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Comparative analysis of Four sample-to-answer influenza A/B and RSV nucleic acid amplification assays using adult respiratory specimens

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ABSTRACT

Background: The use of Sample-to-answer (STA) platforms for the detection of influenza A/B and respiratory syncytial virus (RSV) have greatly improved patient care. These diagnostic assays based on nucleic acid amplification are rapid, accurate and relatively easy to perform.
Objectives: We compared four such platforms for detecting FluA, FluB, and RSV from adult respiratory specimens: Hologic Panther Fusion® Flu A/B/RSV (Fusion), Cobas® Influenza A/B & RSV (Liat), Luminex Aries® Flu A/B & RSV (Aries), and Diasorin SimplexaTM Flu A/B & RSV (Simplexa).
Study Design: Nasopharyngeal (NP) swabs (n = 224) from adults were tested on these platforms and results were compared to Center for Disease Control and Prevention recommended real-time RT-PCR assay for influenza A/B and RSV. Subtyping for FluA and FluB was performed for discrepant analysis where applicable.
Results: Of the 82 FluA, 26 FluB, 15 RSV-positive specimens tested, the positive and negative percentage agreements (PPA and NPA respectively) for FluA detection were 100/100 (Fusion), 95.1/100 (Liat), 92.5/100 (Aries), and 84.1/99.3 (Simplexa); PPA and NPA for FluB detection were 92.3/99.5 (Fusion), 96/99.5 (Liat), 100/99.5 (Aries), and 80.8/100 (Simplexa); and for RSV detection were 100/100 (Fusion), 100/100 (Liat), 88.6/99.5 (Aries), and 73.3/100 (Simplexa). 82 confirmed FluA included 23 pH1N1 and 57 H3N2 strains with 2 strains remaining untyped. Of the 26 confirmed FluB, 25 were of the Yamagata lineage and 1 of unknown lineage.
Conclusion: Only 2 STA platforms demonstrated > 95% PPA for the detection of all three targets while all the 4 platforms demonstrated > 95% NPA for FluA, FluB and RSV.

1. Background

Viral respiratory tract infections cause significant morbidity and mortality in adult patients with underlying cardio-pulmonary disorders, immunocompromised and the elderly [1–3]. Influenza infections reportedly increase the risk of death and hospital admission in older individuals (> 65 years) and in pregnant women [4]. RSV is an important contributor for respiratory illness in adults with comorbidities [3,5] and has also been reported in healthy young adults with illnesses more severe than the average 'cold'. [6]. Rapid and accurate diagnosis of FluA/B and RSV is important for reducing the need for hospitalization, preventing transmission, reducing unnecessary antibiotic usage and initiation of antiviral therapy in patients. [7,8].

The laboratory methods to detect influenza viruses and RSV include culture, serology, direct immunofluorescence assay (DFA), and rapid antigen detection tests (RADT) by immuno-chromatographic methods to the more recent nucleic acid amplification tests (NAAT). Multiple factors such as the type and quality of specimens, time to result availability, level of technical expertise required and variable test performance characteristics influence the utility of the diagnostic methods. RADTs are the most common diagnostic methods used in outpatient settings for Flu A/B and RSV detection mainly because of their rapid TAT [8,9]. However, multiple studies have reported poor sensitivity of RADTs which led to the development and implementation of NAATs for Flu and RSV diagnosis [9,10]. Real-Time PCR assays to detect viral RNA have shorter TAT, superior accuracy, and offer multiplex detection [10]. The Sample to Answer (STA) NAAT systems are automated platforms that are easier to operate with faster TAT. At present, Food and Drug Administration (FDA) approved STA FluA/B and RSV molecular assays are available either as high or moderate complexity assays as in

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Simplexa (Diasorin Molecular, Cypress, CA) [11], Aries (Luminex Corporation, Austin, TX) [12,13], RP (Biofire Diagnostics, Salt Lake City, UT) [14] and Panther Fusion (Hologic, San Diego, CA) [15,16] or as Clinical Laboratory Improvement Amendments (CLIA)-waived point of care (POC) assays as in Alere (Abbott Rapid Diagnostics, IL) [17,18], Liat (Roche Diagnostics, Indianapolis, IN) [19,20], and Xpert (Cepheid, Sunnyvale, CA) [18,21].

