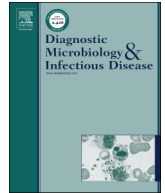




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Development and evaluation of a rapid nucleic acid amplification method to detect influenza A and B viruses in human respiratory specimens



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ABSTRACT

Isothermal nucleic acid amplification methods can potentially shorten the amount of time required to diagnose influenza. We developed and evaluated a novel isothermal nucleic acid amplification method, RT-SIBA to rapidly detect and differentiate between influenza A and B viruses in a single reaction tube. The performance of the RT-SIBA Influenza assay was compared with two established RT-PCR methods. The sensitivities of the RT-SIBA, RealStar RT-PCR, and CDC RT-PCR assays for the detection of influenza A and B viruses in the clinical specimens were 98.8%, 100%, and 89.3%, respectively. All three assays demonstrated a specificity of 100%. The average time to positive result was significantly shorter with the RT-SIBA Influenza assay (<20 min) than with the two RT-PCR assays (>90 min). The method can be run using battery-operated, portable devices with a small footprint and therefore has potential applications in both laboratory and near-patient settings.

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1. Introduction

Influenza viruses are a leading cause of acute respiratory infection and represent a considerable public health burden worldwide (Reed et al., 2015; The PMS, 2016). Humans are commonly infected by influenza A (H1N1 and H3N2 subtypes) and B viruses, which often co-circulate during epidemics. Vaccination against influenza viruses is one of the most effective strategies to minimize the risk of infection. Seasonal influenza vaccines are recommended for young children and the elderly because these groups are at high risk of influenza virus infection and its associated complications (Souty et al., 2015). Despite seasonal vaccination, influenza virus infection remains associated with high morbidity and mortality rates (Simonsen, 1999). This is due to continuous antigenic changes in the surface glycoproteins of influenza virus strains, which reduce the effectiveness of vaccination (de Vries et al., 2016; Webster and Govorkova, 2014).

Antiviral drugs can be used to treat patients with severe influenza virus infection. However, these drugs are only effective when started

within the first 48 h of the illness (Stiver, 2003). Consequently, effective use of antiviral drugs for the treatment of influenza virus infection relies on early and accurate diagnosis. Furthermore, the signs and clinical symptoms of influenza virus infection are similar to those of infections with other bacterial respiratory pathogens. Consequently, clinicians rely on *in vitro* diagnostic methods to confirm influenza virus infection. Therefore, early and accurate diagnosis is not only important for managing the infection but also for minimizing the unnecessary prescription of antibiotics (Low, 2008; Lowe et al., 2017).

Several diagnostic methods are available to detect influenza viruses in patient specimens (Vemula et al., 2016). These include viral culture, direct fluorescent antibody (DFA) tests, serological assays, rapid influenza diagnostic tests (RIDTs), and nucleic acid amplification tests (NAATs). Viral culture is laborious and time-consuming. Although DFA tests, RIDTs, and serology assays have shorter turnaround times (TATs), they lack the required sensitivity (Mahony et al., 2011). NAATs are rapidly replacing viral culture, DFA tests, RIDTs, and serology-based methods as the main method to diagnose influenza due to their improved sensitivity. However, most of these NAATs can only be performed at central laboratory centers due to their complexity and the requirement for sophisticated instrumentation. Isothermal-based NAATs can potentially shorten the amount of time required to diagnose influenza and avoid the need for complex instrumentation. Consequently, these assays could be potentially used for near-patient testing and in low-resource settings. An influenza diagnostic test must be able to detect and differentiate

Abbreviations: CEID₅₀, Chicken Embryo Infectious Dose; DFA, direct fluorescent antibody; IO, invasion oligonucleotide; LOD, limit of detection; NAAT, nucleic acid amplification test; NP, nasopharyngeal; RIDT, rapid influenza diagnostic test; RT-PCR, reverse transcription polymerase chain reaction; RT-SIBA, reverse transcription strand invasion-based amplification; SIBA, strand invasion-based amplification; TAT, turnaround time.

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between influenza A and B viruses because antiviral drugs have different efficacies against these viruses (Allen et al., 2006). However, only a limited number of isothermal-based NAATs can be performed in a multiplex format in a single reaction tube due to the risk of non-specific amplification (Notomi et al., 2000; Piepenburg et al., 2006; Van Ness et al., 2003).

