Detection of Influenza H7N9 Virus: All Molecular Tests Are Not Equal

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The recent emergence of influenza A virus (H7N9) emphasizes the need for its rapid detection. While commercial nucleic acid amplification tests (NAATs) are commonly used to detect seasonal influenza virus, this study demonstrated that the analytical sensitivity of commercial assays is highly variable compared to that of CDC-based in-house NAATs for the detection of H7N9.

An avian influenza A virus (H7N9) was recently linked to 132 human cases and 32 deaths in China (1). Since rapid and accurate detection of novel influenza viruses is the cornerstone of pandemic preparedness (2, 3), many countries have been validating methods to rapidly identify this pathogen (4, 5). While diagnostic options are available, some methods have significant limitations. Growth and propagation of novel influenza viruses require biosafety level 3 containment, and the poor sensitivity of rapid influenza diagnostic tests (RIDTs) limits their usefulness for patient management (3, 6–9). In contrast, nucleic acid amplification tests (NAATs) such as reverse transcription-PCR (RT-PCR) have become the gold standard and a number of commercial assays are available (2, 10). On the other hand, genetic variations in novel influenza viruses could potentially hamper or prevent detection by molecular methods. *In silico* analysis can be used to identify sequence mismatches within the target region amplified by NAATs; however, the target sequence is often proprietary in commercial assays. The ability to detect novel influenza viruses using NAATs must be experimentally evaluated.

Recent publications have confirmed the suspicion that antigen-based RIDTs lack sensitivity for the detection of influenza A virus (H7N9) (11, 12). Surprisingly, two commercial NAATs, Resplex II Plus version 2.0 (Qiagen Inc.) and xTAG RVP Fast (Luminex Molecular Diagnostics), showed analytical sensitivities comparable to those of the RIDTs, with sensitivity values approximately 1,000-fold lower than those of an in-house RT-PCR targeting the highly conserved M gene (12). This is concerning, since commercial NAATs have been considered valuable tools for the detection of newly emerged strains of influenza A virus (13). This study compared the performance of several commercially NAATs to the performance of "in-house" real-time RT-PCR assays employed in public health or reference laboratories across Canada that use primer pairs designed by the Centers for Disease Control and Prevention (CDC) or the World Health Organization (WHO) targeting the M, H7, or N9 genes of influenza A virus (Table 1) (14, 15).

The lower limit of detection (LoD) of each method was determined by testing 10-fold serial dilutions of RNA extracted from influenza A/Anhui/01/2013 virus (H7N9) and tested in five independent runs. To reduce interlaboratory variability, specimen preparation, nucleic acid extraction, and dilutions were carried out at the National Microbiology Laboratory (NML) in Winnipeg (Manitoba, Canada). The virus was grown on embryonated eggs, and the viral stock titer was determined with a 50% tissue culture infective dose ($TCID_{50}$) assay and the Spearman-Kärber calculation method. The viral stock (140 μl) was extracted using a QIAamp Viral RNA Minikit (Qiagen, Mississauga, Ontario, Canada), as recommended by the manufacturer. Each dilution of RNA was prepared as a single batch with five replicates and shipped to participating sites in sufficient volumes to ensure there would be only a single freeze-thaw. Viral RNA was maintained at −70°C until use. All commercial assays were carried out as recommended by the manufacturer. The in-house real-time RT-PCR assays were performed as described by the CDC or WHO (4, 14, 15), and variations and modifications are summarized in Table 1 and Table S1 in the supplemental material, respectively. The LoD was defined by Probit analysis (16) using a probability of 95%, and values were expressed as $TCID_{50}$/ml or copies/ml (Table 1).

The in-house real-time RT-PCRs were consistently more sensitive than all of the commercial assays, despite some variability in protocols, reagents, and equipment (Table 1; see also Table S1 in the supplemental material). Unlike those of the in-house methods, the LoDs of the different commercial assays were highly variable. RealStar Influenza S&T RT-PCR assay version 3.0 (Altona Diagnostics) and the Quidel Molecular Influenza A+B assay

