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Analytical and clinical validation of novel real-time reverse transcriptase–polymerase chain reaction assays for the clinical detection of swine-origin H1N1 influenza viruses

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Abstract

During the early stages of the 2009/2010 swine-origin H1N1 influenza A (S-OIV H1N1 FluA) outbreak, the development and validation of sensitive and specific detection methods were a priority for rapid and accurate diagnosis. Between May and June 2009, 2 real-time reverse transcriptase–polymerase chain reaction (rRT-PCR) assays targeting the hemagglutinin and neuraminidase genes of the S-OIV H1N1 FluA virus were developed. These assays are highly specific, showing no cross-reactivity against a panel of respiratory viruses and can differentiate S-OIV H1N1 from seasonal FluA viruses. Analytical sensitivities of the 2 assays were found to be 10^{-1} tissue culture infectious dose, 50%/ml. Clinical testing showed 99.2% sensitivity and 94.6–98.1% specificity. A large prospective analysis showed that 94.8–95.5% of S-OIV positive specimens were negative by seasonal H1/H3 subtyping. The large-scale validation data presented in this report indicate that these novel assays provide an accurate and efficient method for the rapid detection of S-OIV H1N1 FluA viruses.

Keywords: rRT-PCR; S-OIV H1N1 FluA; Design; Validation

1. Introduction

Shortly after identification of the first infection with swine-origin H1N1 influenza A (S-OIV) in the United States, several patients infected with influenza A (FluA) viruses that could not be typed as human H1 or H3 in origin were reported in the province of Nova Scotia in Canada (Cutler et al., 2009; Dawood et al., 2009). The Canadian National Microbiology Laboratory (Winnipeg, Manitoba) rapidly confirmed that these initial cases were S-OIV H1N1 FluA by sequencing of the matrix (M) gene. In the following weeks, the number of S-OIV infections rapidly spread worldwide (Centers for Disease Control [CDC], 2009; Editorial team, 2009, World Health Organization [WHO], 2009) prompting the development and clinical validation of novel detection assays. The design of adapted detection tests was indispensable for efficient risk assessment, and allowed us to participate in the efforts to minimize the spread of infections, as well as to conduct epidemiologic surveillance (Biere et al., 2009). Compared to viral culture, these novel rapid tests needed to be highly specific, sensitive, and adaptable to high throughput analyses. Here, we describe 2 novel real-time reverse transcriptase–polymerase chain reaction (rRT-PCR) assays, targeting the hemagglutinin (HA) and neuraminidase (NA) genes that were specifically designed for the detection of S-OIV H1N1 FluA and were

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extensively validated in the early stages of the S-OIV outbreak in Ontario.

2. Materials and methods

2.1. Clinical specimens

Nasopharyngeal (NP) swabs were collected from patients across the province of Ontario, Canada, during the initial stage of the pandemic S-OIV H1N1 outbreak between May and June, 2009. Upon collection, NP swabs were suspended in viral transport medium (Starplex Scientific Inc, Etobicoke, ON, Canada) and kept at 4 °C. Total RNA was purified from NP swabs and viral culture supernatant by using the Nucli-SENS® easyMag® (Biomerieux, Montreal, QC, Canada) kit according to the manufacturer's instructions with a 25-μL elution volume. Analytical specificity of each assay was evaluated using a panel of cultured human respiratory viruses (Table 1). Initial clinical validation of each assay was performed with 185 specimens that had been tested for the presence of FluA viruses using a real-time RT-PCR assay developed by the CDC (Dawood et al., 2009) and for the S-OIV H1N1 FluA virus using end-point RT-PCR and sequencing according to the protocol of Cutler et al. (2009).

The large prospective evaluation of the clinical performance of the S-OIV HA and S-OIV NA assays was performed with a total of 2214 specimens. These specimens were simultaneously tested with the CDC rRT-PCR assay to detect seasonal FluA, H1, or H3 and influenza B (Dawood et al., 2009). This method is approved by the US Food and Drug Administration and was distributed through US public health laboratories and the WHO's Global Influenza Surveillance Network.