In this study, we developed a highly sensitive and specific multiplex assay to detect influenza A and B viruses called reverse transcription strand invasion-based amplification (RT-SIBA). Strand invasion-based amplification (SIBA) is an isothermal method that relies on a recombinase-coated single-stranded invasion oligonucleotide (IO) and a polymerase for the rapid and exponential amplification of nucleic acids (Hoser et al., 2014). SIBA is an attractive nucleic acid amplification method owing to its short TAT and high analytical sensitivity and specificity (Eboigbodin and Hoser, 2016; Eboigbodin et al., 2016a, 2016b, 2017). We took full advantage of these characteristics to develop a multiplex test that can detect and differentiate between influenza A and B viruses in a single reaction tube. Furthermore, the clinical performance of the multiplex RT-SIBA Influenza assay was compared with those of two established NAATs for the detection of influenza A and B viruses.

2. Materials and methods

2.1. Viral isolates and clinical specimens

Purified and quantified viral particles from influenza A H1N1 (American Type Culture Collection (ATCC) VR-95PQ), influenza A H3N2 (ATCC VR-544PQ), and influenza B (ATCC VR-1804PQ) were obtained from ATCC (LGC Standards, Germany). These strains were used to determine the limit of detection of the RT-SIBA Influenza assay. Sixteen non-respiratory pathogens were used to evaluate the specificity of this assay. A total of 132 retrospective nasopharyngeal (NP) swab and aspirate specimens were used to evaluate the three assays. Seventy-one NP swab specimens were obtained from the Discovery Life Sciences Biobank (Discovery Life Sciences, USA). These specimens had been previously determined to be positive ($n = 44$) or negative ($n = 27$) for influenza viruses using the eSensor Respiratory Viral Panel (GenMark Diagnostics, USA) or the Simplexa™ Flu A/B & RSV Direct Kit (DiaSorin, Italy). Sixty-one aspirate specimens were obtained from Karolinska University Hospital (Sweden). These specimens had been previously determined to be positive ($n = 40$) or negative ($n = 21$) for influenza viruses using the Simplexa™ Flu A/B & RSV Direct Kit (Diasorin, Italy). All specimens were used in accordance with Karolinska Institutet ethical Review Act 2003 and Discovery Life Sciences Biobank Bioethics Policy.

2.2. Extraction and preparation of viral RNA

Viral RNA was extracted from the ATCC viral particles and clinical specimens using the QIAamp Viral RNA Mini Kit (Qiagen, Germany). Bacterial DNA was extracted using the QiAmp DNA Mini kit (Qiagen). Both types of extraction were performed according to the manufacturer's instructions. The quantitative genome copy numbers and infectious titers of the ATCC viral particles reported by the manufacturer were used to estimate the viral loads (Chicken Embryo Infectious Dose, CEID₅₀) and the amount of viral RNA per reaction. RNA extracted from the clinical specimens was used to evaluate the clinical performance of the RT-SIBA Influenza assay in comparison with those of the RT-PCR assays for detecting influenza A and B viruses.

2.3. Multiplex RT-SIBA Influenza assay

2.3.1. Design

The multiplex RT-SIBA Influenza assay was designed to simultaneously detect all subtypes of influenza A and B viruses in one reaction tube. The sequences of the influenza strains, which were retrieved from

the Virus Pathogen Resource and the National Center for Biotechnology Information database, were aligned (Squires et al., 2012). A total of 4575 non-duplicate influenza A segment 1 and 1495 non-duplicate influenza B segment 3 sequences were aligned. Influenza A assay was designed to detect the highly conserved of segment 1 to allow for the detection of influenza A H1N1 and H3N2 as well as pandemic strains. Likewise influenza B assay was designed to detect the highly conserve region of segment 3 to allow for the detection of both influenza B Yamagata and Victoria lineages. Separate reaction sets comprising a forward primer, a reverse primer, an IO, and a locked nucleic acid (LNA) fluorescent probe were designed to amplify and differentiate between the RNA target regions of influenza A and B viruses. The dual LNA probes were used for detecting and distinguishing influenza A and influenza B multiplex reaction. The influenza A probe was labeled with ROX and Iowa black FQ quencher, whereas the influenza B probe was labeled with Cy5 and Iowa black RQ quencher. The sequences of the oligonucleotides used for the RT-SIBA Influenza assay are listed in Table 1.

