Comparison of Simplexa Flu A/B & RSV PCR with Cytospin-Immunofluorescence and Laboratory-Developed TaqMan PCR in Predominantly Adult Hospitalized Patients

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To compare Simplexa Flu A/B & RSV PCR with cytospin-immunofluorescence and laboratory-developed TaqMan PCR methods, 445 nasopharyngeal samples were tested. Of these, 199 were positive (46 for respiratory syncytial virus [RSV], 120 for influenza A, and 33 for influenza B) and 246 were negative. The direct fluorescent-antibody assay (DFA) detected 132 (66.3%) positive samples, Simplexa direct detected 162 (81.4%), Simplexa using extracts detected 177 (88.9%), and lab-developed TaqMan PCR reference methods detected 199 (100%). The specificities were 99.6%, 100%, 100%, and 100%, respectively. The two Simplexa methods were more sensitive than the DFA ($P = 0.0001$) but less sensitive than the TaqMan reverse transcriptase PCR ($P = 0.0001$).

Influenza virus and respiratory syncytial virus (RSV) cause a substantial disease burden in people of all ages. A rapid laboratory diagnosis can facilitate early antiviral therapy, discontinuation of antibiotics, and implementation of infection control measures (1). Our laboratory uses the cytospin-enhanced direct fluorescent-antibody assay (DFA) as an initial on-demand screen and laboratory-developed real-time TaqMan (LDT) PCR when greater sensitivity is needed (2). In this report, we evaluated Simplexa Flu A/B & RSV PCR as a potential replacement for DFA and LDT PCR.

Nasopharyngeal swab specimens submitted in 3 ml M4 medium (Remel, Lenexa, KS) from February to April 2011 were tested by DFA and LDT PCR as requested by the clinician within 2 to 12 h of collection. The original samples and extracts were then frozen at -70°C , and a subset of the positive and negative samples were retested with the Simplexa PCR within 2 to 4 months.

The cytospin-DFA was performed using SimulFluor reagents (Millipore, Billerica, MA) as previously described (3). For influenza detection and subtyping, the CDC real-time reverse transcriptase (RT) PCR primers and probes were used (2). For RSV, the protocol published by van Elden et al. (4) was used, but the subtypes were not differentiated. Total nucleic acid was extracted from 200 μl of sample and eluted in 55 μl on an EasyMag instrument (bioMérieux, Durham, NC), and 5 μl of extract was added to 20 μl AgPath-ID one-step RT-PCR master mix (Life Technologies, Carlsbad, CA) without an internal control (IC), and the manufacturers' cycling parameters (10 min at 45°C , 10 min at 95°C , 15 s at 95°C , 45 s at 60°C) were used. Amplification was performed for 45 cycles using an ABI 7500 instrument (Applied Biosystems, Foster City, CA). Positive samples with cycle threshold (C_T) values of <38 were accepted as positive. For C_T values of ≥ 38 , amplification was repeated in duplicate and accepted if one replicate was positive.

For Simplexa Flu A/B & RSV PCR (Focus Diagnostics, Cypress, CA), frozen extracts were thawed, vortexed, and pulse spun. To each well of a 96-well disc was added 5 μl of master mix containing primers, an IC, and 5 μl of thawed extract or positive or negative control material. Discs were sealed and placed in a 3 M integrated

cycler (Focus Diagnostics). Amplification was performed for 40 cycles according to the manufacturer's recommendations. Additionally, frozen original samples were thawed and tested directly without extraction in a protocol suggested by the manufacturer, in which 50 μl of vortexed pulse-spun sample was heated to 70°C for 5 min, and then 2 μl was added to 8 μl of master mix into wells in the 96-well disc. When Simplexa testing of an extract did not confirm the original LDT PCR result, it was retested by LDT PCR. If the sample was negative on retesting, it was excluded. Statistical analysis was performed using McNemar's test.

Of 448 samples tested by the four methods, 25 samples that were initially LDT positive tested negative with Simplexa. Retesting with the LDT PCR confirmed 22 positive samples, but 3 were negative and therefore excluded. Thus, the data for 445 samples, 199 positive and 246 negative, were analyzed, and 82.9% were from adults >18 years of age. As shown in Table 1, DFA detected 132 (66.3%), Simplexa direct detected 162 (81.4%), and Simplexa using extracts detected 177 (88.9%) of 199 samples that tested positive with the LDT TaqMan PCR. One RSV DFA-positive sample was negative in the LDT PCR and Simplexa and was deemed a false positive. The specificities were 99.6% for the DFA and 100% for each of the three molecular methods. In total, 69 of 445 samples (15.5%) were inadequate for DFA (<25 columnar epithelial cells) but were counted as negative for this comparison because no results were generated. Fifteen of the DFA-inadequate samples were positive with the LDT PCR (2 RSV, 12 influenza A, and 1 influenza B), 11 were positive with Simplexa using extracted samples, and 9 were positive with Simplexa direct. The DFA was less

