Evaluation of a Single-Step Multiplex RT-PCR for Influenza Virus Type and Subtype Detection in Respiratory Samples

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A single-step multiplex reverse transcription-polymerase chain reaction (RT-PCR) for the simultaneous detection of influenza virus type and subtypes was evaluated for its use in clinical specimens. One part of each specimen was tested using an established standard culture and/or monoclonal antibody-based immunofluorescence assay method, and the other part was tested by

the multiplex RT-PCR method for the presence or absence of the influenza virus, and its type and subtype. Sixty-seven specimens were examined. The results revealed a strong agreement between the data obtained by the established method and those obtained by the multiplex RT-PCR. *J. Clin. Lab. Anal.* 16:163–166, 2002. © 2002 Wiley-Liss, Inc.

Key words: influenza virus; multiplex PCR; typing; subtyping

INTRODUCTION

Three types of influenza viruses (types A, B, and C) cause influenza-related disease (1). The antigenic differences on the nuclear and matrix proteins of the virus are the basis for the types. Type A virus is further subtyped based on changes of the external glycoproteins, the hemagglutinin (H), and the neuraminidase (N). The H and N proteins of type B virus undergo so few changes that they do not result in a definable subtype (2). Type A is responsible for most influenza pandemics. Type B is also prevalent in influenza-related outbreaks (3,4). Type C causes mild illness and is clinically insignificant. During the last several decades, influenza type A subtypes H1N1 and H3N2, and type B viruses have been in circulation in humans, although outbreaks of other strains, such as A (H5N1), occurred in the recent past (5) through a host range jump from avian to human.

Influenza virus detection, and type and subtype identification by standard laboratory methods involve time-consuming and labor-intensive isolation of the virus in tissue culture (6). Other methods, including tests available commercially based on immunofluorescence antibody (IFA) and enzyme-linked immunosorbent assay (ELISA), lack high sensitivity and specificity (7). The reverse transcription-polymerase chain reaction (RT-PCR) allows an alternative rapid detection of the virus (8,9) and has been found to be more sensitive than standard tissue culture-based methods or other commercially available methods (7,9–11). RT-PCR-based methods have also been used for distinguishing A and B types, and subtyping

type A (12,13). However, all previously reported RT-PCR procedures required two steps (RT and then PCR) for amplification of an influenza virus RNA target. Very recently, an optimized one-step multiplex RT-PCR using RNA targets from only laboratory-isolated strains of influenza viruses was reported (14) for the simultaneous detection of virus type (A and B) and subtypes (H and N). In the present study this multiplex RT-PCR method was applied to real samples obtained from patients with respiratory disease. The performance of the method was evaluated by comparing its results with those obtained by standard culture and/or monoclonal antibody-based immunofluorescence methods.

MATERIALS AND METHODS

Stock Virus Strains and Clinical Specimens

The Public Health Laboratory at San Diego provided stocks of influenza virus strains of type A subtype H1N1 and sub-

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TABLE 1. Primers for influenza virus type A, type B, and subtype specific target gene amplification by multiplex RT-PCR

Gene target	Sense primer (5'–3')	Antisense primer (5'–3') ^a	Prototype strain ^b	Amplicon size (bp)
Type A/matrix	(65) CCGAGATCGCACAGAGACTTGAAGAT (90)	(400) GGCAAGTGCACCCAGCAGATAACT (377)	A/Puerto Rico/8/34	311
Type B/NS	(37) ACAAAATTGAGGTGGGTCCG (55)	(145) ACCAGGGTAGTCAAGGGCT (127)	B/Singapore/222/79	108
H1	(490) AATTGCTATGGCTGACGGA (509)	(655) CTACAGAGACATAAGCATTTTC (635)	A/Taiwan/118/96-3 (H1N1)	164
H3	(272) GCAAGCTTACAGCAACTGTT (292)	(503) ATAGTCACGTTACGCGCTG (485)	A/Sydney/1/97 (H3N2)	232
N1	(1059) AAGGGGTTTTCATACAGGTATGTT (1082)	(1165) TCTGTCCATCCATTAGGATCC (1145)	A/Chile/1/83 (H1N1)	106
N2	(779) GGAAATCGTTTCATATTAGCCCCATTG (804)	(955) AGCACACATAACTGGAAACAATGC (932)	A/Johannesburg/33/94 (H3N2)	173

^aAntisense primers are shown 5'–3' as they read from high to low base number on the antisense strand.

^bThe strains whose sequence nucleotide numbers are mentioned at the 5' and 3' ends of the primers.

type H3N2, and influenza type B. Respiratory specimens were obtained from the Children's Hospital at the University of Washington Medical Center, St. Louis; the virology laboratory of the California Department of Health, Berkeley, CA; and the Public Health Laboratory at San Diego, CA. Specimens were collected from patients suspected to have influenza during the flu seasons of 1999–2001 (October 1999–February 2000, and October 2000–February 2001). In all, 67 respiratory samples (40 nasopharyngeal swabs (NPs), and 11 nasal/throat, seven throat, four nasal, and five rhino probes (15)) were examined. One part of a specimen was used for tissue culture isolation, and type and subtyping using the monoclonal antibody-based immunofluorescence method (11,16,17). It should be noted that only the H subtype was determined by the monoclonal antibody-based assay; the N subtype was presumptive (if H was H1 or H3, N was presumed to be N1 or N2, respectively) based on the determined H subtype of an influenza type A virus in a specimen. Eleven out of 40 NPs were only identified as to type by direct fluorescence antibody (DFA) using a commercially available kit (Imagen; DAKO Diagnostics, Buckinghamshire, UK). The remaining part of a specimen was stored frozen at –70°C until it was used for RNA extraction for amplification by multiplex RT-PCR.

