



Performance evaluation of a rapid molecular diagnostic, MultiCode based, sample-to-answer assay for the simultaneous detection of Influenza A, B and respiratory syncytial viruses

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ABSTRACT

Background: Clinical signs and symptoms of different airway pathogens are generally indistinguishable, making laboratory tests essential for clinical decisions regarding isolation and antiviral therapy. Immunochromatographic tests (ICT) and direct immunofluorescence assays (DFA) have lower sensitivities and specificities than molecular assays, but have the advantage of quick turnaround times and ease-of-use.

Objective: To evaluate the performance of a rapid molecular assay, ARIES FluA/B & RSV, using laboratory developed RT-PCR assays (LDA), ICT (BinaxNOW) and DFA.

Methods: Analytical and clinical performance were evaluated in a retrospective study arm (stored respiratory samples obtained between 2006–2015) and a prospective study arm (unselected fresh clinical samples obtained between December 2015 and March 2016 tested in parallel with LDAs).

Results: Genotype inclusivity and analytical specificity was 100%. However, ARIES was 0.5 log, 1–2 logs and 2.5 logs less sensitive for fluA, RSV and fluB respectively, compared to LDA. In total, 447 clinical samples were included, of which 15.4% tested positive for fluA, 9.2% for fluB and 26.0% for RSV, in both LDA and ARIES. ARIES clinical sensitivity compared to LDA was 98.6% (fluA), 93.3% (fluB) and 95.1% (RSV). Clinical specificity was 100% for all targets. ARIES detected 10.6% (4 fluA, 8 fluB, 11 RSV) and 26.9% (7 fluA, 3 fluB, 22 RSV) more samples compared to DFA and ICT, all confirmed by LDA.

Conclusion: Although analytically ARIES is less sensitive than LDA, the clinical performance of the assay in our tertiary care setting was comparable, and significantly better than that of the established rapid assays.

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1. Background

Rapid and accurate detection of influenza A (fluA) and B (fluB) viruses and respiratory syncytial virus (RSV) is important for clinical patient management and infection control purposes. Rapid laboratory diagnostics may result in less antibiotic prescription and more frequent use of antivirals [1–3]. Molecular assays are considered to be the standard for the detection of respiratory viruses, but may be relatively time consuming and need qualified molecular laboratory personnel to execute and for result interpretation. Currently available rapid tests, like direct immunofluorescence

assays (DFA) or immunochromatographic tests (ICT) have quick turnaround times, but are less sensitive and less specific compared to molecular assays.

2. Objective

The aim of the study was to evaluate the performance of the ARIES FluA/B and RSV assay by comparing it to routine molecular laboratory developed assays (LDA), rapid DFA and rapid ICT.

3. Study design

3.1. ARIES influenza A, B and RSV assay

The ARIES system (Luminex) is a FDA and CE/IVD marked molecular diagnostic, sample-to-answer system, based on a

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probe-less RT-PCR using isoguanine and 5-methylisocytosine basepairing (MultiCode) and melt curve analysis for detection [4,5]. 200 µl respiratory sample is pipetted directly into an ARIES cassette, and placed in one of the two independent modules, which can run 1–6 cassettes each, with a sample-to-result time of 2 h (including a hands-on-time of about 5–10 min per 12 samples). The ARIES system runs a nucleic acid extraction, multiplex MultiCode RT-PCR and melting curve analysis, detecting fluA, fluB, RSV and a specimen process control (SPC) in one cassette. Results were generated using assay protocol version A (Luminex).

3.2. Analytical performance evaluation

Genotype inclusivity was assessed using a reference panel, including 16 avian (H1–H16) and 33 human fluA strains (H1N1, H1N1p2009, H3N2, H5N1, H7N7, H2N2), reflecting viruses circulating between 1968 and 2014, 3 fluB strains (2 Yamagata and 1 Victoria) and 2 RSV (A/B) strains. Avian and highly pathogenic fluA strains were inactivated using MagNaPureLC lysisbuffer (200 µl sample + 300 µl lysisbuffer) (Roche, Almere, the Netherlands) before running in the ARIES. Analytical specificity was assessed using high titred virusstocks (median Ct-value 17, range ~11–26) including rhinovirus, enterovirus, parecho 1–3, HMPV, parainfluenza 1–4, HCoV-229e, –OC43, NL63, adenovirus, bocavirus, rubella, measles, mumps, rotavirus, sapovirus, astrovirus, hepatitis B, E and D viruses, Noro-1 and 2, HSV1 and 2, CMV, EBV, VZV, HHV6, HHV7, HHV8, B19, HPV, rhCMV, JC and BK viruses and *Mycoplasma pneumoniae*.

