96.2)	17 (2.5)	3 (0.4)	ND [a]	663 (96.2)
PCR (%)	50 (7.3)	27 (3.9)	611 (88.3)	26 (3.8)	24 (3.5)	ND (0)	635 (92.7)
PCR and culture (%)	50 (7.3)	27 (3.9)	611 (88.3)	27 (3.9)	24 (3.5)	ND (0)	617 (92.6)

[a] - Not detected

Table 4: Number of specimens positive and negative for influenza A and B viruses

Specimen type	No. of specimens				
		Cell culture (%)		RT-PCR (%)	
	Tested	Positive	negative	positive	negative
Throat swab	161	21(13)	141(87)	43(26.7)	118(73.3)
Throat wash	527	4(0.8)	523(99.2)	35(5.5)	492(93.4)
Total	689	25(3.6)	664(96.3)	78(11.3)	610(91.8)

Discussion

Influenza transmission is controlled selectively by vaccinating high- risk groups, timely detection of the first cases in community, and adoption of control measures allow to avoid the spread infection in nursing homes and hospitals (12). Moreover, specific antiviral drugs are effective in prophylaxis of influenza and also in treatment if administered early during the acute phase rational application of antiviral therapies have often been hampered due to the lack of standard rapid laboratory assays for the rapid diagnosis of influenza infection. The rapid systems based on immunoassay can be applied, but only to freshly collected specimens and does not allows diagnosis in frozen samples or after delayed examination of specimens. Recently developed molecular biology techniques are of great interest as they can detect small amounts of viral nucleic acid in pathological specimens rapidly and provide amplified DNA suitable for farther molecular analysis.

In this study we assessed the possibility of introducing RT-PCR as a reference method for routine diagnosis of influenza infection RT-PCR not only detected more than twice as many virus culture overall, but was also more rapid: a result was obtained within 24 h compared to the 7-14 d require for virus isolation. Similar studies showed a 20% to 27% increase in detection rate using PCR, at peak of the influenza season (7, 8, 15), whereas in our study the increase was 24.2%.

The multiplex RT-PCR was able to detect influenza A (A/H_1, A/H_3, A/H_5) and B viruses hemagglutin simultaneously and differentiated

A/H_1, A/H_3, B and very important subtype A/H_5 in two step. The latter hemagglutin subtypes recently have been a very important cause of human infection (1, 14, 15). Although the true numbers of human infections during the H_5N_1 out breaks remain unknown, the 62% mortality rate among human with documented H_5N_1 disease in 2004 and 2005 was markedly higher than the 33% fatality rate among documented human H5N1 cases in 1997 (16). Therefore, multiplex RT-PCR which can rapidly identify type A and B as well as subtypes A/H_1, A/H_3 and A/H_3 and A/H_5, will be very important for control of disease transmission in humans.

In this study, the shelf life of virus in throat swab (virocult) compared to wash throat as shown in result (Table 3) indicates that swab are more suitable for collection of specimens and surviving of the virus than wash throat and PCR contamination in the laboratory is reduced because sample handing is minimized.

Due to the high sensitivity to avian influenza during last year all the specimens that were sent to the lab were suspected cases that only a small positive of the specimens were influenza that is why the percentage of positive cases appears to be low in comparison with similar studies.

We have demonstrated that multiplex RT-PCR can be used for the detection and subtyping of influenza A and B viruses in clinical respiratory samples. The assay described here is both highly sensitive (95.83%) and specific (92.16%) for each individual pathogen and simulated specimens. It should prove to be useful in studies of viral respiratory illness in both surveillance and diagnostic settings.

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