2. Objectives

Our aim was to compare the performance of four STA FluA/B and RSV molecular assays (Fusion, Aries, Liat and Simplexa) to the CDC Influenza rRT-PCR and RSV rRT-PCR assays [16,22,23] for the detection of Flu A/B and RSV from adult respiratory specimens. We selected CDC FluA/B rRT-PCR assays as the reference method based on the evidence of its enhanced performance [24–26] and subtyping of Flu A/B viruses for discrepant analysis.

3. Study Design

3.1. Samples

224 retrospective NP specimens collected from subjects between 18 years to 94 years (median age = 47 years, Inter Quartile Range 35–59) from February to April of 2018 were used for the study. We enrolled salvage specimens on a convenient sampling basis, from adult patients (≥18 years) who were tested for Flu A/B and RSV as standard of care (SOC) procedure at Truman Medical Center, Kansas City. Specimens with a valid SOC test result and adequate volume (minimum 1.2 ml) were selected for the study. Frozen specimens in 3 ml universal transport medium (Becton, Dickinson and Company, NJ) were thawed by a de-identifier at Truman Medical Center and aliquoted by study personnel at Children's Mercy Hospital for testing. The aliquots were distributed to the designated operators who were blinded to the historical data. Fusion, Aries, Liat, and Simplexa testing was performed following manufacturer instructions. This study was reviewed and approved by the institutional review board both at Children's Mercy Hospital and Truman Medical Center.

3.2. STA testing

200 µl of the samples were dispensed in the cassette/cartridge for Aries and Liat, and 500 µl in the transfer tube for Fusion testing before loading onto the instruments. 50 µl each of FluA/B/RSV specific reaction mix and sample was dispensed into the wells of the Direct Assay Disc before loading onto the 3 M integrated cycler for Simplexa testing. Results were obtained at various time points on each platform – 20 min for Liat, 75 min for Simplexa, 2 h for Aries, and 2.4 h for Fusion. Samples with an invalid or error result were excluded from the final analysis.

3.3. Reference testing

Nucleic acid was extracted on the NucliSENS easyMAG extraction system using 200 µl of clinical specimen plus 10 µl of MS2 plasmid (internal control). Aliquots of the extracted material were prepared for specific sets of PCRs on an ABI7500 system– (i) control and RSV PCRs [23], (ii) FluA/B screening PCRs, and (iii) FluA and B subtyping PCRs [22,23]. Samples positive by reference PCR were subtyped using influenza A, H1, H3, pandemic InfA and pandemic H1 primers and probes, and influenza B positive specimens were subtyped as Yamagata and Victoria lineage (https://www.cdc.gov/flu/professionals/diagnosis/table-nucleic-acid-detection.html) [16]. The FluA/B subtyping results were used for discrepant analysis of FluA and FluB. RSV subtyping was not performed. Results from all platforms were compared with CDC rRT-PCR FluA/B and RSV assay for calculating percent agreements.

3.4. Data analysis

The diagnostic yield among different assays was reported in terms of: PPA, NPA and overall percent agreements, calculated by 2 × 2 table analysis. Ct values for FluA, FluB, and RSV was represented by Whisker box plot analysis with median and IQR. A two-tailed *t* test was performed to determine the significance (*P* values) between Ct values from rRT-PCR and other platforms for FluA, FluB and RSV.

4. Results

We performed our final analyses of the 224 samples based on the CDC rRT-PCR FluA/B and RSV assay results which confirmed 82 FluA, 26 FluB, 15 RSV-positives, and 91 negative samples.

4.1. Invalids

A sample was deemed invalid when there was an error or failure to generate a result. Aries demonstrated highest number of invalids (n = 5) followed by Liat and Simplexa (n = 2 on each). Repeat-testing was not performed on the invalid samples due to insufficient volume and they were excluded on the respective platforms from the final data set. Analysis was performed on 224 samples for Fusion, 222 samples for both Liat and Simplexa and 219 samples on Aries (Table 1). All invalid samples had valid results by rRT-PCR method.

4.2. Influenza

Based on the rRT-PCR assay results, 108 influenza-positive specimens (82 FluA and 26 FluB) were tested on the 4 STA platforms. Subtyping of FluA positive specimens with H1N1- and H3N2-specific primers demonstrated 23 pH1N1 and 57 H3N2 strains, while 2 FluA-positive strains remained un-typed after repeated testing. Of the 26 FluB specimens, 25 were subtyped as Yamagata lineage; one FluB specimen's lineage remained undetermined.