Analytical sensitivity and linearity were determined using half log dilution series of cell culture isolate fluA/H1N1/Netherlands/202/95, fluB/Yamagata/Netherlands/022/95, RSV A and RSV B, until negative. Repeatability was assessed using replicates of a process control, positive for fluA, fluB and RSV. Results were compared to an ISO15189:2012 validated laboratory developed automated real-time RT-PCR assays using Aurora FLOW (Roche, Almere, the Netherlands) (LDA) (Supplementary materials).

3.3. Clinical performance evaluation

Clinical performance was evaluated both retrospectively, for inclusion of known positive fluA/B/RSV samples, and prospectively for inclusion of fresh respiratory tracts samples and head-to-head comparison with the routinely used LDA.

For retrospective evaluation stored (–80 °C) pre-treated respiratory tract samples from January 2006–July 2015 were selected; from each year 4 positive samples per species (fluA, fluB, RSV A and RSV B) to include genotypic variances and different sample types. Prospective evaluation was performed on unselected fresh respiratory tract samples from patients presented to our hospital between December 2015–March 2016.

For retrospective evaluation, ARIES results were compared to historical LDA, DFA and ICT results retrieved from the LIMS database. The historical LDA results were obtained using primers and probes identical to those described above; sample pre-treatment, isolation and real-time PCR however, were performed as previously described [6] (Supplementary methods).

Both retrospective and prospective discrepant samples were confirmed by repeating both ARIES and LDA by using Aurora FLOW, as described above. Results after discrepancy testing were used for further analysis.

DFA was performed as previously described [7] and ICT was performed using BinaxNOW® Influenza A and B and BinaxNOW® RSV (Alere health, Tilburg The Netherlands) as described by the manufacturer.

This study was approved by the Medical Ethical Committee of the Erasmus MC (MEC-2015-475).

3.4. Statistical analysis

Statistical analysis was performed using IBM SPSS v21 (Table 2) and Deming regression and Mann-Whitney test for non-parametric non paired data was performed using GraphPad Prism v5.00 for Windows. 95% Confidence intervals were calculated using the Wilcoxon method, Epitools (<http://epitools.ausvet.com.au>).

4. Results

4.1. Analytical performance of ARIES

To determine whether external lysis could be used in the ARIES system, 6 human influenza A virus strains were tested with and without external lysis, resulting in comparable Ct-values (median Δ Ct = 1.1, range 0.6–2.2), taking dilution factor into account. All 54 avian and human fluA, fluB, RSV A and RSV B strains tested for genotype inclusivity were detected by the ARIES. No false positive reactions with non-fluA/fluB/RSV virusstocks were detected.

Analytical sensitivity testing showed comparable results to LDA (≤ 0.5 log) for fluA, but 1 log, 2 logs and 2.5 logs less sensitive for RSV A, RSV B and fluB, respectively. To determine the repeatability, %CV was calculated of 37 replicates of a positive process control (PPC) containing each fluA, fluB and RSV A/B viruses, resulting in a %CV of 2.7–3.6%. The concentration of fluB (LDA Ct-value 26.4) in the PPC was close to the ARIES limit of detection and tested positive 32 out of 37 (86.5%), fluA (LDA Ct-value 27.5) and RSV (LDA Ct-value RSV A 25.1, RSV B 26.4) tested positive in all replicates. T- and F-test showed no significant difference (<0.05) of the data retrieved in three subsequent months, implying PPC can be tested only once a month.

