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In vitro transcribed RNA molecules for the diagnosis of pandemic 2009 influenza A(H1N1) virus by real-time RT-PCR

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A B S T R A C T

The 2009 influenza A(H1N1) outbreak allowed the implementation of new epidemiologic surveillance tools in several countries around the world. A new molecular protocol with appropriate sensitivity and specificity using real-time RT-PCR was developed by the Centers for Disease Control and Prevention (CDC) to identify the pandemic 2009 influenza A (H1N1) virus in human specimens. In the CDC protocol, positive controls are available only upon request and they are taken from cell cultures infected with 2009 influenza A(H1N1) virus, representing a handling risk for laboratory technicians. The poor availability of positive control materials in diagnostic laboratories may limit the public health response. The aim of the work presented in this paper was to develop positive controls for the diagnostic testing of influenza A(H1N1) virus that could be used in the CDC real-time RT-PCR protocol. A series of plasmid constructs bearing partial sequences of the viral genes were created and each construct was used as a template for *in vitro* transcription. RNA molecules were obtained successfully at high yield, *i.e.*, 2×10^7 assays per microliter. Thus, the inclusion of these molecules in the influenza panel as positive controls is proposed. The *in vitro* transcribed RNA could also be used as quality standards in the design of international proficiency studies.

1. Introduction

During the pandemic outbreak of influenza A(H1N1) in 2009, the Ministry of Health in Mexico, the National Microbiology Laboratory of the Public Health Agency of Canada and the Influenza Division at the Centers for Disease Control and Prevention in the United States of America (CDC, USA) collaborated to identify a novel influenza A(H1N1) virus in specimens from Mexican patients (CDC, 2009b). The characteristics and genetic composition of the pandemic 2009 influenza A(H1N1) virus were reported (Garten et al., 2009) and have been commented broadly on and discussed (Evseenko et al., 2011; Shinde et al., 2009; Smith et al., 2009). After the genetic information was available, the CDC developed and placed online a protocol for the molecular detection of 2009 influenza A(H1N1) virus in human samples (CDC, 2009a). This protocol facilitated the epidemiologic surveillance programs for the early detection of influenza around the world. The assay uses real-time RT-PCR to identify three viral genes that encode the matrix (M), nucleoprotein (NP), and haemagglutinin (HA) proteins and one human gene encoding the ribonuclease P/MRP 30 kDa subunit (RPP30, but called RP or RNase P in the CDC protocol). The viral amplification profile permits discrimination between the A pandemic and seasonal influenza. Human RPP30 detection serves as an effective control for nucleic acid extraction (CDC, 2009a).

To assess the accurate diagnosis of 2009 influenza A(H1N1) virus in specimens by clinical laboratories, the molecular assay included negative and positive controls. The positive controls were taken from cell cultures with high titers of 2009 influenza A(H1N1) virus and are available only upon request (CDC, 2011). Although specialized laboratory technicians handled the infected cell cultures in appropriate areas, there remains a risk of virus infection. Furthermore, it has been reported that, due to the difficulty of acquisition,

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Table 1
Primers and probs used for RT-PCR assays.

Name of primers	Primer sequences (5′-3′)	Probes sequences (5′-3′)[a]
InfA forward	5′-GAC CRA TCC TGT CAC CTC TGA C-3′	5′-TGC AGT CCT CGC TCA CTG GGC ACG-3′
InfA reverse	5′-AGG GCA TTY TGG ACA AAK CGT CTA-3′	
Sw InfA forward	5′-GCA CGG TCA GCA CTT ATY CTR AG-3′	5′-CYA CTG CAA GCC CA"T" ACA CAC AAGCAG GCA-3′
Sw InfA reverse	5′-GTG RGC TGG GTT TTC ATT TGG TC-3′	
Sw H1 forward	5′-GTG CTA TAA ACA CCA GCC TYC CA-3′	5′-CA GAA TAT ACA "T"CC RGT CAC AAT TGG ARA A-3′
Sw H1 reverse	5′-CGG GAT ATT CCT TAA TCC TGT RGC-3′	
RnaseP forward	5′-AGA TTT GGA CCT GCG AGC G-3′	5′-TTC TGA CCT GAA GGC TCT GCG CG-3′
RnaseP reverse	5′-GAG CGG CTG TCT CCA CAA GT-3′	

[a] TaqMan® probes are labeled at the 5′-end with FAM dye and at the 3′-end with BHQ1 quencher. "T" means a modified T with BHQ1 internally to prevent probe extension by Taq polymerase.

the lack of positive control materials in diagnostic laboratories could limit the public health response (Wang et al., 2011; Whiley et al., 2010). Moreover, the implementation of standardized procedures and materials among diagnostic laboratories is necessary to assess the quality of epidemiologic surveillance networks (Gao et al., 2011). The aim of this study was to use a plasmid approach to synthesize innocuous RNA molecules that would be useful as positive controls for the diagnosis of 2009 influenza A(H1N1) virus by real-time RT-PCR.