4.3. FluA

Fusion detected maximum number of FluA specimens with 82 positives followed by Liat and Aries with 78 and 74 positive samples respectively. Simplexa reported the lowest FluA positives with 69 samples. The PPA, NPA and overall percent agreement between the respective assays and rRT-PCR for FluA detection are shown in Table 1. 3 FluA samples were negative on 3 platforms (Aries, Liat, Simplexa) but positive by rRT-PCR and discrepant analysis for these samples identified 2 as H3N2 and 1 as NT (Table 1). 3 different FluA samples were negative on 2 platforms (Liat and Simplexa, n = 1; Simplexa and Aries, n = 2) but positive by rRT-PCR. Discrepant analysis confirmed 2 of those specimens to be H3N2 and one as pH1N1 strain. 7 samples failed detection on Simplexa alone (6 H3N2 and 1 N T strains). NPA was 100% across all platforms except Simplexa (99.3%) which had 1 additional positive result (Table 1).

4.4. FluB

PPA, NPA and overall percent agreements between the different STA platforms and RT-PCR for detecting FluB are listed in Table 1. Of the 26 FluB specimens, Fusion, Liat, Aries, were all positive for 24 samples whereas Simplexa, was positive for 21 samples. (Table 1). 3 FluB specimens were undetected by Simplexa alone; one FluB specimen tested negative on 2 assays (Panther and Simplexa) and one on 4 assays (Panther, Simplexa, Liat, and Aries). All the discrepant samples were of Yamagata lineage, except the single untypeable strain which was negative across all platforms.

Table 1
Performance of STA platforms for detection of FluA, FluB and RSV.

	Categories*	Fusion (n = 224)	Aries (n = 219)	Liat (n = 222)	Simplexa (n = 222)
FluA	A	82	74	78	69
	B	142	139	140	139
	C	0	0	0	1
	D	0	6	4	13
	PPA (95% CI)	100% (94.4 – 100)	92.5% (83.8 – 96.9)	95.1.0% (87.3 – 98.4)	84.1% (70.4 – 90.9)
	NPA (95% CI)	100% (96.7 – 100)	100% (96.7 – 100)	100% (96.8 – 100)	99.3% (95.9 – 99.9)
	Overall agreement	100%	97.3%	98.2%	93.7%
	Discrepant Subtype	NA	H3N2 (4), pH1N1 (1), NT (1)	pH1N1 (1), H3N2 (2), NT (1)	H3N2 (10), pH1N1 (1), NT (2)
FluB	A	24	24	24	21
	B	197	193	196	196
	C	1	1	1	0
	D	2	1	1	5
	PPA (95% CI)	92.3% (73.4 – 98.6)	96% (77.7 – 97.7)	96% (77.7 – 99.7)	80.8% (60.0 – 92.7)
	NPA (95% CI)	99.5% (96.8 – 99.9)	99.5% (96.4 – 99.9)	99.5% (96.4 – 99.9)	100% (97.6 – 100)
	Overall agreement	98.7%	99.2%	99.1%	97.7%
	Discrepant Subtype	Yamagata (2)	Yamagata	Yamagata	Yamagata (5)
RSV	A	15	13	15	11
	B	209	203	207	207
	C	0	1	0	0
	D	0	2	0	4
	PPA (95% CI)	100% (74.6 – 100)	88.6% (58.3 – 97.6)	100% (74.7 – 100)	73.3% (44.8 – 91.0)
	NPA (95% CI)	100% (97.7 – 100)	99.5% (96.9 – 99.9)	100% (97.7 – 100)	100% (96.3 – 99.9)
	Overall agreement	100%	98.6%	100%	98.2%

NT- Non typeable. RSV subtyping was not performed.
* A = CDC and STA PCR positive; B = CDC and STA PCR negative; C = STA positive only; D = STA negative only.

4.5. RSV

Of the 15 RSV positive specimens, Fusion and Liat detected 15 positives each, whereas Aries detected 13 RSV positives. Simplexa demonstrated the lowest PPA (Table 1) with 11 positive results. PPA and NPA were highest in Fusion followed by Liat (Table 1). 2 RSV positive samples by rRT-PCR were negative on 2 platforms (Aries and Simplexa) while 2 additional RSV-positive specimens remained undetected on Simplexa alone.

