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Direct Multiplex Reverse Transcription–Nested PCR Detection of Influenza Viruses Without RNA Purification

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This paper describes the development a of direct multiplex reverse transcription–nested polymerase chain reaction (PCR) method, devised for simultaneous detection and typing of influenza viruses. This method combines the direct reverse transcription reaction without RNA purification with the enhancement of sensitivity and specificity of nested PCR. The method successfully detected three major human influenza viruses: influenza virus A subtype 1 (H1N1) and subtype 3 (H3N2), and influenza B virus (B). The minimum number of virus particles (pfu/ml) necessary for detection in spiked saliva samples was 200 (H1N1), 140 (H3N2), and 4.5 (B). The method's sensitivity and simplicity will be convenient for use in clinical laboratories for the detection and subtyping of influenza and possibly other RNA viruses.

Keywords: Influenza virus, multiplex RT–PCR

Influenza is an important infectious disease that is responsible for considerable morbidity and mortality, particularly in elderly and compromised individuals [3, 4]. Influenza types A and B are two of the most important causes of human respiratory infection. Rapid diagnosis of influenza viruses is important for surveillance, prevention, and treatment of the associated illnesses, and eventually for differential diagnosis of other respiratory viruses.

Although virus isolation by cell culture and culture in embryonated eggs remain the standards for influenza virus detection, many novel diagnostic techniques have been developed in the past few years to obtain more sensitive and rapid diagnostic results. These include immunofluorescence [9], enzyme immunoassay [7], reverse transcription–polymerase chain reaction (RT–PCR) [1, 2, 6, 8], real-time RT–PCR [12, 13], nucleic acid sequence-based amplification (NASBA) [5], and reverse transcriptase–loop-mediated isothermal amplification (RT–LAMP) [10, 11].

RT–PCR is a rapid, sensitive, and specific molecular biological method for the detection of influenza viruses from clinical samples. RT–PCR protocols typically employ Moloney murine leukemia virus (MMLV) reverse transcriptase for first-strand cDNA synthesis, followed by second-strand synthesis and DNA amplification with *Taq* DNA polymerase. However, conventional RT–PCR requires the isolation of RNA from clinical samples, which is time-consuming and is accompanied by an unavoidable loss of material. The use of clinical samples directly in RT–PCR without prior isolation of RNA or pretreatment of the sample would circumvent some of the problems associated with RNA extraction.

An alternative to RNA purification would be to lyse the viruses present in clinical samples using a heating step, since heating of clinical samples is a very simple way to release nucleic acids [15]. Here, we have developed a simple and highly sensitive method to detect influenza viruses in clinical samples by direct multiplex RT–nested PCR without RNA extraction, in which influenza-containing material was used directly as a template without RNA isolation and the resultant cDNA was detected by nested PCR.

Materials and Methods

Virus Strains

The experiments were performed with three crude isolates from embryonated egg culture: influenza A/New Caledonia/20/99 (H1N1), A/Sydney/5/97 (H3N2), and B/Yamagata/16/88 (B). Each was used at a respective stock concentration of 4.0×10^7, 2.8×10^6, and 9.1×10^5 plaque forming units (pfu)/ml. The titrated viruses were spiked in phosphate-buffered saline (PBS) and human saliva for clinical specimens.

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Ten-fold dilutions of each viral stock solution were prepared in each medium, and was directly used for RT–PCR. Three independent experiments were carried out for each virus. To compare the sensitivity differences between RT–PCR assay with and without RNA purification, viral RNA was extracted from each medium using the QIAamp viral RNA kit (Qiagen, Valencia, CA, U.S.A.) according to the manufacturer's instructions.

RT–PCR Primers

Sequences of the primers used in this study are listed in Table 1. Primers used for reverse transcription and first amplification were described previously [6]. Primers used for second nested PCR were designed from conserved regions of the hemagglutinin (HA) portion within the sequence of the first amplification product. The nucleotide sequences of HA genes of over 10 strains of influenza viruses were obtained from the GenBank database. Multiple sequences were aligned using the CLUSTAL W software program (http://www.ebi.ac.uk/Tools/clustalw2/).