TABLE 1 Protocol summaries and analytical sensitivity

Assay[a]	Protocol summary				Estimated LoD[b]	
	Equipment	RT-PCR reagent	Primer and probe source	Target gene	No. of copies/ml	TCID$_{50}$/ml
CDC M* (NS)	ABI7500 Fast (Life Technologies)	TaqMan Fast Virus 1-Step Master Mix (Life Technologies)	CDC (modified)	M	617	0.20
CDC H7* (MB)	CFX 96 (Bio-Rad Laboratories)	TaqMan Fast Virus 1-Step Master Mix (Life Technologies)	CDC (modified)	H7	1,334	0.37
CDC M* (BC)	ABI7500 Fast (Life Technologies)	TaqMan Fast Virus 1-Step Master Mix (Life Technologies)	CDC (modified)	M	1,340	0.37
CDC M* (MB)	CFX 96 (Bio-Rad Laboratories)	TaqMan Fast Virus 1-Step Master Mix (Life technologies)	CDC (modified)	M	1,429	0.40
CDC*M (QC)	IQ5 (Bio-Rad Laboratories)	Quantitect RT-PCR probe (Qiagen Inc.)	CDC	M	1,496	0.42
CDC M (SK)	BioMark HD (Fluidigm Corp.)	Quantitect RT-PCR probe (Qiagen Inc.)	CDC	M	3,388	0.95
CDC M* (SK)	BioMark HD (Fluidigm Corp.)	Quantitect RT-PCR probe (Qiagen Inc.)	CDC (modified)	M	3,891	1.10
CDC M (PE)	LightCycler 2.0 (Roche Diagnostics)	Roche Master mix HybProbe (Roche Diagnostics)	CDC	M	5,754	1.60
WHO N9 (SK)	BioMark HD (Fluidigm Corp.)	Quantitect RT-PCR Probe (Qiagen Inc.)	WHO	N9	5,754	1.60
CDC*H7 (SK)	BioMark HD (Fluidigm Corp.)	Quantitect RT-PCR probe (Qiagen Inc.)	CDC	H7	5,808	1.62
CDC M* (QC2)	IQ5 (Bio-Rad Laboratories)	Quantitect RT-PCR probe (Qiagen Inc.)	CDC	H7	5,998	1.98
CDC M* (AB)	ABI7500 Fast (Life Technologies)	TaqMan Fast Virus 1-Step Master Mix (Life Technologies)	CDC	M	6,761	2.00
WHO H7 (SK)	BioMark HD (Fluidigm Corp.)	Quantitect RT-PCR Probe (Qiagen Inc.)	WHO	H7	7,161	2.04
CDC M (NS)	ABI7500 Fast (Life Technologies)	Invitrogen SuperScript III Platinum One-Step qRT-PCR system (Life Technologies)	CDC (in duplex with influenza B virus)	M	7,762	2.19
RealStar (ON)	Rotor Gene 6000 (Corbett Research)	RealStar Influenza S&T kit v.3 (Altona Diagnostics)	Undisclosed	M	11,482	3.20
Quidel (ON)	Rotor Gene 6000 (Corbett Research)	Molecular Influenza A+ B (Quidel Corp.)	Undisclosed	M	13,335	3.72
Simplexa (ON)	3 M cycler (Focus Diagnostics)	Simplexa FluA/B & RSV (Focus Diagnostics)	Undisclosed	M	118,850	33.11
Seegene RVI5 (NS)	DNA Engine (Bio-Rad)	Seeplex RV15 One Step ACE detection kit (Seegene) with agarose gel electrophoresis	Undisclosed	M	288,403	79.43
xTag RVP Fast (QC)	LiquiChip 200 (Qiagen Inc.)	XTag RVP Fast v. 1	Undisclosed	M	1,496,236	416.87
Seegene RVI5 (MB)	ABI Veriti 96 well (Life Technologies)	Seeplex RV15 One Step ACE detection kit (Seegene) with capillary electrophoresis (QIAxcel, Qiagen Inc.)	Undisclosed	M	2,985,383	1,659.59
xTag RVP Classic	Luminex 200 (Luminex Corp.)	XTag RVP Classic (Luminex Corp.)	Undisclosed	M	ND	ND

[a] An asterisk (*) in the assay name denotes a modification from the CDC protocols that are summarized in Table S1 in the supplemental material. Abbreviations are as follows: AB, Alberta; BC, British Columbia; CDC, Centers for Disease Control and Prevention; H7, hemagglutinin 7; LoD, limit of detection; M, matrix; MB, Manitoba; N9, neuraminidase 9; ND, not detected; NS, Nova Scotia; ON, Ontario; PE, Prince Edward Island; QC, Québec; SK, Saskatchewan; TCID$_{50}$, 50% tissue culture infectious dose; U.K., United Kingdom; WHO, World Health Organization.
[b] The estimated LoDs are ranked in descending order of the analytical sensitivity determined at a probability of 95%.