2.3.2. Reaction conditions

The multiplex RT-SIBA Influenza assay was performed using the SIBA® reagent kit (Orion Diagnostica Oy, Finland) with the reaction supplemented with 16 units of GoScript™ Reverse Transcriptase (Promega, UK). The forward primer, reverse primer, IO, and probe were used at concentrations of 300, 200, 300, and 150 nM, respectively, to detect influenza A virus and at concentrations of 250, 175, 200, and 150 nM, respectively, to detect influenza B virus. The UvsX and Gp32 enzymes were used at concentrations of 400 and 300 ng/ml, respectively. A total of 2 µl of each RNA extract was used in a total reaction volume of 20 µl. Products were detected using fluorophore-labeled probes and SYBR Green (1:100,000 dilution). Reactions were incubated at 43 °C for 45 min, and fluorescence was measured every 60 s using a Bio-Rad CFX96 system (Bio-Rad Laboratories, United Kingdom). A melting curve was generated after amplification at 40–95 °C to verify the specificity of the reaction products.

2.4. Multiplex reverse transcription polymerase chain reaction (RT-PCR) influenza assays

One previously published and one commercially available a CE-certified RT-PCR assay to detect influenza A and B viruses were performed for comparison. The previously published RT-PCR assay was performed using CDC influenza A and B-specific primers and probes labeled with the 6-carboxyfluorescein and 4',5'-dichloro-2',7-dimethoxyfluorescein dyes (Selvaraju and Selvarangan, 2010). Each reaction (total volume of 20 µl) contained 10 µl 2× EXPRESS qPCR Supermix, Universal (Thermo Fisher Scientific, Finland), 2 µl Express SuperScript® Mix for One-Step qPCR (Thermo Fisher Scientific), 1 µM of each primer, 0.04 µM probes, and 2 µl RNA extract. The reaction conditions were as follows: reverse

Table 1
Oligonucleotides used in the multiplex RT-SIBA Influenza assay.

Name	Sequence 5'–3'
FluA-F primer	AATTGATGGCCATCCCAAT
FluA-R primer	AACGGGACT CTAGCAT
FluA-IO	CCCCCCCCCT GAATTCITTTGGTCGCTGTCTGGCTGCA mGmUmAmAmGmUmAmUmGmC
FluA-probe	/56-ROXN/AA+A + CGG + GA + CT + C + T/3IABkFQ/
FluB-F primer	AGATGTTGAATAGCATT
FluB-R primer	AGGCCAAAAACAAATG
FluB-IO	CCCCCCCCCCCC TTGCTGGTTGAATTCAGGATCTTCACTA mAmAmUmUmCmUmGmCmCmA
FluB-probe	/cy5/A + GG + CC + AA+A + AA+C + A/3IAbRQSp

For invasion oligonucleotides (IOs), bold sequences denote non-homologous seeding area sequences. mA, mC, mG, and mU denote 2'-O-methyl RNA nucleotides. F, forward; R, reverse; +, locked nucleic acid bases; IABkFQ, Iowa Black FQ quencher; IAbRQSp, Iowa Black RQ quencher; FluA, Influenza A; FluB, Influenza B; RT-SIBA, reverse transcription strand invasion-based amplification.

transcription at 50 °C for 30 min, Taq activation at 95 °C for 2 min, and 45 cycles of denaturation at 95 °C for 15 s and amplification at 55 °C for 30 s. Analysis was also performed using the commercial RealStar® Influenza RT-PCR Kit 2.0 (Altona Diagnostics, Germany) according to the manufacturer's instructions. The two RT-PCR assays were performed as duplicates in a Bio-Rad CFX96 system (Bio-Rad Laboratories).

3. Results

3.1. Optimization of the multiplex RT-SIBA Influenza assay

The multiplex RT-SIBA Influenza assay was optimized for rapid and specific detection of influenza A and B virus RNA. Gp32 and UvsX are key components of RT-SIBA. While Gp32 prevents the formation of secondary structures in the single-stranded DNA (IO and primers), UvsX cooperatively binds to the single-stranded IO and displaces Gp32 to form an UvsX-IO filament complex. This complex catalyzes strand separation of the homologous target duplex, which enables primers to bind and extend the target via the action of a polymerase (Formosa and Alberts, 1986; Hoser et al., 2014; Liu and Morrical, 2010). The affinity of UvsX for a single-stranded DNA is dependent on the nucleotide composition, particularly the ratio of purines to pyrimidines (Formosa and Alberts, 1986). Because the influenza A and B assays were designed to detect different target sequences, we reasoned that their requirement for UvsX and Gp32 may differ. We sought to determine the optimal concentrations of UvsX and Gp32 to efficiently amplify both the influenza A and B virus targets in the same reaction tube.