RT–PCR Conditions

RT was carried out by mixing 1 μl of diluted influenza virus or purified RNA template with the subtype-specific reverse primers (H1-F272, H3-F64, B-F281) in a reaction tube. Virus RNA and primers were annealed by heating at 94°C for 5 min followed by cooling to 4°C for 2 min. Tubes were then placed in a cold block before addition of the remaining reaction components including MMLV reverse transcriptase (Mbiotech, Seoul, Korea), reaction buffer, and 20 units of RNase inhibitor (Mbiotech), to make a total volume of 10 μl. Reverse transcription was then completed by heating the samples to 42°C for 60 min.

After the RT reaction, first PCR was performed. One μl of the synthesized cDNA was added to 19 μl of the reaction mixture containing 1× PCR buffer, 0.25 μM of each primer, 0.25 mM of each dNTP, and 1 unit of *Taq* DNA polymerase (Bioquest, Seoul, Korea). The tubes were heated at 94°C for 5 min and then subjected to 35 cycles of amplification: 30 s at 94°C for denaturation, 30 s at 54°C for annealing, and 30 s at 72°C for extension.

After the first PCR reaction, nested PCR was performed to detect the resultant amplification product. The first-step PCR product (1 μl) was mixed with 19 μl of the second-step reaction mixture consisting of 1× PCR buffer, 0.125 μM of each primer, 0.25 mM of each dNTP, and 1 unit of *Taq* DNA polymerase (Bioquest). PCR was performed in a thermal cycler (Clemens, Germany) preheated to 94°C. The cycling conditions consisted of an initial denaturation for 5 min at 94°C followed by 35 cycles of 30 s at 94°C, 30 s at 56°C, 30 s at 72°C, and an additional 5 min at 72°C for final elongation. After the reaction, 5 μl of each reaction was run on a 2% agarose gel and stained with ethidium bromide.

RESULTS AND DISCUSSION

A direct multiplex RT–nested PCR method for detecting influenza viruses has been developed that combines the direct RT reaction without RNA purification with the enhancement of sensitivity and specificity of nested PCR. Our method comprises three steps: direct RT reaction, PCR amplification of cDNA, and nested PCR amplification. First, RT primer is directly hybridized to the heated influenza virus sample without RNA isolation and then reverse transcribed with MMLV reverse transcriptase. Next, the RT products are amplified by first PCR followed by a second nested PCR step.

The developed direct multiplex RT-nested PCR method was applied for the detection of three major human influenza viruses: influenza virus A subtype 1 (H1N1) and subtype 3 (H3N2), and influenza B virus (B). Fig. 1 shows a typical result. Three nested primer pairs were used to generate a PCR product with base pair (bp) lengths of 312, 96, and 160, corresponding to influenza H1N1, H3N2, and B, respectively. A product of the expected size was obtained for each viral template by the multiplex assay in the presence of all the primers (Fig. 1). The specificity of the reaction was demonstrated by the presence of a single band having the expected size of the PCR product. The product specificities of the amplicons obtained from the multiplex RT–nested PCR assay were confirmed by

Table 1. RT–PCR primers used in this study.

Primer (5'→3')	Product size (bp)	Reference
First Amplification		
H1-F272 AATCATGGTCCTACATTG	585	[6]
H1-R856 TTTGAGGTGATGATTCCTGA		
H3-F64 AACGGAACGCTAGTGAA	402	[6]
H3-R466 GGTGCAACCAATTCAATC		
B-F281 GCTTTCCTATAATGCACG	290	[6]
B-R571 CCCAAACAGTAATTTGGT		
Second Amplification		
H1 nest-F AAATTTGCTATGGCTGACGG	312	This work
H1 nest-R GCTATTAGATTTCCATTTGCCTCA		
H3 nest-F GCACACTGATAGATGCTCTATTGG	96	This work
H3 nest-R TGCTGTAGGCTTTGCTGCGTTCAAC		
B nest-F CCCAATCTTCTCAGAGGATATGAA	160	This work
B nest-R GGACAGCCCAAGCCATTGT		