4.2. Clinical performance of ARIES

In total 447 clinical respiratory tract samples were included, of which 162 belonged to the retrospective study arm, specifically 133 nasal washings, 14 throat swabs, 6 bronchial alveolar lavages (BAL), 9 sputa and 1 nose swab, of which 23.5% were positive for fluA (9.9% H1N1p09, 1.2% H1N1, 11.7% H3N2 and 0.6% H1N1 + H3N2) 19.8% for fluB (8.6% Victoria, 10.5% Yamagata, 1 sample (0.6%) non-typeable) and 50.6% for RSV (26.5% RSV A and 24.1% RSV B). Additionally, 285 prospective samples were included (55 nasal washings), 170 throat swabs, 41 BAL, 15 sputa, 2 nose swabs, 1 mouth swab and 1 pleural fluid of which 10.8% was positive for fluA (all H1N1p09 strains), 3.5% for fluB (all Victoria strains) and 11.9% for RSV (5.6% RSV A and 6.3% RSV B).

The 447 clinical samples were collected from 406 patients (Table 1). In total for both study arms 15.4%, 9.2% and 26% of the samples were positive for fluA, fluB and RSV (13.2% RSV A and 12.8% RSV B), respectively for both LDA and ARIES. Confirmed discrepant results were found in 10 samples: 1 fluA (LDA Ct-value 36.0, H1N1p09 strain), 3 fluB (LDA Ct-values 32.5–33.9, all Victoria strains) and 6 RSV A (LDA Ct-values 29.3–34.5). In 6 (all nasal washings) of these 10 discrepant samples two viruses were detected using LDA (Table 1), having a common result in ARIES: the viral RNA with the highest concentration (LDA Ct-values 15.8–23.7) was detected, the viral RNA with the lowest concentration (LDA Ct-values 31.5–34.5) was not detected. LDA was also not confirmed by DFA or ICT for these samples, although 1 nasal washing (RSV A LDA Ct-value 29.3) was propagated by virus culture. One positive LDA result (throat swab, fluA LDA Ct-value 36.0) was confirmed by a sample from the same patient, drawn earlier in the infection, which tested positive in both ARIES and LDA.

Table 1
Characteristics of clinical samples.

Characteristics		Total		LDA FluA+		LDA FluB+		LDA RSV+	
		n	% of all	n	% of all	n	% of all	n	% of all
Sample type	nose wash	189	42.3%	34	7.6%	27	6.0%	94	21.0%
	throat swab in UTM	183	40.9%	26	5.8%	11	2.5%	22	4.9%
	BAL	48	10.7%	3	0.7%	2	0.4%	3	0.7%
	sputum	22	4.9%	4	0.9%	4	0.9%	3	0.7%
	nose swab	3	0.7%	2	0.4%	1	0.2%	0	0.0%
	pleural fluid	1	0.2%	1	0.2%	0	0.0%	0	0.0%
	mouth swab	1	0.2%	0	0.0%	0	0.0%	0	0.0%
Sex	Male	242	54.1%	47	10.5%	24	5.4%	68	15.2%
	Female	205	45.9%	23	5.1%	21	4.7%	54	12.1%
Age	0–1 years	106	23.7%	11	2.5%	0	0.0%	77	17.2%
	1–5 years	46	10.3%	13	2.9%	9	2.0%	12	2.7%
	5–18 years	61	13.6%	14	3.1%	18	4.0%	7	1.6%
	18–65 years	175	39.1%	25	5.6%	16	3.6%	23	5.1%
	65+ years	59	13.2%	7	1.6%	2	0.4%	3	0.7%
Species_genotype	A_H1N1pdm09	46	10.3%	46	10.3%				
	A_H1seasonal	2	0.4%	2	0.4%				
	A_H3N2	18	4.0%	18	4.0%				
	A_H3N2 + H1N1seasonal	1	0.2%	1	0.2%				
	B_Victoria	22	4.9%			22	4.9%		
	B_Yamagata	17	3.8%			17	3.8%		
	B_unknown	1	0.2%			1	0.2%		
	RSV_A	59	13.2%					59	13.2%
	RSV_B	57	12.8%					57	12.8%
	RSV_A + B	1	0.2%					1	0.2%
	A_H1N1pdm09 + B_Victoria	2	0.4%	2	0.4%	2	0.4%		
	A_H3N2 + RSV_A	1	0.2%	1	0.2%			1	0.2%
	B_Victoria + RSV_A	2	0.4%			2	0.4%	2	0.4%
	B_Yamagata + RSV_A	1	0.2%			1	0.2%	1	0.2%
	negative	217	48.5%						

