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Protocols

Evaluation of the Seegene Allplex™ RV master assay for one-step simultaneous detection of eight respiratory viruses in nasopharyngeal specimens

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ABSTRACT

Background: The Seegene Allplex™ RV Master (RVM) assay is a one-step multiplex real-time reverse transcription polymerase chain reaction (RT-PCR) system for detecting eight viral respiratory pathogens from nasopharyngeal swab, aspirate, and bronchoalveolar lavage specimens. The eight RVM targets are: severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), Influenza A (Flu A), Influenza B (Flu B), Human respiratory syncytial virus (RSV), adenovirus (AdV), rhinovirus (HRV), parainfluenza virus (PIV), and metapneumovirus (MPV). The assay is based on Seegene's multiple detection temperature (MuDT) technology and provides cycle threshold (Ct) values for each of its viral targets upon PCR completion.

Objective: We aimed to evaluate the diagnostic performance of the RVM assay by calculating sensitivity, specificity, accuracy, Positive Predictive Value (PPV), Negative Predictive Value (NPV), Positive Percent Agreement (PPA), Negative Percent Agreement (NPA), and Overall Percent Agreement (OPA) compared to definite diagnosis and analogous reference assays.

Study design: Diagnostic sensitivity, specificity, accuracy, PPV, and NPV were calculated by comparing the results of the RVM assay to that of definite diagnosis assays; while PPA, NPA, and OPA were calculated by comparing results of the RVM assay to that of analogous reference products. Definite diagnosis and reference methods comprised whole genome sequencing and PCR genotyping, the Allplex™ SARS-CoV-2/FluA/FluB/RSV and Respiratory Panels 1, 2, and 3 assays (Seegene), and the Xpert® Xpress SARS-CoV-2/FluA/FluB/RSV Plus assay (Cepheid). Reproducibility of the RVM assay using fully-automated and semi-automated nucleic acid (NA) extraction workflows and as performed by independent operators was also assessed. In total, 249 positive respiratory specimens and at least 50 negative specimens for each target tested were used for this evaluation study.

Results: Sensitivity, specificity, accuracy, PPV, NPV, PPA, NPA, and OPA ranged from 95.7 % to 100 % for detecting all eight targets tested on the RVM assay. Reproducibility PPA, NPA, and OPA between automated and semi-automated NA extraction workflows were all >97.9 %, while the reproducibility PPA, NPA and OPA between independent operators were all 100 %.

Conclusion: These results demonstrate a high level of sensitivity, specificity, accuracy and diagnostic predictive value of the RVM assay and high agreement with comparable reference assays in identifying all eight of its targets. Taken together, our study underscores the diagnostic utility of the RVM assay in detecting eight viral respiratory pathogens.

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1. Background

Respiratory infections remain a leading cause of morbidity and mortality globally (Madhi and Klugman, 2006; GBD, 2016 Lower Respiratory Infections Collaborators, 2018; Paget et al., 2019). Although treatment can vary depending on the underlying causative agent, infections from a wide range of respiratory pathogens can result in similar symptoms, such as cough, fatigue, headache, nausea, congestion, and sore throat. Thus, accurately identifying the aetiology of respiratory infections is imperative for proper clinical management and can also help reduce unnecessary antibiotic use. Molecular diagnostics (MDx) techniques detect genetic fingerprints of potential pathogens in specimens and offer the most sensitive and accurate method of identifying respiratory infections, with polymerase chain reaction (PCR) tests being the most commonly used MDx method. The utility and immense potential of MDx in disease diagnosis was recently highlighted by the widespread use of PCR testing during the novel coronavirus disease 2019 (COVID-19) pandemic to rapidly detect its etiological agent, SARS-CoV-2. Advances in MDx technology now enable multiplex-PCR tests, which detect multiple pathogens simultaneously from a single specimen in a single reaction (Caliendo, 2011; Han et al., 2017; Lee et al., 2014).