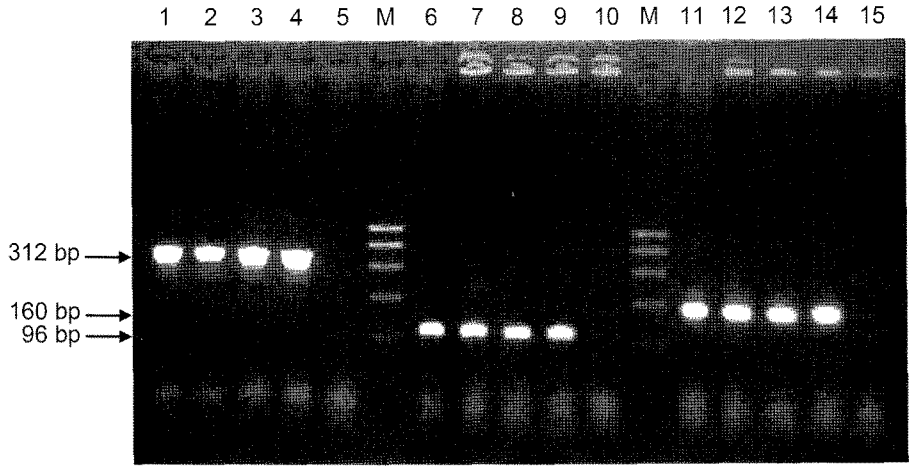

Fig. 1. Detection of PBS serially diluted influenza viruses by direct multiplex RT–nested PCR.
The experiments were performed with influenza H1N1 (lanes 1–5), H3N2 (lanes 6–10), and B (lanes 11–15) at a respective stock concentration of 4.0×10^7, 2.8×10^6, and 9.1×10^5 pfu/ml. In each lane, all three primer sets were used. Virus dilutions were 10^{-1} (lanes 1, 6, 11), 10^{-2} (lanes 2, 7, 12), 10^{-3} (lanes 3, 8, 13), 10^{-4} (lanes 4, 9, 14), and 10^{-5} (lanes 5, 10, 15). Lane M contains size markers (100–500 bp).

sequencing. Furthermore, the specificity of this assay was also confirmed by the simultaneous detection of all three PCR products from mixed virus samples of influenza H1N1, H3N2, and B in a single reaction tube (Fig. 2).

The sensitivity of the direct multiplex RT–nested PCR assay was evaluated by serial dilution of sample virus in PBS. As shown in Fig. 1, a 312-bp PCR product was detected in up to a 10^{-4} dilution of H1N1 virus stock, corresponding to a detection limit of 200 pfu/ml. When influenza H3N2 virus was used, the expected 96-bp PCR product was also detected in up to a 10^{-4} dilution of the virus stock, corresponding to a detection limit of 14 pfu/ml (Fig. 1). When influenza B virus was used, the expected 160-bp PCR product was likewise detected in up to a 10^{-4} dilution of the virus stock, corresponding to a detection limit of 4.5 pfu/ml (Fig. 1). These detection sensitivities are comparable to previous reports. Chi *et al.* [2] reported detection of influenza viruses from purified virus RNA by one-step RT–PCR with a detection limit of $1.1–2.1\times10^3$ virus particles. Ito *et al.* [11] reported a detection level of 10 focus-forming units of influenza viruses per ml by RT–LAMP [11].

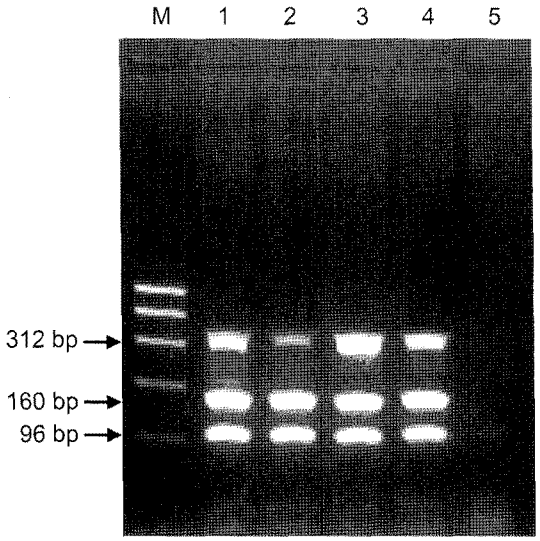

Fig. 2. Simultaneous detection of PBS serially diluted influenza viruses by direct multiplex RT–nested PCR.
Influenza H1N1, H2N2, and B viruses were 10-fold serially diluted, mixed, and subjected to direct multiplex RT–nested PCR in a single reaction tube. The stock concentrations of the viruses were the same as indicated in Fig. 1. The viral dilutions were 10^{-1} (lane 1), 10^{-2} (lane 2), 10^{-3} (lane 3), 10^{-4} (lane 4), and 10^{-5} (lane 5). Lane M contains size markers (100–500 bp).