Clinical sensitivity and specificity were assessed using both retrospective and prospective samples with LDA as the golden standard and compared to DFA and ICT (Table 2, Fig. 1). This resulted in a superior clinical sensitivity for ARIES fluA, fluB and RSV compared to the other two rapid tests, DFA and ICT. Clinical specificity was quite equal for ARIES, DFA and ICT, ranging from 95.5% up to 100% in the present study. Six ARIES positive samples (3 fluA (2x BAL, 1 sputum), 1 fluB (nasal washing) and 2 RSV (throat swabs), ARIES Ct-values 38.1–40.1) could initially not be confirmed by LDA, but after sample pre-treatment for sputum, nasal washing and BALs (10x dilution) and subsequent retesting in both ARIES and LDA yielded equivalent negative results. To gain insight and to analyze the difference in Ct-value of the LDA positive samples, which were missed in the rapid tests (ARIES, ICT and DFA) Fig. 1 was plotted. Mann-Whitney test showed a statistical difference in LDA Ct-values of ARIES missed samples compared to ICT and DFA in all cases, meaning that both ICT and DFA are significantly less sensitive than ARIES. Compared to DFA (n = 217) and ICT (n = 117), ARIES detected

in 23 (10.6%; 4 fluA, 8 fluB, 11 RSV) and 33 (28.2%; 7 fluA, 4 fluB, 22 RSV) more samples viral RNA respectively, all confirmed by LDA (Ct-value range 16.5–31.6) (Fig. 1).

Fig. 2 shows the distribution of paired LDA and ARIES Ct-values, using LDA as golden standard. Positive LDA samples only were taken into account. Deming regression analysis of all targets shows a good correlation between the two methods, although ARIES had an average Ct-delay of 5.2 ± 1.6 for fluA, 10.8 ± 1.7 for fluB and 6.1 ± 1.9 for RSV, which was confirmed in the analytical performance evaluation (data not shown).

To assess robustness of the ARIES, a failed cassette analyses was performed. In total 669 cassettes were run, 180 pre-research use only (RUO) and 489 optimised RUO cassettes, of which 2.44% (pre-RUO) and 1.95% (RUO, FDA approved in June 2016) failed respectively. Of the 286 prospective fresh samples 10 cassettes failed: 3 nasal washings, 4x BAL and one sputum were viscous. Of these samples, 150 μ l was diluted in 900 μ l DMEM containing 40% FBS, vortexed and centrifuged. 200 μ l of the supernatant was

Table 2
Clinical sensitivity and specificity of influenza A, B and RSV for ARIES, ICT and DFA using LDA as golden standard.

Virus	Assay	N	Sensitivity		Specificity	
			% [95%CI]	N	% [95%CI]	N
Influenza A	ARIES	447	98.6 [92.3–99.8]	69/70	100.0* [99.0–100.0]	377/377
	DFA	217	88.2 [73.4–95.3]	30/34	99.5 [97.0–99.9]	182/183
	ICT (Binax)	116	74.1 [55.3–86.8]	20/27	95.5 [97.0–99.9]	85/89
Influenza B	ARIES	447	93.3 [82.1–97.7]	42/45	100.0* [99.1–100.0]	402/402
	DFA	216	63.0 [44.2–78.5]	17/27	100.0 [98.0–100.0]	189/189
	ICT (Binax)	116	37.5 [13.7–69.4]	3/8	99.1 [94.9–99.8]	107/108
RSV	ARIES	447	95.1 [89.7–97.7]	116/122	100.0* [98.8–100.0]	323/325
	DFA	217	84.9 [76.3–90.8]	79/93	100.0 [97.0–100.0]	124/124
	ICT (Binax)	117	70.2 [59.8–79.0]	59/84	100.0 [89.6–100.0]	33/33

* First round testing (before discrepancy analysis) showed a specificity of 99.2% for fluA, 99.4% for RSV and 99.8% for fluB.