The RVM assay (Seegene Inc., Seoul, South Korea), is a semi-quantitative, real-time, one-step RT-PCR test that allows simultaneous detection of eight different respiratory pathogens (AdV, Flu A, Flu B, MPV, PIV, RSV, HRV, and SARS-CoV-2) from nasopharyngeal swabs, aspirates, and bronchoalveolar lavage specimens (Seegene Inc; Houwen et al., 2023). The RVM assay is based, in part, on Seegene's proprietary MuDT analytical technique, which allows for multiple pathogen target detection in a single fluorescence channel. In conjunction with the Seegene Viewer analysis software, the assay provides a Ct value for each of the eight targets upon completion of RT-PCR on a real-time thermal cycler instrument (Lee et al., 2014). The RVM assay was commercially released in 2022 as a CE-IVD marked molecular diagnostic test (Seegene's AllplexTM RV Master Assay receives Australian TGA approval; Coronavirus Test Tracker, 2021).

2. Objectives

The main objective of this study was to evaluate the diagnostic performance of the RVM assay (candidate test product) in detecting each of its eight targets compared to definite diagnosis and commercially available reference assays (comparator test products). Primary study outputs included measurements of diagnostic sensitivity, specificity, accuracy, PPV, NPV, PPA, NPA, and OPA between candidate and comparator test products, while secondary study outputs included measurements of reproducibility when the candidate test product was used on different extraction platforms or by different operators on the same extraction platform.

3. Study design

3.1. Study location and methodology

This was a single-center comparative study performed at Ndlovu Laboratories and Research Centre, Elandsdoorn, Dennilton, Limpopo Province, South Africa, between January and December 2023. The study was designed to evaluate the diagnostic sensitivity, specificity, accuracy, PPV, NPV, PPA, NPA and OPA of the Allplex™ RVM assay in identifying AdV, Flu A, Flu B, MPV, PIV, RSV, HRV, and SARS-CoV-2 in relation to other comparator tests in line with US FDA diagnostic evaluation guidelines (Health, C. for D. and R, 2020). Additionally, the reproducibility of the RVM assay on different extraction platforms and between different independent operators was also evaluated. Diagnostic performance of the RVM assay on SARS-CoV-2 External Quality Assessment (EQA) samples received in the laboratory for diagnostics quality control/quality assurance (QC/QA) purposes was also evaluated.

3.2. Specimen selection

Nasopharyngeal (NP) swabs received for laboratory diagnostics between 2020 and 2023 were retrospectively used for this study. NP swab types used comprised the ALL⊕CARE Dry Flocked Swab (CliniHealth®, South Africa) and eNAT® Flocked Swab in transport medium (Copan Diagnostics, Murrieta, CA, USA). Dry swabs were resuspended in 2 mL saline (0.9 % NaCl) prior to testing. Briefly, after routine diagnostic testing, aliquots of samples that tested positive, and a subset of those that tested negative, were stored long-term at −80°C. In addition, SARS-CoV-2 positive samples were further genotyped by either whole genome sequencing or PCR variant typing prior to storage at −80°C. Archived sample aliquots that were positive for the targets of the RVM assay with Ct < 35 and known negative samples for RVM targets were retrieved and thawed for evaluation on candidate and reference test products for this study. Only anonymized samples with no personal identifiable information were used, thus ethics approval was not required. Stored positive NPs were thawed from −80°C and retrospectively tested using fully-automated or semi-automated nucleic acid (NA) extraction and PCR setup workflows. In total, 249 positive samples comprising 224 laboratory-confirmed positive samples (10 AdV, 39 Flu A, 18 Flu B, 53 HRV, 19 MPV, 11 PIV, 23 RSV, and 51 SARS-CoV-2) and 25 positive-control saline-spiked samples were used for this evaluation. In addition, ≥50 laboratory-confirmed negative samples for each of the 8 RVM targets of interest were used. Specificity (negative) samples contained non RVM target isolates including Human Enterovirus, *Haemophilia influenza*, *Streptococcus pneumonia*, and Coronavirus 229E.

