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Comparison of a laboratory-developed RT-PCR and the CDC RT-PCR protocol with rapid immunodiagnostic testing during the 2009 H1N1 influenza A pandemic

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Abstract

We evaluated the performance of a laboratory-developed multiplex real-time reverse transcription-PCR assay (LDT rRT-PCR), the Centers for Disease Control and Prevention (CDC) 2009 H1N1 rRT-PCR protocol using the LightCycler 480 II, the multiplex xTAG Respiratory Virus Panel (xTAG RVP), and rapid immunodiagnostic testing (RIDT) using the BinaxNOW Influenza A & B to detect 2009 H1N1 with 426 nasopharyngeal swab specimens during the 2009 H1N1 pandemic. The specificity of the methods tested was ≥98%, and the individual test sensitivities were RIDT at 42.3% [95% confidence interval (CI), 31.4–54.0], LDT rRT-PCR at 98.9% (95% CI, 92.9–99.9), CDC 2009 H1N1 rRT-PCR at 78.2% (95% CI, 67.8–86.0), and xTAG RVP at 93.1% (95% CI, 85.0–97.2). A negative RIDT result should not be used to make decisions with respect to treatment or infection prevention. rRT-PCR is the preferred first-line diagnostic test for detecting 2009 H1N1 influenza A.

Keywords: Influenza; H1N1; PCR; Antigen; RIDT; xTAG; RVP

1. Introduction

In spring 2009, a novel influenza A causing acute respiratory illness was identified in North America (Novel Swine-Origin Influenza A (H1N1) Virus Investigation Team 2009). In the United States, an estimated 60 million infections, 270,000 hospitalizations, and 12,270 deaths had been caused by the 2009 H1N1 virus as of mid-March 2010 (Centers for Disease Control and Prevention, 2010). Rapid laboratory detection of pandemic A/H1N1 must rely upon detection with 1) direct fluorescent antigen testing; 2) rapid antigen or immunodiagnostic testing formats (RIDT); or 3) conventional or real time reverse-transcriptase polymerase chain reaction (rRT-PCR) testing (Storch, 2003). During the 2009 H1N1 pandemic, a laboratory-confirmed diagnosis was frequently obtained to help with the early initiation of antiviral therapy and the implementation of interventions by infection prevention and control. The 2009 H1N1 was the first pandemic influenza virus to be tested by many of the molecular diagnostic advances in medical microbiology since 1968. In order to evaluate the importance of obtaining rRT-PCR as the first-line diagnostic test for detecting 2009 H1N1, we studied 426 NP swab specimens by RIDT and 3 different PCR-based detection methods, including a laboratory-developed rRT-PCR assay (LDT rRT-PCR), the Centers for Disease Control and Prevention (CDC) 2009 H1N1 rRT-PCR protocol, and the xTAG Respiratory Virus Panel (Luminex, Austin, TX, USA) (xTAG RVP).

2. Material and methods

2.1. Specimens

From May to October 2009, 448 NP swab specimens, stored in 3.0-mL M4/M4RT viral transport media, were

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received by our laboratory for testing. A total of 426 of those were tested using the BinaxNOW Influenza A & B RIDT in the laboratory setting. Patient samples were kept refrigerated at 2–8 °C until analysis within 24 h of collection. Post-analysis, the specimens were archived at −70 °C. Archived samples were later thawed, vortexed, and aliquoted in preparation for total nucleic acid extraction. Each aliquot of 200 μL was combined with 20 μL of MS2 phage internal control solution (1:100,000 dilution in M4/M4RT media, ATCC catalog no. 15597) and extracted on the NucliSENS EasyMag system (Generic Protocol 2.0.1, BioMérieux, Durham, NC, USA). Each 100-μL eluate was split into 4, single-use, 25-μL aliquots and was immediately frozen at −70 °C for future use in influenza A rRT-PCR testing.