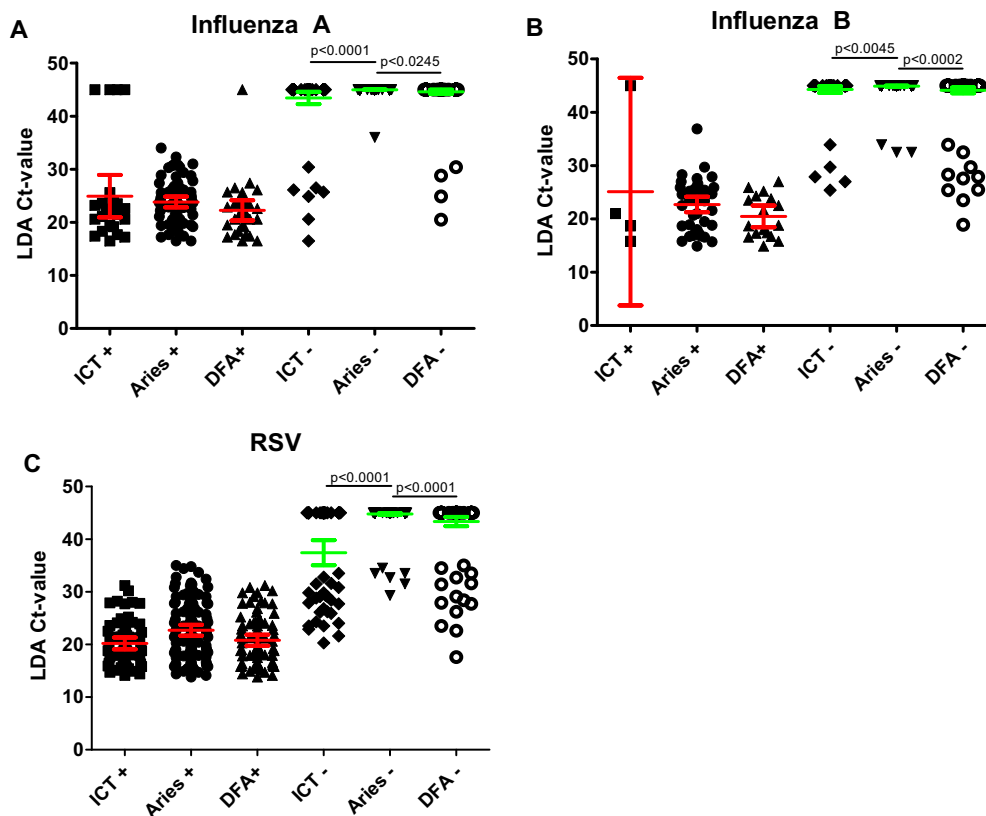


Fig. 1. Comparison of ARIES, ICT and DFA influenza A virus (A), influenza B virus (B) and RSV (C) positive and negative samples using LDA Ct-values as golden standard. LDA Ct-value ≥ 45 was considered negative. Mann-Whitney test for non-parametric non-paired data showed significant differences in LDA Ct-values for fluA, fluB and RSV between ARIES negative sample group and ICT or DFA negative samples, indicating a higher sensitivity of ARIES.