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TABLE 1 Performance characteristics of cytospin-DFA and Simplexa Flu A/B & RSV kit with unextracted and extracted samples compared to those of LDT TaqMan RT-PCR

Test	Total no. of samples ^a	RSV			Influenza A			Influenza B			Overall		
		No. positive (test/LDT) ^b	Sensitivity (% [95% CI])	Specificity ^c (% [95% CI])	No. positive (test/LDT) ^b	Sensitivity (% [95% CI])	Specificity (% [95% CI])	No. positive (test/LDT) ^b	Sensitivity (% [95% CI])	Specificity (% [95% CI])	No. positive (test/LDT) ^b	Sensitivity ^d (% [95% CI])	Specificity ^c (% [95% CI])
Cytospin-DFA	445 ^d	34/46	73.9 (59.6 to 84.5)	99.8 (98.5 to >99.9)	75/120	62.5 (53.6 to 70.7)	100 (98.6 to 100)	23/33	69.7 (52.5 to 82.8)	100 (98.9 to 100)	132/199	66.3 (59.5 to 72.5)	99.6 (97.5 to >99.9)
Simplexa Flu A/B & RSV Direct, 2-μl sample	445	37/46	80.4 (66.6 to 89.6)	100 (98.9 to 100)	100/120	83.3 (75.6 to 89.0)	100 (98.6 to 100)	24/33	72.7 (55.6 to 85.1)	100 (98.9 to 100)	162/199	81.4 (75.3 to 86.2)	100 (98.2 to 100)
5-μl extract	445	42/46	91.3 (79.1 to 97.1)	100 (98.9 to 100)	107/120	89.2 (82.3 to 93.7)	100 (98.6 to 100)	28/33	84.9 (68.6 to 93.8)	100 (98.9 to 100)	177/199	88.9 (83.8 to 92.7)	100 (98.2 to 100)

^a Sixty-nine of 445 samples tested by DFA were reported as inadequate but were counted here as DFA negative; 15 DFA-inadequate samples were positive by LDT, 11 were positive by Simplexa, and 9 were positive by Simplexa direct.
^b Reference method was LDT TaqMan PCR using a 5-μl extract. The values shown are the number of positive results with the indicated test/number of positive results with the LDT.
^c One additional RSV DFA-positive result was deemed false positive; all other DFA-positive samples and all Simplexa-positive samples confirmed by LDT RT-PCR.
^d All PCR methods were more sensitive than DFA ($P = 0.0001$), even when DFA-inadequate samples were excluded from analysis. LDT TaqMan PCR was more sensitive than either of the two Simplexa methods ($P = 0.0001$, McNemar's test).

sensitive than the three molecular test options ($P = 0.0001$), even when the 69 inadequate samples were excluded from the analysis ($P = 0.0001$, data not shown). The DFA began to miss PCR-positive samples beginning at LDT C_T values of 25 to 26 and missed almost every PCR-positive sample with a C_T value of >30 .

Simplexa direct failed to detect the majority of samples with an LDT C_T value of >30 , whereas Simplexa testing of extracts missed many samples with an LDT C_T value of >33 . No samples tested as invalid using Simplexa, and no samples were Simplexa positive and LDT negative. Of 112 influenza A-positive samples that were subtyped, 53 (47.3%) were the pandemic H1 strain, and 59 (52.7%) were the seasonal H3 strain; thus, both subtypes were equally represented. Simplexa direct detected only 162 (91.5%) of 177 samples deemed positive by Simplexa using extracts ($P = 0.0003$). Testing 2 μl of unextracted sample versus 5 μl of 4-fold-concentrated extract resulted in a 10-fold-higher nucleic acid input when extracts were tested. Thus, paired mean C_T values were ≥ 4 cycles lower for extracts than for unextracted samples, corresponding to $>1 \log_{10}$ difference by real-time PCR (data not shown).