Nucleotide sequence analysis of HA end-point RT-PCR products and whole genome S-OIV used BigDye Terminator Cycle Sequencing Ready Reaction kit v3.1 (Applied Biosystems, Fosters City, CA) and was analyzed using an ABI 3130xl automated DNA sequencer. Sequence analysis was performed using Vector NTI (Invitrogen, Carlsbad, CA). Sequences were compared with all entries in the data bank of the National Center for Biotechnology Information and were analyzed for nucleotide sequence similarities using the Basic Local Alignment Search Tool.

Table 1
Analytical specificity of S-OIV HA and NA rRT-PCR evaluated by testing against a panel of cultured viruses

Virus	S-OIV HA	S-OIV NA
FluA/Toronto/3141/2009(H1N1)	+	+
FluA/Toronto/3184/2009(H1N1)	+	+
FluA/Toronto/C2781/2009(H1N1)	+	+
FluA/Toronto/0462/2009(H1N1)	+	+
FluA virus A/Toronto/T9842/2009(H1N1)	+	+
FluA virus A/Toronto/T5362/2009(H1N1)	+	+
FluA virus A/Toronto/T5294/2009(H1N1)	+	+
FluA virus A/Toronto/T0106/2009(H1N1)	+	+
FluA virus A/Toronto/C2781/2009(H1N1)	+	+
FluA virus A/Toronto/C2781/2009(H1N1)	+	+
FluA virus A/Toronto/C0270/2009(H1N1)	+	+
FluA virus A/Toronto/R8564/2009(H1N1)	+	+
FluA virus A/Toronto/R8557/2009(H1N1)	+	+
FluA virus A/Toronto/T5308/2009(H1N1)	+	+
FluA human-OIV H1	− (12)	− (10)
FluA human-OIV H3	− (13)	− (10)
Influenza B human-OIV	− (10)	− (6)
Adenovirus	− (2)	− (2)
Enterovirus/rhinovirus	− (3)	− (3)
Human respiratory syncytial virus	− (6)	− (3)
Parainfluenza virus type 1	− (4)	− (4)
Parainfluenza virus type 2	− (3)	− (3)
Parainfluenza virus type 3	− (4)	− (4)
Parainfluenza virus type 4	− (9)	− (8)

The number of samples tested is indicated in parentheses.

2.2. Cultivation of S-OIV H1N1 viruses

FluA/Toronto/3141/2009(H1N1), FluA/Toronto/3184/2009(H1N1), FluA/Toronto/C2781/2009(H1N1), and FluA/Toronto/0462/2009(H1N1) virus strains were cultured from NP swabs that had been confirmed positive for the presence of S-OIV H1N1 viruses and characterized by whole genome sequencing according to a protocol distributed by the WHO and the CDC (http://www.who.int/csr/resources/publications/swineflu/GenomePrimers_20090512.pdf). Strains were cultured with Rhesus macaque cells in minimum essential medium/4% bovine serum albumin at 37 °C with 5% CO_2 in roller drums. After 10 days of incubation, all cultures showed cytopathic effects. Cultures were pooled, serially diluted, and quantitated by measuring the tissue culture infectious dose, 50%/ml ($TCID_{50}$) using the Reed-Muench methods (Reed and Muench, 1938).

2.3. Primers and probes of the S-OIV NA and S-OIV HA rRT-PCRs

Design of primers and probes was based on S-OIV H1N1 HA and NA RNA sequences available from April 1, 2009, to May 14, 2009 at the GISAID database (http://www.platform.gisaid.org/). Oligonucleotides and probes to HA and NA genes were designed with Primer Express 3.0 software (Applied Biosystems) and purchased from Eurofins MWG Operon (Huntsville, AL). The probes consisted of oligonucleotides labeled at the 5′ end with 6-carboxyfluorescein as the reporter and at the 3′ end with Black Hole Quenchers (BHQ1). The 5′ to 3′ sequences of oligonucleotides designed for the S-OIV HA assay targeting the HA RNA were as follows: S-OIV HA F primer, 5′-CCTGGG AAATCCAGAGTGTGA-3′; S-OIV HA R primer, 5′-CG TTCCATTGTCTGAACTAGRTGTT-3′, and S-OIV HA probe, 5′-TCACTCTCCACAGCAAGCTCATGG-3′. The 5′ to 3′ sequences of oligonucleotides designed for the S-OIV NA assay targeting the NA RNA were as follows: S-OIV NA F primer, 5′-AGCCACTCAATTCAACTTGGG AAT-3′; S-OIV NA R primer, 5′-GCCCGCTAATTTCACGG