2.2. Respiratory viral panel by RT-PCR

xTAG-RVP is a multiplex RT- PCR system for detection of influenza A (H1/H3), influenza B, RSV A and B, parainfluenza 1 to 4, human metapneumovirus, enterovirus/rhinovirus, adenovirus, and coronaviruses 229E, OC43, NL63, and HKU1. Detection was performed using the Luminex 200, a micro-fluidic bead array platform, following the manufacturer's instructions. Interpretation of mean fluorescence intensities to establish positivity was performed with the Tag-It Data Analysis Software (TDAS) utilizing the International Use Only (IUO) template (TDAS RVP-I, Luminex). Results that were positive for the influenza A universal target (influenza A non-specific) but negative for the H1 (seasonal) and H3 targets were considered positive for 2009 H1N1.

2.3. CDC 2009 H1N1 LC480 rRT-PCR

An in-house adaptation of the CDC protocol for detection and characterization of influenza A 2009 H1N1 (World Health Organization, 2009a) was accomplished with the use of a 96-well format LightCycler 480 II instrument (Roche, Indianapolis, IN, USA). Primer and probe reagents (TIB MOL BIOL, Adelphia, NJ, USA), reaction master-mix setup, and cycling parameters were as described by the protocol. Amplification curves of sigmoid shape and C_t value <40 that were observed for SWInfA or SW H1 targets were considered positive for 2009 H1N1.

2.4. InfA M2/InfA H1 duplex by rRT-PCR (LDT rRT-PCR)

Two 2-step rtPCR assays for the detection of the influenza A *matrix 2* (InfA M2) and 2009 H1N1 *hemagglutinin* (InfA H1) genes were validated on the LightCycler 480 II instrument as a laboratory-developed test (LDT). For cDNA synthesis, 10 μL of RNA eluate was added to a 10-μL volume of master mix (First Strand cDNA synthesis kit; Roche) composed of 1.0 μL molecular grade water, 0.5 μL reverse transcriptase, 4.0 μL reverse transcriptase reaction buffer, 0.5 μL RNase inhibitor, 2.0 μL deoxynucleotide mix (10 mmol/L), and 2.0 μL random hexamer primer. Cycling conditions were as follows: 10 min at 25 °C, 30 min at 55 °C, 5 s at 85 °C and then held at 4 °C.

Primer and probe sequences reactive to Lambda bacteriophage DNA were included to control for the inhibition of the InfA M2 detection. Master-mix setup utilizing the FastStart DNA Master kit (Roche) included per reaction the following: 2.6 μL molecular grade water, 2.4 μL Mg^{2+} 25 mmol/L, 4.0 μL InfA M2 primer/probe mix, 4.0 μL internal control (Lambda DNA), and 2.0 μL FastStart. Oligonucleotide primers and probes for the M2 reaction were obtained pre-mixed at the following concentrations: FluA_F, TAACCgAggTCgAAACgTATgTTCT, 15.15 μmol/L; FluA_R, ggCATTTTggACAAAgCgTCTA, 12.12 μmol/L; FluA_MAT1, CgAAATCgCgCAgAgACTTgAAgATgT–FL, 6.06 μmol/L; FluA_MAT2, LC640-TTgCTgggAAA-AACACAgATCTTgAggC–ph, 6.06 μmol/L; Lambda_F, ATgCCACgTAAgCgAAACA, 9.09 μmol/L; Lambda_R, gCATAAACgAAgCAgTCgAgT, 9.09 μmol/L; Lambda_FL, CACTTCCCgAATAAC- FL, 4.54 μmol/L; and Lambda_690, LC690-CggATATTTTTgATCTgACC-gAAgCg-ph, 4.54 μmol/L (TIB MOL BIOL).

A second master mix for the InfA H1 target utilizing the FastStart DNA Master kit included per reaction the following: 8.6 μL molecular grade water, 2.4 μL Mg^{2+} 25 mmol/L, 2.0 μL InfA H1 primer/probe mix, and 2.0 μL FastStart. Oligonucleotides and their concentrations for the InfA H1 reaction were as follows: susH1_SE, CTCATggTCCTACATTgTggAAAC, 15.15 μmol/L; susH1_A, TCAAACCTTTCAAATgATgA-CACTg, 15.15 μmol/L; susH1_FL, AgCTCCTCATAATC-gATgAATCTCC-FL, 9.09 μmol/L; and susH1_LC, LC640-ggTAACACgTTCCATTgTCTgAACTAg-ph, 9.09 μmol/L (TIB MOL BIOL).