3.3. Diagnostic performance metrics and definitions

For the purpose of this study, definite diagnosis assays are defined as assays that unambiguously establish the true positive (or negative) status of a sample by providing underlying sequence, variant, or subtype information for the given sample. In contrast, reference diagnosis assays, for the purpose of the study, are defined as previously evaluated commercial PCR diagnostic assays that test for a target of interest being compared to the candidate assay. Briefly, the definite diagnosis and reference assays used in this study included whole genome sequencing and variant genotyping for SARS-Cov-2, the Xpert® Xpress CoV-2/Flu/RSV plus (CoV2FR+) (Cepheid, Sunnyvale, CA, USA) and Allplex™ SARS-CoV-2/FluA/FluB/RSV (SCV2FabR) (Seegene Inc., Seoul, South Korea) assays, both of which test for SARS-CoV-2, Flu A, Flu B, and RSV; and the Allplex™ Respiratory Panels 1, 2, and 3 (RP1, RP2, RP3) (Seegene Inc., Seoul, South Korea) assays, the three of which collectively test for AdV, Flu A, Flu B, HRV, PIV, MPV, and RSV (Houwen et al., 2023; Gimferrer et al., 2018; Lee et al., 2018; Huh et al., 2017; Lade et al., 2022; Leung et al., 2021; Mostafa et al., 2021; Noble et al., 2022; Wabe et al., 2021). Definite diagnosis assays were used to calculate sensitivity, specificity, accuracy, PPV, and NPV of the candidate test product while reference diagnosis assays were used as comparators for calculating PPA, NPA, and OPA in relation to the candidate test product. In cases where definite diagnosis was not available for a given target (AdV, HRV, and MPV), sensitivity, specificity, accuracy, PPV, and NPV were not calculated and only reference diagnosis comparisons (PPA, NPA, and OPA) were calculated. Due to limited available published data on actual disease prevalence in the study area, disease prevalence rates for calculating PPV, NPV and specificity, were estimated from anonymized internal laboratory diagnostic testing positivity rates within a period overlapping when samples were collected for the study (October 2021-April 2023). The following disease prevalence estimates were used for SARS-CoV-2, Flu A, Flu B, RSV, and PIV, respectively: 10.60 %, 3.80 %, 0.35 %, 2.53 %, and 2.46 % (Supplemental Dataset Link 1, disease_prevalance_estimates). Supplemental Fig 1 depicts the overall study scheme and all calculation formulas used in our analysis.

Supplemental Tables 1 and 2 provide a comprehensive breakdown of the specific definite and reference diagnosis assays used for each target evaluation and the underlying rationale, sample sizes, and the performance metrics and outputs calculated by each.

Inter-platform reproducibility of the RVM assay was evaluated by comparing the assay workflow between a fully-automated (Hamilton NIMBUS Microlab IVD) and a semi-automated (Seegene SEEPREP32) system (Supplemental Fig 1B). Inter-operator reproducibility of the RVM assay was evaluated by comparing results from two independent operators both using the semi-automated workflow system (Supplemental Fig 1 C). Contingency Table (2×2) were derived for each RVM target against each comparator method and used to calculate sensitivity, specificity, PPA, NPA, and OPA in Google Sheets (Google Sheets). Where applicable, accuracy, PPV, and NPV were derived from the same contingency tables using online statistical software and all diagnostic metrics were expressed as percentages with Clopper-Pearson 95 % confidence intervals using the same software (Mercaldo et al., 2007; Schoonjans, F.; 2x2 Contingency Calculator). All anonymized raw data with Ct values used for calculations and deriving diagnostic metrics and the results of all analyses are freely available as an open source Google Sheets document at Supplemental Dataset Link 1.

3.4. Nucleic acid extraction and PCR

Fully-automated NA extraction and PCR set-up was performed using the Microlab NIMBUS IVD automated liquid handler (Hamilton, USA) with the STARMag 96 ×4 Universal Cartridge Kit (Cat No. 744300.4. UC384, Seegene Inc., South Korea) according to the manufacturer's instructions, with 500 µL starting sample volume and 100 µL final eluted RNA volume. Semi-automated extraction and manual PCR set-up was performed on the SEEPREP32 NA extraction instrument (Seegene Inc., South Korea) using the STARMag 96 ProPrep C Extraction Plate Kit (Cat No. EX0723A01, Seegene Inc., South Korea) according to the manufacturer's instructions, with 200 µL starting volume and 100 µL final eluted RNA volume. Fully-automated NA extraction and PCR set-up workflow was used for all performance comparisons between the test candidate and all definite diagnosis and reference test products (inter-assay comparisons). Semi-automated NA extraction and PCR set-up workflow was used for inter-platform and inter-operator reproducibility performance comparisons on the test candidate assay. Supplemental Fig 1 details which specimens were tested using either the Nimbus automated liquid handler and/or the SEEPREP32 semi-automated system. Real-time one-step RT-PCR for all Allplex™ assays were performed on the CFX96™ DX Touch™ thermal cycler system (Bio-Rad Laboratories, Hercules, CA, USA) according to the manufacturer's instructions, and cycling conditions for the RVM assay are shown in Supplemental Table 3. All CFX96 ™ DX Touch™ results were analysed by the Seegene Viewer software (Seegene Inc., Seoul, South Korea). Xpert® Xpress CoV2FR+ PCR was performed on the GeneXpert GXII-2-D-10C module (Cepheid, Sunnyvale, CA, USA), according to the manufacturer's instructions.