Twenty-two extracts tested negative with Simplexa but positive with the LDT (4 RSV, 13 influenza A, and 5 influenza B). Of the 13 influenza A samples missed by Simplexa, 10 were the pandemic H1 strain, 2 were the H3 strain, and 1 was untyped. The disproportionate number of H1-positive samples missed by Simplexa raises concern about its sensitivity for this subtype. However, we also noted that discrepant samples had very high C_T values (mean, 36.0; 95% confidence interval [CI], 35.0 to 37.0). Twenty-one of 22 discrepant samples (95.5%) were from adults, and chart reviews revealed that all but one of them were collected late in the illness when the patients presented with bacterial superinfections, dehydration, or decompensation of cardiopulmonary disease. Three were repeat samples collected over 1 month in a known-positive immunocompromised patient. Only one patient presented early after the onset of symptoms, and that patient received Tamiflu.

Fourteen discrepant samples with sufficient sample remaining were sent to Focus Diagnostics, extracted using MagNA Pure, and tested with the Simplexa assay. Clear amplification curves were observed for 11 of 14 samples but crossed the threshold to positive only if the preset threshold was lowered. The instrument threshold was originally set using fresh samples, and it was postulated that freezing might have led to a lower signal amplitude, but this theory was untested.

Several papers were recently published on Simplexa Flu A/B & RSV using one or more of three approaches, extracted nucleic acid (5–7) and/or direct testing of either 2 μl of sample in a 96-well disc (6, 8) or 50 μl of sample in an 8-well disc (7, 9). The 50-μl option is now available as an *in vitro* diagnostic (IVD) kit. Testing was performed on either fresh (7–9) or frozen (5, 6) samples. The comparator tests were conventional methods (7) or a variety of molecular tests (5, 6, 8, 9). Not surprisingly, the results varied. Two studies using fresh samples found Simplexa direct testing (2 and 50 μl) to be similar in sensitivity to their LDT PCRs (8, 9). The proportion of samples with low viral loads was not reported in those studies. One study had a number of false-positive Simplexa results, ultimately attributed to a malfunctioning integrated cyclor. These two studies reported similar invalid rates (2.3% to 2.8%), whereas studies using frozen direct or extracted samples generally did not report invalid results.

While one study using frozen samples found Simplexa performance to be similar to that of commercial PCR tests conducted in parallel, all the samples had high viral loads with C_T values of <27 (6). These researchers also found C_T values that were 6 cycles higher for direct testing than for extracted nucleic acid. A comparison of Simplexa and Verigene reported inferior Simplexa results, but mostly frozen extracts were used, the extraction method was not given, and the discrepant samples had high C_T values. The attempt by the authors to conduct prospective testing of fresh samples yielded only 3 samples that were positive for influenza virus. Instructions for the Simplexa kit now specify the use of only Focus-qualified lots of MagNA Pure and dilution of EasyMag silica beads for optimal sensitivity. These recommendations may not have been followed in the published studies.

In our study, the retesting of discrepant samples at Focus Diagnostics raised concern that using frozen samples may bias against Simplexa. In contrast, with our LDT assays, we find that C_T values are usually very reproducible after freezing. Nevertheless, it is possible that the need of clinical laboratories to archive frozen samples during peak influenza seasons and to conduct comparative studies when the clinical workload decreases introduces bias even for molecular tests. Also, 45 (22.6%) of 199 LDT positive samples in our study had C_T values of >30 (low viral loads), thus accentuating test sensitivity differences.

In summary, Simplexa was more sensitive than cytospin-DFA for unextracted and for extracted samples. Since cytospin-DFA in our laboratory detects >90% of conventional culture-positive samples, we also expect Simplexa to be more sensitive than culture, but this was not tested. However, Simplexa is not an on-demand test like DFA, and tests are done in batches, albeit multiple times a day if needed. Although Simplexa was less sensitive than our current LDT assays, it was simpler, required minimal hands-on time, included an internal control, and had a shorter assay time. For nonmolecular laboratories transitioning from traditional methods, Simplexa provides the option of direct testing and uses an instrument with a small footprint that can be obtained on reagent rental, circumventing the requirement for expensive equipment purchases. Samples missed by Simplexa using extracted samples had very low viral loads. More than 95% of the discrepant results were from adults and, with one exception, were tested late in the course of the illness, when patients presented with secondary complications. Thus, it appears the clinical impact of missing these low-viral-load samples might be minimal. Our study is the first to provide a direct comparison of Simplexa to

cytospin-DFA and the highly sensitive CDC real-time PCR and to provide clinical correlation for discrepant samples. A significant portion of the samples tested in our study had low viral loads, accurately reflecting our tested patients but accentuating sensitivity differences between the methods. In clinical settings where samples from young children or from adults presenting early in illness are tested, a higher detection rate with Simplexa is anticipated.

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