4.6. Cycle threshold (Ct)

The median and IQR of Ct values for FluA, FluB, and RSV detections on Fusion, Aries, Simplexa and CDC rRT-PCR are depicted as box-plots in Fig. 1. Liat does not report Ct values. The mean Ct ± SD for CDC rRT-PCR assays was 28.1 ± 6.13 for FluA, 27.5 ± 6.1 for FluB, and 28.1 ± 6.5 for RSV. Fusion demonstrated the lowest mean Ct ± SD (27.7 ± 5.48 for FluA, 27.8 ± 4.94 for FluB and 26.8 ± 6.88 for RSV), with significantly lower Ct compared to other assays (Fig. 1). The highest mean Ct ± SD for FluB and RSV was on Aries (FluB: 33.03 ± 4.9, RSV: 29.4 ± 4.97), while Ct ± SD for FluA was comparable on both Aries (30.93 ± 5.42) and Simplexa (30.5 ± 5.27).

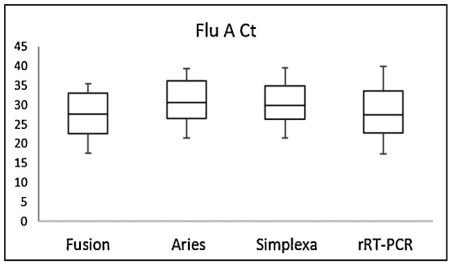
Analysis of discrepant samples revealed that mean Ct values for samples undetected on the different platforms for FluA, FluB and RSV were 36.5, 36 and 37.6 respectively. The high Ct values could be attributed to lower viral load in the specimens thereby accounting for the lack of detection.

5. Discussion

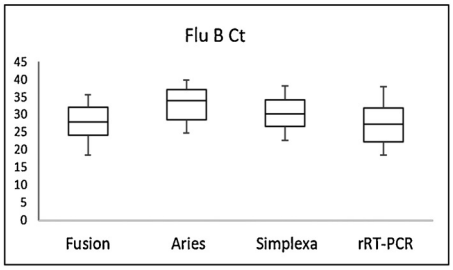
Our study compares 4 STA assays for FluA/B and RSV detection in adult population. Previous studies have reported the superior diagnostic performance of Aries, Liat, Fusion and Simplexa for the rapid diagnosis of respiratory pathogens in both children and in adults [11–13,15,27,28]. In an earlier retrospective study, Aries reported 96–100% sensitivity and 99.3–100% specificity for Flu A/B and RSV detection [27]. In a prospective study, a 96.6% PPA for FluA, 100% PPA for FluB and RSV and 98.9% negative percent agreement (NPA) was reported [13]. A multicenter clinical trial of Aries system reported final sensitivities of 98.8%, 98.0%, and 97.7% for FluA, FluB and RSV respectively, and specificities for all targets ranged from 98.6% to 99.8% [12]. In a recent pediatric retrospective study, Aries demonstrated sensitivities of 98.6%, 93.7% and 94.4% for FluA, FluB and RSV respectively, with specificities of > 99% for all three targets [16]. We now report > 99% NPA for the same targets and < 95% PPA for FluA and RSV with Aries using adult specimens. Cobas Liat Flu A/B and RSV assays have earlier demonstrated superior accuracy with sensitivity and specificity ranging between 96–100% [16,19,28]. Other than a lower PPA for FluA (92.5%), findings from our study show comparable PPA for FluB and RSV with > 99% NPA for all three targets. Simplexa Direct assay has shown variable sensitivities ranging from 90.0% to 98.6% for FluA, 85.4% to 100%, for FluB and 87% to 99.3% for RSV; specificities range from 97.9% to 100% for FluA, 99.4% to 100% for FluB and 97.9% to 99% for RSV in previous studies [11,16]. In our study, Simplexa demonstrated low (< 85%) PPA for all three targets but > 99% NPA. Overall the assays in our study panel demonstrated > 90% overall percent agreements for all analytes.

We observed significant numbers of overall negative results compared to those from RT-PCR but more so with FluA in our study (13 samples failed to get detected 23 times). Typing results confirm 10/13 samples to be H3N2, 1 as pH1N1 and 2 as non-typeable. All samples were obtained in the 2017–2018 influenza season which was a high severity season across all age groups, predominated by H3N2 circulating strains [29]. H3N2 strains are known to mutate and have high antigenic variability similar to H1N1 (pdm09) strains thereby affecting performance of commercial FluA assays [30,31]. Antigenic variability is rare but occasionally demonstrated in both influenza B and RSV, leading to a probe-target mismatch which impact the assay performance of diagnostic tests resulting in false-negatives [32–37].