To determine the optimal concentrations of Gp32 and UvsX, the multiplex RT-SIBA Influenza assay was conducted using 200–500 ng/ml of each protein. The effects of these various concentrations of Gp32 and UvsX on the assay were determined. Fig. 1 shows the amount of time taken to obtain a positive result with 200 copies of influenza A and B virus RNA per reaction. A positive result was obtained when the fluorescence signal of the probe exceeded the background signal. The amount of time taken to obtain a positive result for influenza A H1N1 decreased as

the concentration of UvsX increased from 200 to 500 ng/ml, but increased as the concentration of Gp32 increased from 200 to 500 ng/ml. Similar findings were made with respect to the amount of time taken to obtain a positive result for influenza A H3N2. This is not surprising because the same primers and IO were used to detect these two strains. Variation of the UvsX concentration had less of an effect on the detection of influenza B virus. By contrast, an increase in the Gp32 concentration significantly decreased the amount of time taken to obtain a positive result for influenza B virus, opposite to its effect on the detection of influenza A virus. Influenza B virus was not detected in reactions containing 200 ng/ml Gp32 and 500 ng/ml UvsX. The difference in the amount of time taken to detect influenza A and B viruses could be due to a difference in the affinity of UvsX for DNA nucleotides. Based on these findings, subsequent experiments were performed using UvsX and Gp32 at final concentrations of 400 and 300 ng/ml, respectively.

3.2. Analytical sensitivity of the multiplex RT-SIBA Influenza assay

The analytical sensitivity of the multiplex RT-SIBA Influenza assay was determined using RNA extracted from influenza A H1N1, influenza A H3N2, and influenza B viral particles of known viral loads (CEID₅₀) and RNA copy numbers. The sensitivity of the assay was determined in three independent experiments by adding serial dilutions of RNA extracted from viral particles with a CEID₅₀ of 10⁶ to one per reaction. Four replicates of each dilution were used and the results are shown in Fig. 2. The multiplex RT-SIBA Influenza assay reproducibly detected all influenza virus strains tested within 20 min using 10 CEID₅₀ per reaction. When CEID₅₀ was higher than 100 per reaction, it took as little as 7 min to obtain a positive result. These results demonstrate that the multiplex RT-SIBA Influenza assay is faster than most RT-PCR methods previously reported to detect influenza viruses. The limit of detections (LODs) at which influenza viruses were detected 95% of the time were further determined by Probit regression analysis. The LODs of the RT-SIBA Influenza assay for influenza A H1N1, influenza A H3N2, and influenza B viruses were 5, 1, and 9 CEID₅₀ per reaction, respectively