3.5. Whole genome sequencing and PCR genotyping

Whole genome sequencing (WGS) and bioinformatics analysis were performed on the Genexus Ion Torrent sequencer (Thermo Scientific, USA), using the SARS-CoV-2 Ion AmpliSeq™ Research Panel according to the manufacturer's instructions. PCR variant genotyping was performed using Allplex™ SARS-Cov-2 Variant assays I-VII (Seegene Inc., South Korea), according to the manufacturer's instructions, and analysed using the Seegene Variant Viewer software. Supplemental Dataset Link 1, SCV2, contains a breakdown of SARS-CoV-2 samples analysed by WGS or PCR genotyping as well as variants identified for each.

4. Results

The complete set of sensitivity, specificity, accuracy, PPV, NPV, PPA, NPA, and OPA results with corresponding 95 % confidence intervals for the RVM assay in identifying AdV, Flu A, Flu B, HRV, MPV, PIV, RSV, and SARS-CoV-2 compared to definite and reference diagnosis assays are systematically summarized in Table 1. In sum, the RVM assay's diagnostic sensitivity, specificity, accuracy, PPV, NPV, PPA, NPA, and OPA were all >96 % in detecting each of the 8 targets when compared to corresponding definite and reference diagnosis assays (Table 1). Of note, PPV and NPV of SARS-CoV-2, Flu A, Flu B and RSV, respectively, were 100 % and 98.4 %, 100 % and 99.9 %, 100 % and and 100%100 % and 100 %100 % (Table 1). In comparison to Seegene's previous generation Allplex™ SCV2FabR assay, the average PPA, NPA and OPA of the RVM assay for the collective detection of SARS-CoV-2, Flu A, Flu B, and RSV, was 98.4 % ±1.95 %, 100 % and 99.2 % ±0.95 %, respectively (Supplemental Dataset 1, SCV2). Similarly, when compared to the GeneXpert Cepheid CoV2FR+ assay, the average PPA, NPA and OPA of the RVM assay for the collective detection of SARS-CoV-2, Flu A, Flu B, and RSV, was 98.3 % ±2.12 %, 99.2 % ±1.52 % and 98.5 % ±1.11 %, respectively (Supplemental Dataset 1, SCV2). We also analysed 15 prospective clinical samples of unknown status, obtained for diagnostic testing, in parallel on the RVM and SCV2FabR assays. We analysed positive/negative outcomes for SARS-CoV-2, FluA, FluB, and RSV using the Nimbus automated workflow. Clinical diagnosis PPA, NPA, and OPA between the two assays for all 4 targets were 100 %, 98.1 %, and 98.3 %, respectively, closely mirroring our diagnostics performance observations (Supplemental Dataset 1, clinical_comparison).

Inter-platform reproducibility of the RVM assay was evaluated by testing nucleic acid extracted on the Microlab Nimbus fully automated workflow compared to the SEEPREP32 semi-automated workflow. A total of 93 samples, comprising positive and negative specimens for SARS-CoV-2, Flu A, Flu B, RSV, HRV, and PIV, were evaluated in parallel using Nimbus and SEEPREP32 extraction workflows followed by PCR. PPA, NPA, and OPA were all above 98 % (Table 2).

Inter-operator reproducibility was evaluated by comparing manual PCR prep workflows performed by two independent operators on an identical set of 48 nucleic acid samples, comprising positive and negative specimens for SARS-CoV-2, Flu A, Flu B, RSV, HRV, and PIV, extracted using the SEEPREP32 system only. PPA, NPA, and OPA were all 100 % between both operators. We also evaluated the performance of the RVM assay in detecting all eight targets from saline samples spiked with assay positive control. Twenty-five saline samples, each 190uL, were spiked with 10uL RVM assay positive control, for 200uL total volume per sample, and processed using the Nimbus automated workflow. Twenty-six negative samples from previous diagnostic testing were also included. OPA, PPA, and NPA between observed and expected results were all 100 % (Table 2).