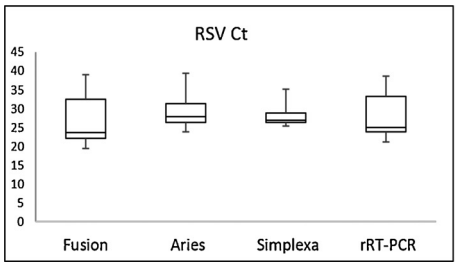
The design (primers and probes, amplification protocol) or assay parameters of the different *t*-tests (volume of specimens, extraction protocol, variations in platform setup, assay cut-off for target detection and turn-around time) may explain differences in assay performance. In case of Simplexa, the input volume is 50 µl compared to 500 µl for Panther Fusion and 200 µl for Liat and Aries which could potentially explain the comparatively lower sensitivity of Simplexa assay. Additionally, Simplexa does not have a complete extraction process which could also compound to the issue of varied performance.



Flu A Assay	N	Median	IQR	*P* values
Fusion	82	27.4	22.2 – 33.1	0.5032
Aries	74	30.7	26.5 – 36.3	**0.0032**
Simplexa	69	29.9	26.3 – 34.9	**0.0128**
CDC rRT-PCR	82	27.5	22.8 – 33.6	



Flu B Assay	N	Median	IQR	*P* values
Fusion	24	27.9	24.3 – 32	0.8183
Aries	24	30.65	28 – 37.3	**0.0008**
Simplexa	21	29.9	26.7 – 34.2	0.0948
CDC rRT-PCR	26	27.5	22.2 – 31.9	



RSV Assay	N	Median	IQR	*P* values
Fusion	15	23.7	19.5 – 32.6	0.6277
Aries	13	27.8	26.4 – 31.4	0.5283
Simplexa	11	27	26.5 – 28.8	0.9914
CDC rRT-PCR	15	25.4	23.8 – 33.3	

Fig. 1. Box plot diagrams and tables depicting the Ct values for FluA (top), FluB (middle), and RSV (bottom) detection on STA platforms and the reference method, rRT-PCR. Significant *P* values comparing the three assays (Fusion, Aries and Simplexa) with CDC rRT-PCR are also reported in bold.

The quality of specimen characterized by the viral load and time of collection from onset of illness also influence the sensitivity of an assay. Respiratory specimens should ideally be collected in less than 4 days after illness onset when influenza viral shedding is highest. Viral shedding generally declines after 4 days in immunocompetent patients, although infants and young children may harbor detectable influenza viruses for longer periods. Studies have reported that duration of sH1N1 shedding was longer in children than in adults [38–40]. In a meta-analysis of rapid influenza antigen detection assays, pooled sensitivities of RADTs have been shown to have better performance in children compared with adults (approximately 13% higher), which may be attributed to higher viral loads and longer viral shedding in children compared with adults [8]. Other studies have also reported lower sensitivities for both Flu and RSV detection in adults with RADTs [10,41–43].

Moreover, diagnosing and distinguishing influenza or RSV infections from other respiratory infections on the basis of signs and symptoms alone are difficult, especially in adults. In separate studies, the positive predictive value (PPV) of typical influenza symptoms such as fever and cough in adults > 60 years is reported to range within 30% to 53% as compared to 64% (in children < 5 years) and 83% (children aged between 5–12 years) [43,44]. Thus, molecular diagnostics by PCR testing provides more accurate and timely diagnosis of Flu and RSV infections, and is especially preferred choice in adults due to atypical clinical findings in the elderly and lack of sensitive diagnostic assays. Rapid PCR detection have reportedly resulted in the early initiation of antiviral therapy in elderly population with influenza and RSV, in turn leading to reduced risk of extra medical visits, hospitalization and death [45,46].

Our study is an effort to evaluate and compare the performance of some of the popular STA platforms for FluA/B and RSV detection with few limitations, one of which is the relatively small number of RSV and Flu B positive specimens used in our study. Additionally, we did not subtype the RSV positive specimens, thereby failing to incorporate a conclusive discrepant analysis for this group. We used aliquots of frozen samples for the study and the freeze-thaw cycle, although uniform across all the platforms, could have affected the assay performance.

Despite the limitations, data from our study provide important information on the performance features of these assays for detection of FluA/B and RSV in respiratory specimens collected from adults.

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Hologic Inc. CA, USA

CRediT authorship contribution statement

Dithi Banerjee: Conceptualization, Project administration, Methodology, Writing - original draft. **Neena Kanwar:** Methodology. **Ferdaus Hassan:** Methodology. **Kamani Lankachandra:** Writing - review & editing. **Rangaraj Selvarangan:** Conceptualization, Funding acquisition, Writing - review & editing.

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