The sensitivity of the direct multiplex RT–nested PCR assay was then compared by use of purified RNA as a template. When purified RNA from influenza viruses was used as a template, the detection limit was 100-fold lower than that of the direct multiplex RT–nested PCR assay using influenza virus directly without RNA purification (data not shown). This most likely reflected RNA loss during the purification step, suggesting that the direct multiplex RT–nested PCR assay without RNA extraction is a more sensitive approach, because the untreated virus sample is used directly as a template in RT–PCR, eliminating the step of virus recovery or RNA extraction. Direct use of intact influenza virus has the advantage that the nucleic acid is protected by proteins and can, therefore, be added to the diagnostic assay before the lytic step, preventing loss of trace samples during RNA purification [14].

To examine whether our method could be applicable to the detection of influenza viruses in clinical samples, spiked saliva samples were examined. When saliva was used to dilute influenza H1N1 and B viruses, the PCR

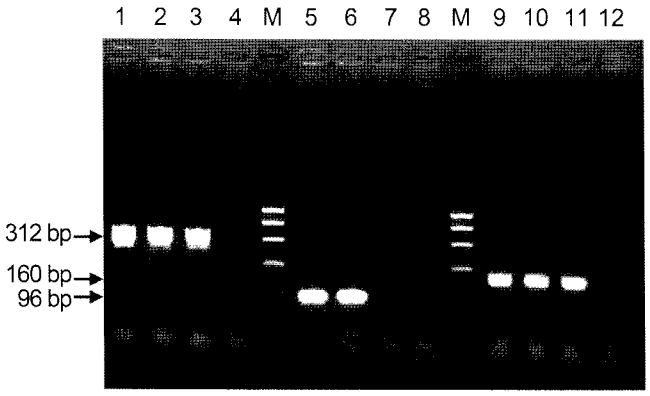

Fig. 3. Detection of saliva serially diluted influenza viruses by direct multiplex RT–nested PCR.
Influenza H1N1 (lanes 1–4), H3N2 (lanes 5–8), and B (lanes 9–12) viruses were 10-fold serially diluted in saliva and subjected to direct multiplex RT–nested PCR. The stock concentrations of each virus were the same as noted in Fig. 1. In each lane, all three primer sets were used. The dilutions of virus were 10^{-2} (lanes 1, 5, 9), 10^{-3} (lanes 2, 6, 10), 10^{-4} (lanes 3, 7, 11), and 10^{-5} (lanes 4, 8, 12). Lane M contains size markers (100–500 bp).

products were detected in up to a 10^{-4} dilution of the virus stock, similar to the use of PBS as the diluting medium (Fig. 3). In contrast, the sensitivity of the assay for H3N2 in saliva was reduced 10-fold and the PCR product was detected in up to a 10^{-3} dilution of the original sample. However, this detection limit, which corresponded to 140 pfu/ml, was still high enough to detect influenza virus in clinical samples compared with a previous report [2], indicating that our assay could be useful for the direct detection of influenza viruses in clinical samples.

Our method was performed directly using influenza virus samples without RNA purification. This was successfully carried out using enhanced sensitivity of nested PCR. RNA purification, which is an essential step for RT–PCR, is laborious and impractical for routine procedures. Although the recent development and retailing of products enabling the extraction of RNA from human specimens has made RNA purification far easier and faster than the standard procedures, these new methods still require considerable time and expense, especially for a large number of samples. RT–PCR performed directly on clinical samples without RNA purification is preferred because of the time-saving, convenience, reduced cost, personnel safety in lessening the risk of exposure to infectious material, and possible automation for large-scale diagnosis. To our knowledge, this is the first report showing the efficacy of multiplex RT–nested PCR in detection of influenza viruses from spiked clinical samples without RNA purification.

In conclusion, the sensitivity level achieved in the present study and the simplicity of our method will make it convenient for use in clinical laboratories for the detection and subtyping of influenza viruses. The method could be applicable to detection of other RNA viruses as well.

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