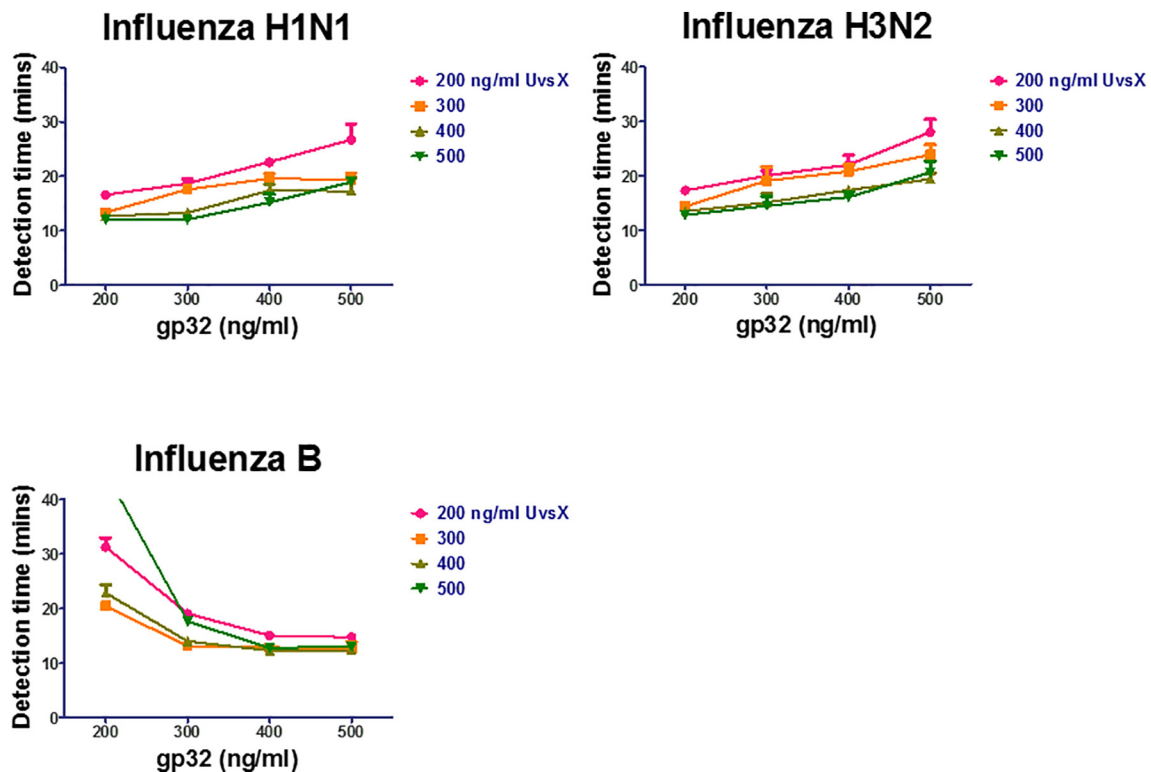


Fig. 1. Optimization of the multiplex RT-SIBA Influenza assay. Impact of the concentrations of Gp32 and UvsX on the detection of influenza A and B viruses. Influenza B virus was not detected in reactions containing 200 ng/ml Gp32 and 500 ng/ml UvsX.

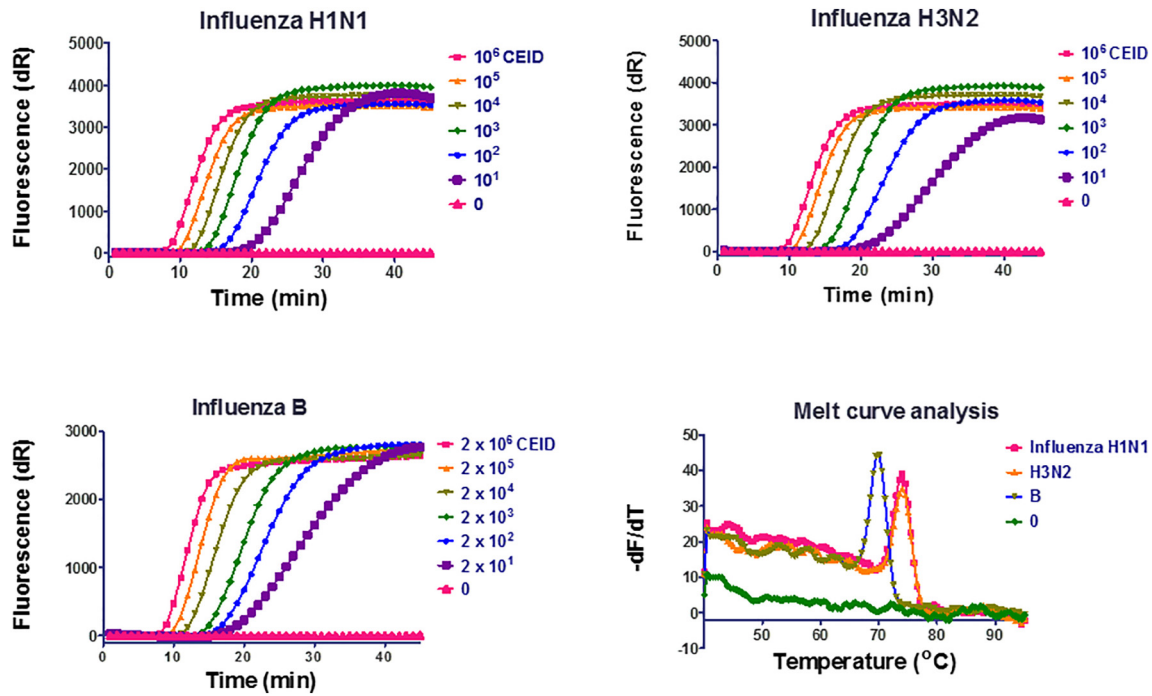


Fig. 2. Sensitivity of the multiplex RT-SIBA Influenza assay for the detection of influenza viruses. (a) Influenza A H1N1 virus. (b) Influenza A H3N2 virus. (c) Influenza B virus. (d) Melting curve profiles of influenza viruses (200 copies of influenza A H1N1, influenza A H3N2, and influenza B RNA).