For both assays, 5-μL aliquots from the previously described cDNA product were used. Cycling conditions on the LightCycler 480 II were as follows: a single hold of 95 °C for 10 min [ramp rate (°C/s) = 4.40]; 45 cycles of 95 °C for 5 s [ramp rate (°C/s) = 4.40], 62 °C for 5 s [acquisition mode = single, ramp rate (°C/s) = 2.20, sec target = 55 °C, step size = 0.5 °C, step delay = 1 cycle], 72 °C [ramp rate (°C/s) = 4.40]; a hold of 40 °C for 30 s (ramp rate (°C/s) = 1.50). InfA M2 and InfA H1 targets were run as parallel but separate reactions on the 96-well plate with detection at 640 nm. The InfA M2 master-mix internal control was detected in the 660-nm channel. The required color compensation file was generated by using the Universal Color Compensation kit (TIB MOL BIOL, Cat no. 40-0318-00). Amplification curves of sigmoid shape and C_t value <40 that were observed for both InfA M2 and InfA H1 targets were considered positive for 2009 H1N1.

3. Results

The mean age of the 448 patients was 38.7 years and the mean age of the patients positive for 2009 H1N1 in this study was 22.7. All 448 specimens were tested with the LDT rRT-PCR, CDC H1N1 LC480 rRT-PCR, and the xTAG RVP. A positive influenza A 2009 H1N1 specimen was defined as requiring 2 positive results obtained from any of 4 different

Table 1
Analytic performance of rRT-PCR, Luminex xTAG RVP, and BinaxNOW Influenza A & B RIDT[a]

Influenza A 2009 H1N1 test method	Sensitivity	Specificity	PPV[c]	NPV	Prevalence	TP	TN	FP	FN
BinaxNOW Influenza A & B RIDT	42.3 (31.4–54.0)[b]	98.0 (95.7–99.1)	82.5 (66.6–92.1)	88.3 (84.6–91.3)	18.3 (14.8–22.4)	33	341	7	45
LDT SOIA rRT-PCR	98.9 (92.9–99.9)	98.9 (97.0–99.7)	95.6 (88.4–98.6)	99.7 (98.2–100)	19.4 (15.9–23.4)	86	358	4	1
CDC 2009 H1N1 LC480 rRT-PCR	78.2 (67.8–86.0)	99.7 (98.2–100)	98.6 (91.1–99.9)	95.0 (92.2–96.9)	19.4 (15.9–23.5)	68	360	1	19
Luminex xTAG RVP	93.1 (85.0–97.2)	99.5 (97.8–99.9)	97.6 (90.8–99.6)	98.4 (96.3–99.3)	19.4 (15.9–23.5)	81	359	2	6

[a] Positivity is defined as requiring 2 or more positive results obtained by rRT-PCR or xTAG RVP.
[b] 95% Confidence intervals.
[c] PPV = positive predictive value; NPV = negative predictive value; TP = true positive; TN = true negative; FP = false positive; FN = false negative.

PCR-based detection assays, which included LDT rRT-PCR, xTAG RVP, the CDC 2009 H1N1 rRT-PCR performed at the Oregon State Public Health Laboratory (OSPHL) or the Washington State Department of Health (WSDOH), or the CDC 2009 H1N1 LC480 rRT-PCR performed using the LightCycler 480 II (Roche) instrument. Conversely, a negative result was defined as any result where all PCR-based detection assays were negative or where only a single positive result was obtained by rRT-PCR or xTAG. No result was obtained where a specimen was RIDT positive and also positive by only a single PCR-based method.