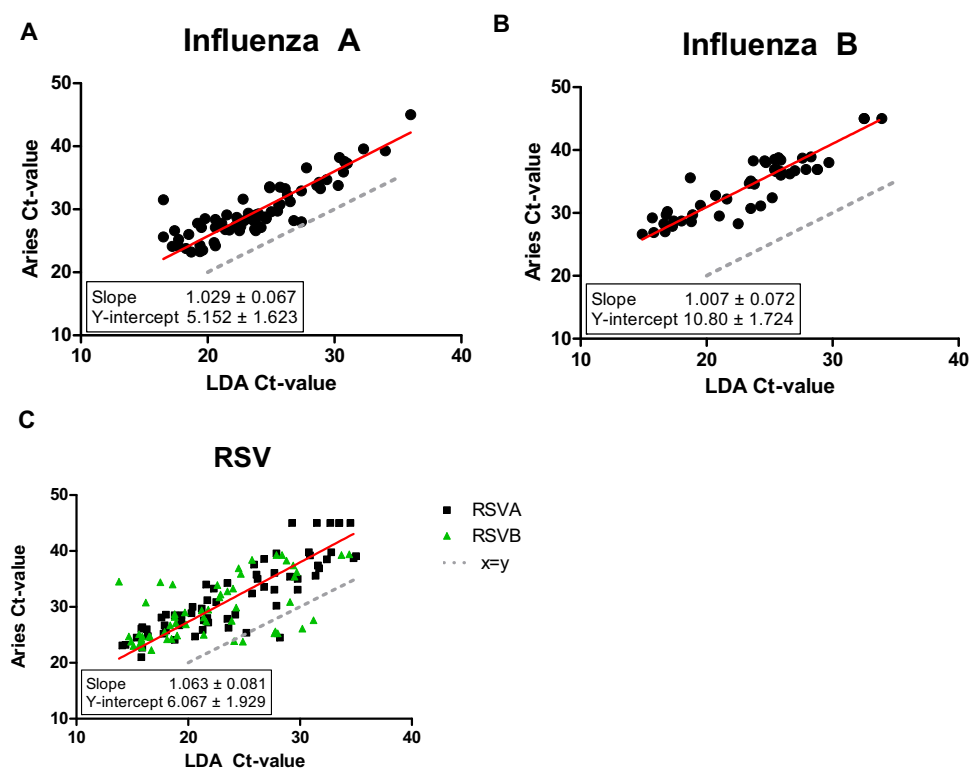


Fig. 2. Correlation plot of paired LDA and ARIES Ct-values of LDA positive fluA (A), fluB (B) or RSVA/B (C) samples. Deming regression (red line, in case of RSV: plotted for total RSV data) of the data showing a significant deviation from zero for all viruses, slope and Y-axis intercept is shown in the figure. X=Y line is plotted for reference (dotted grey line).

successfully retested in ARIES. Furthermore, one pre-treated nasal washing and one untreated throat swab failed.

5. Conclusions

In this study we evaluated the performance of the ARIES Flu A/B and RSV assay, a molecular diagnostic rapid test and compared it to LDA and two established, non-molecular rapid assays. The analytical performance, containing genotype inclusivity, specificity, linearity and repeatability showed comparable results to the LDA assays. The analytical sensitivity of fluA is also comparable to that of the LDA, though the fluB and RSV assays were up to 2.5 logs less sensitive.

However, though probe-less real-time PCRs are generally considered as less specific, the increased specificity due to the isoguanine/5-methylisocytosine chemistry used in ARIES results in a clinical sensitivity which exceeds that of ICT and DFA, and is comparable to that of the LDA in the present study. ARIES was not able to detect 10 samples (2.2%) containing high LDA Ct-values (LDA Ct-value 28.8–36.0). Though, these Ct-values are expected to be undetectable in the ARIES as interpolated from the detectable range measured in the analytical sensitivity experiment (data not shown). Whereas the number of samples, which could not be detected by DFA and ICT was much higher and contained significantly lower Ct-values (Fig. 1). 6 of the 10 samples missed by the ARIES system were double infections; in all 6 cases ARIES only detected the viruses with the lower LDA Ct-value (Table 1).