Isolation of RNA

RNA from a stock virus strain or a clinical specimen was extracted using the QIamp kit (Qiagen, Inc., Chatsworth, CA) following the manufacturer's protocol.

Multiplex RT-PCR

Multiplex RT-PCR was performed following essentially the same protocol as described previously (14). In brief, a 50 µL reaction mixture in 1× RT-PCR buffer (Qiagen, Inc.) contained the following components: 10 µL of the extracted RNA, 2 µL of the enzyme mix (a mixture of heterodimeric recombinant reverse transcriptases Omniscript, Sensiscript, and HotStar Taq DNA polymerase, supplied by Qiagen, Inc.), 400 µM of each dNTPs (dATP, dCTP, dGTP, and either dTTP or dUTP), 10 µL of 5× Q solution (a buffer agent to adjust the melting temperature of primers) (Qiagen, Inc.) and 0.5 µM each of the sense and antisense primer (provided in Table 1) for each type- and subtype-specific targets. The reaction was subjected to a precycle condition consisting of 30 min at 50°C (for RT), and 15 min at 95°C, followed by 40 cycles of amplification. Each cycle consisted of 94°C for 15 sec, 55°C for 15 sec, and 72°C for 30 sec.

Agarose Gel Electrophoresis

Ten percent of the amplified product was loaded in an agarose gel (2.5%) containing 0.5 µg/mL ethidium bromide, and electrophoresis was conducted in TBE (89 mM Tris-borate, 2

mM EDTA, pH 8.0) buffer. After electrophoresis the DNA bands were visualized through UV transillumination.

RESULTS

Figure 1 shows the analysis of the product amplified in a single-step RT-PCR using RNA from a set of representative specimens. RNA extracted from each of the control isolates of type A strains (A (H3N2) and A (H1N1)), and type B were amplified and used as marker DNA fragments specific for types and subtypes. The type and subtype of a specimen were determined by analyzing and comparing the sizes of amplified DNA fragments with that of the control marker fragments. DNA fragments of size 311 bp of type A specific matrix protein gene was commonly produced for any type A virus, and a 108 bp type B specific nonstructural (NS) gene fragment indicated the presence of a type B virus. Subtypes H (H1 or H3) and N (N1 or N2) specific DNA fragments were of sizes H (164 bp or 232 bp) and N (106 bp or 173 bp), respectively. An absence of specific amplified DNA product indicated an absence of influenza virus in the specimen. By culture and monoclonal antibody-based assay, seven samples were type A (H1N1), 26 were type A (H3N2), eight were type B, and 15 samples were negative for influenza virus. One sample (NP) negative by culture was positive for type B by RT-PCR. This positive PCR result may have been due to the increased sensitivity of the PCR-based method (8,9,12). Another sample (rhinoprobe), which was type A (H1N1) by culture and monoclonal antibody-based assay, was negative by RT-PCR. This negative PCR may have been due to the presence of a speci-

men inhibitor (18). Eleven NP samples identified only as type A by the Imagen kit were all found to be A (H3N2) by the RT-PCR method. The presence/absence of influenza virus, and its type and subtype in other specimens (54 out of 56 samples) determined by the RT-PCR method were exactly in agreement with those determined by the established procedure.

DISCUSSION

The method of single-step multiplex RT-PCR for the amplification and detection of influenza virus type- and subtype-specific targets has been reported only for laboratory isolates of the virus, and has not previously been tested in actual clinical specimens (14); thus, the present evaluation of the method in real specimens was much warranted. The results of this study verified that the primer sets and the reaction conditions of multiplex RT-PCR are potentially applicable for successful type and subtype detection of clinical specimens suspected to contain influenza virus of type A, with subtype H1N1 or H3N2, or type B virus. Presently, routine H subtyping of influenza type A virus is performed by subtype-specific monoclonal antibodies or by the cumbersome hemagglutinin inhibition (HI) test, and N subtyping is not done at all. The present RT-PCR method was rapid, involved less hands-on time, and generated information relating to both H and N subtypes (simultaneously) of influenza viruses in specimens. Also, since it is a single-tube, single-step procedure, there is low or no risk of cross contamination. This rapid and highly sensitive method can greatly assist in better management of patients, particularly those at high risk. Also, such rapid detection

Fig. 1. Agarose gel analysis of the amplified product of multiplex RT-PCR simultaneously targeting type- and subtype-specific genes of influenza viruses, using RNA from a representative set of respiratory samples. Sample numbers are as indicated at the top of the lanes. By size analysis of the products of RT-PCR, samples 0460 and ph05 were determined to be type A

(H1N1); samples 0112, 0697, and 0104 were type A (H3N2); samples ph16 and 0125 were type B, and samples 0919 and ph20 were negative for influenza viruses. The amplified product of control isolates of type A (H1N1 and H3N2), and type B were loaded in the lanes as shown. Ladder: 100 bp DNA size marker.

can help to avoid unnecessary hospitalization, and prevent ineffective and inappropriate antiviral and/or antibiotic treatment. The rapid subtyping of the virus can be reassuring to a patient diagnosed to have influenza, when a lethal subtype is also in circulation (such as the situation in Hong Kong during the 1997–1998 influenza outbreak (5)). It can facilitate epidemiological surveillance, and thus can be of immense importance in the control of disease transmission as well.

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