From 448 specimens, 82 (18.3%) were confirmed H1N1 positives (2 or more RT-PCR results positive) and 347 (77.5%) were negative with all methods. Twelve (2.7%) were considered to be negative as only one of the RT-PCR assays gave a positive result. Out of the total 448 NP swab specimens, a subset of 72 had confirmation testing by OSPHL and WSDOH with the CDC H1N1 rRT-PCR. Similarly, 48 specimens that were tested with xTAG RVP and were influenza A with no type result were referred to the OSPHL and WSDOH laboratories and were determined to be positive for influenza A 2009 H1N1 with the CDC 2009 H1N1 rRT-PCR. Overall, xTAG RVP influenza A positives without a type report had a 97.6% positive predictive value (PPV) for 2009 H1N1 (Table 1).

Three hundred eighty-six of the 426 NP swab specimens tested were negative by RIDT testing, of which 42 (10.9%) were shown to be a false-negative result. The increased sensitivity of rRT-PCR (Table 1) was demonstrated by showing that there was significantly lower influenza A virus RNA target detected in rRT-PCR–positive specimens that were RIDT negative compared to those that were RIDT positive ($P < 0.0001$). Interestingly, 108 of the true-negative results contained another respiratory virus (2 coinfections) as determined with xTAG RVP, consisting of 51 enterovirus/rhinovirus, 20 parainfluenza 3, 15 human metapneumovirus, 10 adenovirus, 4 parainfluenza 2, 2 parainfluenza 1, 1 influenza B, 2 influenza A H1 (seasonal), and 3 influenza A H3 (seasonal).

4. Discussion

In April 2009, the rRT-PCR Swine Flu Panel assay (CDC H1N1 rRT-PCR) for 2009 H1N1 was made available by the CDC and authorized for emergency use by the FDA (WHO, 2009a). Viral RNA detection by PCR is described by the WHO as the method of choice for the detection of 2009 H1N1 virus infection (WHO, 2009b). The use of rRT-PCR testing permits the highest sensitivity for the detection of influenza in respiratory specimens and uniquely allows for the specific identification of the influenza A 2009 H1N1. RIDTs have been widely adopted because they are rapid, convenient, and require less expertise to perform. As a consequence, a great deal of attention has been placed on the accuracy of RIDT for detecting 2009 H1N1. RIDT has been previously demonstrated to have a lower sensitivity than RT-PCR in the detection of seasonal influenza, often ranging from 41% to 87% depending on the prevalence, specimen type, and age of the patient (Liao et al., 2009). The analytical sensitivities of several RIDT assays have been shown to be comparable between 2009 H1N1 and seasonal influenza H1N1 (Chan et al., 2009). The clinical sensitivity for the 2009 H1N1 virus was reported to be 11–70% (Writing Committee of the WHO Consultation on Clinical Aspects of Pandemic (H1N1) 2009 Influenza, 2010) or, compared with only RT-PCR as the reference, in the range of 45–63% when 30 positive specimens were included (Karre et al., 2010; Velasco et al., 2010; Writing Committee of the WHO Consultation on Clinical Aspects of Pandemic (H1N1) 2009 Influenza, 2010). Similar to these findings, the Binax NOW FluA/B RIDT in this study had a sensitivity of 42.3% compared to PCR (Table 1). The low negative predictive value of RIDT for influenza A has serious implications for the continued reliance of RIDT as the first-line diagnostic test for influenza when a laboratory diagnosis is required for patient management. This observation agrees with the consensus that a negative result should not be used to make decisions with respect to treatment or infection prevention (Writing Committee of the WHO Consultation on Clinical Aspects of Pandemic (H1N1) 2009 Influenza, 2010).

29.5% (106/367) of the NP swabs sent to our laboratory during the 2009 H1N1 pandemic that tested negative for influenza 2009 H1N1 were shown to contain a respiratory virus by xTAG RVP testing. These findings highlight the importance of accurate laboratory testing to support the diagnoses of patients with signs and symptoms of influenza. The xTAG RVP has a valuable capability to detect novel influenza A virus strains and it was utilized during the early

months of the 2009 H1N1 pandemic before an H1N1 2009–specific PCR could be validated.