The ARIES fluA/B/RSV assay was officially FDA-approved for nasopharyngeal swabs. However, the most common respiratory tract sample types in our routine diagnostic setting are throat swabs in universal transport medium (adults) and nasal washings (children). Hence, these latter sample types and sputa and BALs were included in this study. Nasal washings, BAL and sputa were pre-treated as part of routine work-up for the retrospective study arm prior to testing on the ARIES system, resulting in these cases in successful runs. In the prospective study on the contrary, these sample types were tested untreated and 10 of 285 cassettes failed, probably due to viscosity of the samples as the internal specimen control was either negative or out of range. One may argue if viscosity/human cells of these fresh (untreated) samples may have been the cause for 6 samples (1.3%) of the prospective study arm to initially test (false) positive without LDA or DFA confirmation. These samples were either fluA (n = 3), fluB (n = 1) or RSV (n = 2) positive by ARIES only and had high ARIES Ct-values ranging from 38.1 to 40.1. On the other hand, 26 (11.5%) of the LDA confirmed ARIES (true) positive samples also had ARIES Ct-values of >38. If we compared these Ct-values to those of the LDA assays, the range varied between LDA Ct-value 25.7–34.8 for RSV, Ct-value 24.7–29.7 for fluB and Ct-value 30.4–32.3 for fluA. Therefore, high ARIES Ct-values should be considered as positive.

All LDA assays are routinely performed on pre-treated samples [6]. During this pre-treatment cells are removed from the fresh sample and the supernatant is diluted ~10 times. Since the ARIES analytical sensitivity for fluA is comparable to LDA, it is possible that the 3 ARIES+/LDA- fluA samples were missed by the LDA due to pre-treatment. This scenario is less likely for the 7 RSV or fluB, since the ARIES analytical sensitivity was 1–2.5 log less sensitive than LDA. Indeed, ARIES false positivity cannot be ruled out in these cases. All failed cassettes and “false-positive” non-throat swab samples were pre-treated and repeated in both ARIES and LDA. No failing cassettes or false positivity was observed hereafter. Throat swabs were repeated untreated in both assays and positivity could not be confirmed. Therefore, we recommend to dilute viscous nasal washings, sputa and BAL sample types prior to running on the ARIES system, so failure of cassettes by inhibition as well as possible false

positivity can be avoided, which may positively affect the specificity of the ARIES.

The BinaxNow influenza and RSV (ICT) assays are commonly used and many clinical studies have addressed sensitivity and specificity before, showing a lot of variation. [8–20] The published sensitivity varied between a modest 59%–83% for influenza A virus, 72%–90% for RSV and a poor sensitivity for influenza B virus (33%–69%). The ICT sensitivity observed in our study confirms these findings. Others suggest to confirm ICT or DFA rapid tests with techniques like RT-PCR or virus culture to conduct proper results [8,10,11,14,15,20], due to low sensitivities. We suggest that confirmatory testing for fluA, fluB or RSV may no longer be needed when using ARIES, because of the superior sensitivity to antigen rapid tests. Testing for other viral or bacterial respiratory pathogens may be of higher clinical importance.

In terms of ease-of-use, is ARIES equal to systems like GeneXpert (Cepheid) or other molecular rapid test platforms, however most platforms have runtimes of < 1 h, where ARIES' runtimes are up to 2 h. Nevertheless, other platforms do not have the possibility to run in-house developed real-time assays, which generic ARIES cassettes do allow the user to develop.

In conclusion, the ARIES assay is a rapid molecular based assay for detection of influenza A virus, influenza B virus and RSV. The platform showed a high clinical specificity and sensitivity, which is comparable to LDA and better than those of established rapid assays such as DFA and ICT. Other respiratory tract samples than throat swabs can be run on the ARIES, but pre-dilution is recommended to gain more reliable results.

Conflicts of interest

The fluA/B/RSV cassettes for this study were provided by Luminex Corp. SP is consultant for ABL, Luxembourg and member of the scientific board for ARIES, Luminex Corp, USA. She did not receive money personally, all expenses were paid to her institution. PLAF receives funding from the EU FP7 project PREPARE (602525). In addition, he participates in the IRIS trial, sponsored by Hoffmann–La Roche and has received speakers fees from GSK. He did not receive money personally, all expenses were paid to his institution. MPKG is the head of the Viroscience department of the Erasmus MC Rotterdam. In this role as in all previous roles, it is necessary to liaise with industry on a range of topics, including vaccines and antivirals, occasionally involving collaborative research and development agreements with fund laboratory staff and consumables. She did not receive money personally, all expenses were paid to her institution. JV, AE and SSD have no conflicts of interest.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.jcv.2016.10.019>.

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