(Table 2), which correspond to 5, 10, and 19 RNA copies per reaction, respectively.

3.3. Analytical specificity of the multiplex RT-SIBA Influenza assay

Melting curve analysis was performed to analyze the specificity of the multiplex RT-SIBA Influenza assay. A single specific amplicon was observed with influenza A and B virus templates (Fig. 2d). This suggests that the assay can detect and differentiate between influenza A and B viruses. The analytical specificity of the multiplex RT-SIBA Influenza assay was further elucidated by including nucleic acids extracted from 16 common respiratory pathogens (Table 3). Nucleic acids were extracted from microbes at concentration higher than 10^6 CFU or TCID per ml. These nucleic acids did not cross-react with the multiplex RT-SIBA Influenza assay. Furthermore, the assay could efficiently detect low amounts of influenza virus RNA in the presence of high amounts of respiratory pathogens.

3.4. Evaluation of the multiplex RT-SIBA Influenza assay using NP swab and aspirate specimens

The ability of the RT-SIBA Influenza assay to detect influenza viruses was further evaluated using 132 retrospective NP swab and aspirate specimens. A total of 44, 40, and 48 of these specimens were determined

to be positive for influenza A virus, positive for influenza B virus, and negative for influenza viruses, respectively. These specimens were re-analyzed using the RT-SIBA Influenza assay and two established RT-PCR multiplex influenza assays. The results were compared with the previous data (Table 4), and the sensitivity and specificity of each assay for the detection of influenza A and/or B viruses were determined. By comparison with the previous results, the sensitivities and specificities to detect influenza A/B viruses were calculated to be 98.8% (95% CI: 93.6–99.8%) and 100% (95% CI: 92.6–100%) for the RT-SIBA Influenza assay, respectively; 100% (95% CI: 95.6–100%) and 100% (95% CI: 92.6–100%) for the RealStar® Influenza RT-PCR assay, respectively; and 89.3% (95% CI: 80.9–94.3%) and 100% (95% CI: 92.6–100%) for the CDC Influenza RT-PCR assay, respectively. The CDC Influenza RT-PCR assay had the lowest sensitivity, while the sensitivity of the RealStar® Influenza RT-PCR assay was slightly higher than that of the RT-SIBA Influenza assay.

The sensitivities of the RT-SIBA Influenza, RealStar® Influenza RT-PCR, and CDC Influenza RT-PCR assays were 100%, 100%, and 95.5% for the detection of influenza A virus alone, respectively, and 97.6%, 100%, and 80.5% for the detection of influenza virus B alone, respectively. Moreover, the specificities of the RT-SIBA Influenza, RealStar® Influenza RT-PCR, and CDC Influenza RT-PCR assays for the detection of influenza virus A alone were 98.9%, 100%, and 100%, respectively. The specificities of the RT-SIBA Influenza, RealStar® Influenza RT-PCR, and CDC Influenza RT-PCR assays for the detection of influenza virus B alone were 100%, 96.7%, and 100%, respectively.

The RT-SIBA Influenza assay simultaneously detected influenza A and B viruses in a clinical specimen previously reported to be only positive for influenza B virus. In addition, the RealStar® Influenza RT-PCR assay simultaneously detected influenza A and B viruses in one clinical specimens previously reported to be only positive for influenza A virus. The detection of both viruses in these specimens was presumed to be a false positive result since it differed from the results from the sending laboratory. Alternatively, these discrepancies could reflect the superior sensitivity of the RT-SIBA and RealStar® Influenza RT-PCR assays over the CDC Influenza RT-PCR assay and the previously used method. These data demonstrate that the RT-SIBA Influenza assay had high analytical sensitivity and specificity for the detection of influenza

Table 2
Limit of detection of the multiplex RT-SIBA Influenza assay.