(Quidel Corp.) were only slightly less sensitive than the in-house RT-PCR assays. With a LoD estimated at 6.17 log10 copies/ml, xTagRVP Fast was approximately 200- to 2,000-fold less sensitive than the CDC-based assays (Fig. 1), which is consistent with the findings by Chan et al. (12), who reported a LoD of 7.2 log10 copies/ml for the same strain and 6.8 log10 copies/ml for A/Zhejiang/DTID-ZJU01/2013 virus. Of note, the xTagRVP Classic assay generated only indeterminate results, inconsistently, for the first three most concentrated dilutions of RNA. For this study, indeterminate values were plotted as positive in Fig. 1 but would require confirmation for diagnostic purposes. The poor sensitivity of the xTag RVP Classic assay is concerning, given that this assay is approved by the Food and Drug Administration (FDA) and used in many front-line laboratories in North America. Seeplex RV15

(Seegene) was also 4,800-fold less sensitive than most in-house real-time RT-PCR assays, and this observation was consistent between different laboratories (Fig. 1).

Many factors can influence the performance of NAATs, including specimen type, transport, and the nucleic acid extraction method. The panel used in this study consisted of extracted RNA, removing the extraction variable. While freeze-thaw could reduce the overall sensitivity of the molecular assays, each RNA dilution was limited to a single freeze-thaw, and thus, the various methods could be directly compared. Although this study did not compare the clinical performances of specimens collected from patients, our results highlight the analytical variability of the different commercial assays and the importance of verifying the ability to detect newly emerged influenza viruses in reference laboratories. This
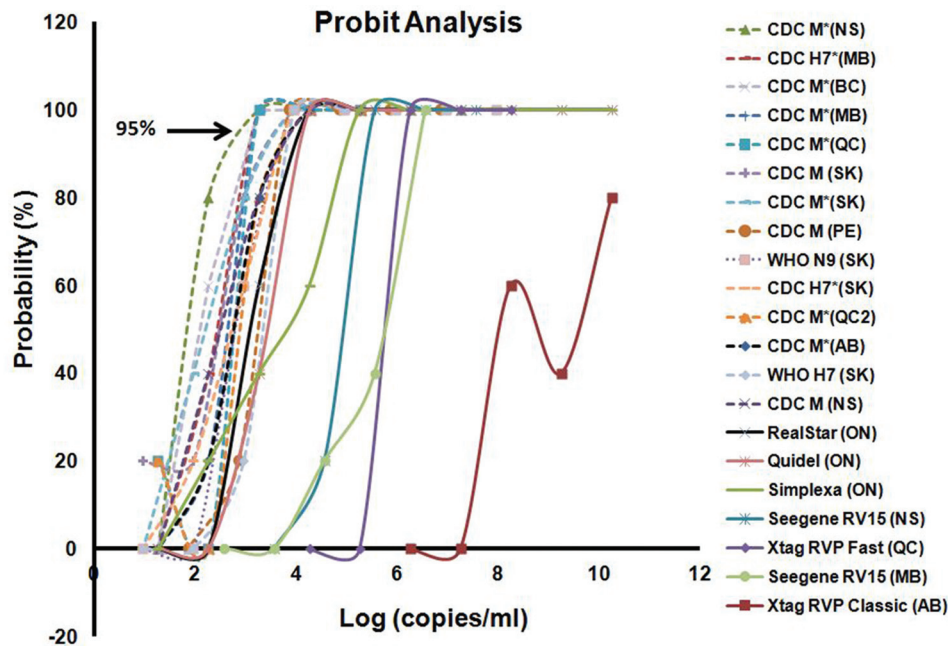
FIG 1 The analytical sensitivity was estimated using Probit analysis, and values were determined at a probability of 95%. An asterisk (*) in the assay name denotes a modification from the CDC protocols that are summarized in Table S1 in the supplemental material.

finding will have major impacts on health care systems, as the methods used in different laboratories may not have equivalent sensitivities. Given the poor sensitivity of some commercial assays, front-line-laboratory personnel, clinicians, and other stakeholders such as public health authorities need to understand the limitations of these assays and that a negative result cannot reliably rule out infection in circumstances in which there is a high index of suspicion for H7N9 infection. In these cases, further testing at a local public health laboratory or other reference laboratories that use the CDC- or WHO-based M, H7, or N9 assays is essential. We believe that specimens from individuals with risks factors for novel influenza virus strains should be tested by laboratories with these in-house reference NAATs or with assays with equivalent performance characteristics. This could be facilitated with specialized screening tools (e.g., education, surveillance, and requisitions) for patients who have animal contacts or a history of travel to regions with emerging influenza A virus strains.

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