2. Materials and methods

2.1. RNA extraction

The specimen processing was performed in accordance with pertinent national biological safety regulations and all of the procedures were performed by trained personnel. A pool of human nasopharyngeal swab samples, which were determined previously to be positive for influenza, was used for the RNA extraction according to the QIAamp® Viral RNA Mini Kit procedure (Qiagen, Hilden, Germany). Briefly, an aliquot of 280 μL of the positive-sample pool was inactivated in 560 μL of AVL buffer-carrier, mixed by pulse-vortexing for 15 s, and incubated for 10 min at room temperature. Then, 560 μL of absolute ethanol was added to the sample and the sample was mixed by pulse-vortexing for 15 s. The solution was applied in two parts to a QIAamp mini spin column and centrifuged at 6000 *g* for 1 min. The washing buffers AW1 and AW2 were added consecutively to the column and centrifuged at 6000 and 20,000 *g* for 1 and 3 min, respectively. The RNA was eluted with 70 μL of AVE buffer.

2.2. PCR and cloning of PCR products

PCR mixtures containing 12.5 μL PCR Master Mix, 0.5 μL SuperScript™ III RT/Platinum® Taq (Invitrogen, Carlsbad, USA), and 40 μM each of the forward and the reverse primers were set up in a 25 μL final volume. The reaction mixtures were dispensed into a 96-well PCR plate. The primers used for amplifying the fragment of M, NP and HA viral genes, and the human RPP30 gene are shown in Table 1. The PCR assays were conducted in a real-time PCR 7500 Fast System (Applied Biosystems, Foster City, USA) using the following conditions: reverse transcription for 30 min at 50 °C, Taq inhibitor inactivation for 2 min at 95 °C, and 45 cycles of denaturation for 15 s at 95 °C and annealing/extension for 30 s at 55 °C. The PCR products were visualized on 2% agarose gels stained with ethidium bromide to confirm their integrity and size. For absolute the quantitation assays, the PCR was set up in a final volume of 25 μL, which included 10 μM fluorogenic probes for each gene, and performed as mentioned above (Table 1). Sequence Detection Software version 1.4 (Applied Biosystems, Foster City, USA) was used for data analysis.

Fragments of the viral M, NP, and HA and the human RPP30 genes from the PCR assays were inserted individually into the pEXP5-CT-Topo® vector (Invitrogen, Carlsbad, USA) according to the manufacturer's instructions. Briefly, 4 μL of fresh PCR product and 1 μL of salt solution were incubated with 1 μL of pEXP5-CT-Topo® for 5 min at room temperature. The ligation mixture was transformed into OneShot® TOP10 competent *E. coli* cells (Invitrogen, Carlsbad, USA). The candidates obtained from a single ampicillin-resistant colony were characterized by PCR to determine the insert orientation using one appropriate primer listed in Table 1 and a combination of primers T7 forward (5′-TAA TAC GAC TCA CTA TAG GG-3′) and T7 reverse (5′-ATC CGG ATA TAG TTC CTC CTT TC-3′). DNA sequencing was performed using the BigDye® terminator v3.1 cycle sequencing kit in a 3130*xl* Genetic Analyzer (Applied Biosystems, Foster City, USA). The vectors pEXP5-CT-InfA, pEXP5-CT-swInfA, pEXP5-CT-swH1 and pEXP5-CT-RP, which contain fragments of the viral M, NP, and HA genes and the human RPP30 gene, respectively, were obtained.

2.3. Synthesis of RNA molecules

In vitro transcription was performed using the MAXIscript® kit (Invitrogen, Carlsbad, USA) according to the manufacturer's instructions. Approximately 3×10^{11} molecules of each plasmid with viral or human gene fragments were used as templates. Briefly,

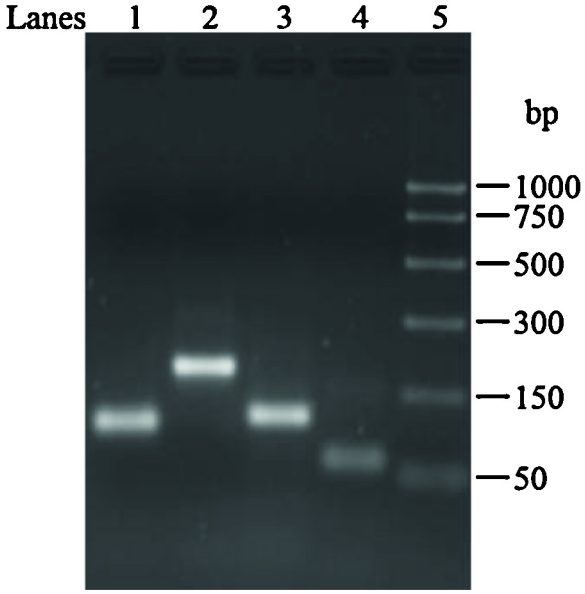

Fig. 1. Representative gel image of PCR products from the influenza A(H1N1) molecular diagnostic test. Lanes 1–4 correspond to the matrix (106 bp), nucleoprotein (195 bp), haemagglutinin (116 bp) and RPP30 (65 bp) PCR products, respectively. Lane 5 is a DNA ladder (PCR marker®, Promega), and the molecular weights are shown on the right.