Strain	ATCC number	Influenza A subtype or influenza B genetic lineage	Limit of detection (CEID ₅₀ /reaction)	Limit of detection (genome equivalents/reaction)
Influenza A virus	ATCC VR-95	H1N1	5	5
Influenza A virus	ATCC VR-544	H3N2	1	10
Influenza B virus	ATCC VR-1804	B, Yamagata	9	19

Table 3
Potential cross-reactive organisms in the multiplex RT-SIBA Influenza assay.

Strains	Multiplex RT-SIBA Influenza result (min)
<i>Influenza A virus</i> ATCC VR-95	10
<i>Influenza A virus</i> ATCC VR-544	10
<i>Influenza B virus</i> ATCC VR-1804	11
<i>Escherichia coli</i> ATCC 25922	ND*
<i>Klebsiella pneumoniae</i> ATCC 13883	ND*
<i>Staphylococcus aureus</i> ATCC 6538	ND*
<i>Staphylococcus epidermidis</i> 2954	ND*
<i>Streptococcus agalactiae</i> ATCC 12386	ND*
<i>Streptococcus dysgalactiae</i> ATCC 12388	ND*
<i>Streptococcus pneumoniae</i> ATCC 6305	ND*
<i>Streptococcus pyogenes</i> ATCC 19615	ND*
<i>Parainfluenza virus 1</i> ATCC - VR-94	ND*
<i>Coronavirus</i> ATCC-VR-740	ND*
<i>Adenovirus 1</i> ATCC VR-1	ND*
<i>Adenovirus 7</i> ATCC VR-7	ND*
<i>Enterovirus 71</i> ATCC VR-1432	ND*
<i>Rhinovirus 17</i> ATCC VR-1663	ND*
<i>Human respiratory syncytial virus A</i> ATCC VR-1540	ND*
<i>Human respiratory syncytial virus B</i> ATCC VR-1400	ND*

ND* = Not detection through the time of performing the reaction. Nucleic acids were extracted from microbes at concentration higher than 10⁶ CFU or TCID per ml.

A and B viruses. Furthermore, it took significantly less time to obtain a positive result with the RT-SIBA Influenza assay (less than 20 min) than with either of the RT-PCR assays (more than 90 min; Ct, ~25).

4. Discussion

Timely diagnosis of influenza virus infection is important to manage patients, control infection, and prevent the inappropriate use of antibiotics (Low, 2008; Lowe et al., 2017). Molecular methods, such as isothermal nucleic acid amplification, offer significant advantages such as relatively short TATs and simple setups. However, most isothermal amplification

methods have mainly been used to detect a single target region, and there is limited evidence of their multiplexing capability (Notomi et al., 2000; Piepenburg et al., 2006; Van Ness et al., 2003). This has hampered the adoption of such methods in clinical settings, especially for the diagnosis of influenza, which requires the detection and differentiation of influenza A and B viruses. We previously reported an isothermal method to detect influenza A and B viruses in two different reaction chambers (Eboigbodin et al., 2016b). In the present study, we developed and evaluated an isothermal nucleic acid amplification method called RT-SIBA for the rapid detection and differentiation of influenza A and B viruses in a single reaction tube. This assay relies on a recombinase-coated oligonucleotide, a reverse transcription enzyme, and target-specific primers to exponentially amplify the target sequence of viral RNA (Eboigbodin and Hoser, 2016; Eboigbodin et al., 2017; Hoser et al., 2014). The reactions are performed at a low and constant temperature (43 °C), and therefore do not require complex instrumentation.

The multiplex RT-SIBA Influenza assay displayed high analytical sensitivity and specificity for the detection of influenza A and B viruses. Moreover, this assay detected 100 copies of influenza A H1N1, influenza A H3N2, and influenza B viral RNA within 15 min. This is significantly faster than most nucleic acid amplification methods, which take more than 1 h to detect such a low number of RNA copies. Furthermore, the RT-SIBA Influenza assay did not detect other common respiratory pathogens, suggesting that it is highly specific. The concentrations of UvsX and Gp32 proteins and the UvsX:Gp32 ratio can affect the sensitivity and speed of RT-SIBA, especially in the case of a multiplex assay. This was true for the multiplex RT-SIBA Influenza assay, in which the optimal concentrations of UvsX and Gp32 differed for the detection of influenza A virus versus influenza B virus. This is likely because the affinity of UvsX for an oligonucleotide is dependent on the length and nucleotide composition (Formosa and Alberts, 1986; Liu and Morrical, 2010). Although both UvsX and Gp32 are crucial for SIBA amplification, they also compete with themselves for binding to oligonucleotides (Formosa and Alberts, 1986; Hoser et al., 2014; Liu and Morrical, 2010). Therefore, the optimal concentrations of UvsX and Gp32

Table 4
Clinical performance of the multiplex RT-SIBA Influenza assay in comparison with two RT-PCR assays for the detection of influenza A and B viruses.