The CDC H1N1 rRT-PCR protocol for the detection and characterization of the pandemic influenza A 2009 H1N1 is specified to be used with 96-well format real-time-PCR instruments. Performance of this assay when applied on a LightCycler 480 II instrument (CDC H1N1 in-house LC480 rRT-PCR) demonstrated a comparatively low sensitivity (78.2%) but retained a very high PPV which is ideal for confirmation testing (Table 1). The protocol for the CDC H1N1 rRT-PCR assay which has been distributed worldwide by the World Health Organization is published to be compatible with a diverse number of different instruments, but each laboratory needs to thoroughly validate its in-house performance.

A limitation of this study is that the molecular testing was performed retrospectively. An additional limitation is that the definition used for a true positive, whereby 2 positive results were required from any of the 4 different PCR-based detection assays, could have penalized an individual assay with superior performance. This bias would lead to the individual assay appearing to have poorer specificity by detecting influenza in specimens that were determined to be negative by the other molecular assays.

In summary, during the 2009 H1N1 pandemic, we evaluated a number of PCR-based assays using a group of NP swabs that were tested by RIDT. We describe the significantly higher sensitivity of all rRT-PCR assays compared to RIDT. The higher sensitivity of rRT-PCR for both seasonal and pandemic influenza A reduces the usefulness of RIDT for the diagnosis and treatment of patients with influenza-like illness. The convenience of relying on RIDT as a first-line diagnostic test comes at the high cost of poor sensitivity.

References

Centers for Disease Control and Prevention (2010) CDC estimates of 2009 H1N1 influenza cases, hospitalizations and deaths in the United States, April 2009–March 13, 2010. Retrieved July 31, 2010, from http://www.cdc.gov/h1n1flu/estimates/April_March_13.htm.

Chan KH, Lai ST, Poon LL, Guan Y, Yuen KY, Peiris JS (2009) Analytical sensitivity of rapid influenza antigen detection tests for swine-origin influenza virus (H1N1). *J Clin Virol* 45:205–207.

Storch GA (2003) Rapid diagnostic tests for influenza. *Curr Opin Pediatr* 15:77–84.

Karre T, Maguire HF, Butcher D, Graepler A, Weed D, Wilson ML (2010) Comparison of Becton Dickinson Directigen EZ Flu A+B test against the CDC real-time PCR assay for detection of 2009 pandemic influenza A/H1N1 virus. *J Clin Microbiol* 48:343–344.

Liao RS, Tomalty LL, Majury A, Zoutman DE (2009) Comparison of viral isolation and multiplex real-time reverse transcription-PCR for confirmation of respiratory syncytial virus and influenza virus detection by antigen immunoassays. *J Clin Microbiol* 47:527–532.

Novel Swine-Origin Influenza A (H1N1) Virus Investigation Team (2009) Emergence of a novel swine-origin influenza A (H1N1) virus in humans. *N Engl J Med* 360:2605–2615.

Velasco JM, Montesa-Develos ML, Jarman RG, Lopez MN, Gibbons RV, Valderama MT, Yoon IK (2010) Evaluation of QuickVue influenza A+B rapid test for detection of pandemic influenza A/H1N1 2009. *J Clin Virol* 48:120–122.

World Health Organization (2009a) CDC protocol of realtime RTPCR for influenza A (H1N1); 2009. Retrieved October 13, 2009, from http://www.who.int/csr/resources/publications/swineflu/realtimeptpcr/en/index.html.

World Health Organization (2009b) WHO information for laboratory diagnosis of pandemic (H1N1) 2009 virus in humans — revised; 2009. Retrieved July 31, 2010, from http://www.who.int/csr/resources/publications/swineflu/WHO_Diagnostic_RecommendationsH1N1_20090521.pdf.

Writing Committee of the WHO Consultation on Clinical Aspects of Pandemic (H1N1) 2009 Influenza (2010) Clinical aspects of pandemic 2009 influenza A (H1N1) virus infection. *N Engl J Med* 362: 1708–1719.