	RT-SIBA Influenza					
	Influenza A/B virus		Influenza A virus		Influenza B virus	
	Positive	Negative	Positive	Negative	Positive	Negative
Positive	83	1	44	0	40	1
Negative	0	48	1	87	0	91
Total no. of samples	132		132		132	
Sensitivity (95% CI)	98.8% (93.6–99.8%)		100% (92.0–100%)		97.6% (87.4–99.6%)	
Specificity (95% CI)	100% (92.6–100%)		98.9% (93.8–99.8%)		100% (95.6–100%)	
Average detection time (minutes)	16.9		15.6		18.4	
	RealStar® Influenza RT-PCR (Altona)					
	Influenza A/B virus		Influenza A virus		Influenza B virus	
	Positive	Negative	Positive	Negative	Positive	Negative
Positive	84	0	44	0	41	0
Negative	0	48	0	88	1	90
Total no. of samples	132		132		132	
Sensitivity (95% CI)	100% (95.6–100%)		100% (92.0–100%)		100% (91.4–100%)	
Specificity (95% CI)	100% (92.6–100%)		100% (95.9–100%)		96.7% (94.0–99.8%)	
Average detection time (Ct)	24.2		24.5		24.6	
	CDC Influenza RT-PCR					
	Influenza A/B virus		Influenza A virus		Influenza B virus	
	Positive	Negative	Positive	Negative	Positive	Negative
Positive	75	9	42	2	33	8
Negative	0	48	0	88	0	91
Total no. of samples	132		132		132	
Sensitivity (95% CI)	89.3% (80.9–94.3%)		95.5% (84.9–98.8%)		80.5% (66.0–89.8%)	
Specificity (95% CI)	100% (92.6–100%)		100% (95.8–100%)		100% (96.0–100%)	
Average detection time (Ct)	24.1		24.9		23.5	

required for SIBA amplification may differ between target regions. Consequently, it is crucial to empirically determine the optimal concentrations of these proteins that allow multiple targets to be efficiently detected within the same reaction tube.

The analytical performance of the multiplex RT-SIBA Influenza assay was further elucidated by testing 132 retrospective NP swab and aspirate specimens that were previously determined to be positive or negative for influenza viruses. Moreover, the performance of this assay for the detection of influenza A and B viruses was compared with those of two established RT-PCR assays. The sensitivity of the RT-SIBA Influenza assay (98.8%) was significantly better than that of the CDC Influenza RT-PCR assay (89.3%), but slightly lower than that of the RealStar® Influenza RT-PCR assay (100%). The specificities of all three assays were 100%. Meanwhile, the sensitivities of the RT-SIBA Influenza assay for the detection of influenza A and B viruses were 100% and 97.5%, respectively. This slightly reduced sensitivity suggests that further optimization is required. Indeed, the assay was markedly improved by optimization of the Gp32 and UvsX concentrations. Nonetheless, the RT-SIBA Influenza assay displayed a high sensitivity and specificity for the detection of influenza A and B viruses and its findings correlated well with those of the reference methods. Furthermore, the average amount of time taken to obtain a positive result was significantly shorter with the RT-SIBA Influenza assay (<20 min) than with the CDC and RealStar® Influenza RT-PCR assays (>90 min). Moreover, the RT-SIBA Influenza assay is performed at a low and constant temperature, and can therefore be run using battery-operated, portable devices with a small footprint. Thus, this assay has potential applications in both laboratory and near-patient settings.

Disclosures/Conflict of Interest

SE, PA, KL, SS, MM and KE are employees of Orion Diagnostica Oy. All SIBA patents/patent applications are owned by Orion Diagnostica Oy. SE and KE are named inventors in SIBA patents applications.

Authors' contributions

KE conceived the study; SE, PA, LJ and KL designed and performed the experiments; All authors analyzed results, wrote and approved the manuscript.

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