Finally, the RVM assay was used for SARS-CoV-2 QA/QC testing in 3 EQA proficiency testing cycles, provided by LGC Standards (London, UK) (About Us, and achieved a 100 % success rate (not shown). Table 2 summarises all the results for reproducibility, spiked, and real-time clinical comparisons, including corresponding confidence intervals.

5. Discussion

We performed a comprehensive evaluation of the Allplex™ RVM assay by assessing standard diagnostic metrics including sensitivity, specificity, accuracy, PPV, NPV, PPA, NPA, and OPA (Health, C. for D. and R, 2020). These metrics were analysed in comparison to definite diagnosis and/or comparable reference assays for all eight RVM assay targets. We also analysed the reproducibility of the RVM assay with NA extracted on the semi-automated Seegene SEEPREP32 system compared to the fully-automated Hamilton Nimbus extraction and PCR-setup system. In addition, we performed reproducibility evaluations across independent operators and, finally, we evaluated the performance of the

Table 1
Sensitivity, Specificity, Accuracy, and Predictive and Percent Agreement Values (Positive, Negative, Overall) of the RVM assay in comparison to Definite and Reference Diagnostic Assays.

	pos n	neg n	Assay	Comparison	Metric	Observed Value	95 % C.I
SARS-CoV−2	51	82	Sequencing/ Variant Genotyping Allplex SCV2FabR/ Xpert CoV2FR+	Definite Diagnosis	Sensitivity	96.1 %	86.54 % - 99.52 %
					Specificity	100.0 %	95.6 % - 100 %
					Accuracy	98.82 %	95.16 % - 99.91 %
					PPV	100.0 %	92.75 % - 100 %
					NPV	98.35 %	93.86 % - 99.57 %
	51	51	Allplex SCV2FabR	Reference Diagnosis	PPA	96.1 %	86.54 % - 99.52 %
					NPA	100.0 %	93.02 % - 100 %
					OPA	98.0 %	93.1 % - 99.5 %
	46	31	Xpert CoV2FR+	Reference Diagnosis	PPA	95.7 %	85.16 % - 99.47 %
					NPA	100.0 %	88.78 % - 100 %
					OPA	97.4 %	91.0 % - 99.3 %
Flu A	39	50	Allplex RP 1	Definite Diagnosis	Sensitivity	97.4 %	86.52 % - 99.94 %
					Specificity	100.0 %	92.89 % - 100 %
					Accuracy	99.90 %	Not calculated
					PPV	100.0 %	90.75 % - 100 %
					NPV	99.90 %	99.30 % - 99.99
	39	50	Allplex SCV2FabR	Reference Diagnosis	PPA	97.4 %	86.52 % - 99.94 %
					NPA	100.0 %	92.89 % - 100 %
					OPA	98.9 %	93.9 % - 99.8 %
	39	33	Xpert CoV2FR+	Reference Diagnosis	PPA	97.4 %	86.52 % - 99.94 %
					NPA	100.0 %	89.42 % - 100 %
					OPA	98.6 %	92.5 % - 99.8 %
Flu B	18	50	Allplex RP 1	Definite Diagnosis	Sensitivity	100.0 %	81.47 % - 100 %
					Specificity	100.0 %	92.89 % - 100 %
					Accuracy	100.0 %	94.72 % - 100 %
					PPV	100.0 %	81.47 % - 100 %
					NPV	100.0 %	92.89 % - 99.96 %
	18	50	Allplex SCV2FabR	Reference Diagnosis	PPA	100.0 %	81.47 % - 100 %
					NPA	100.0 %	92.89 % - 100 %
					OPA	100.0 %	93.9 % - 99.8 %
	17	32	Xpert CoV2FR+	Reference Diagnosis	PPA	100.0 %	86.52 % - 99.94 %
					NPA	97.0 %	89.42 % - 100 %
					OPA	98.0 %	92.5 % - 99.8 %
RSV	23	50	Allplex RP 1	Definite Diagnosis	Sensitivity	100.0 %	86.52 % - 99.94 %
					Specificity	100.0 %	92.89 % - 100 %
					Accuracy	100.0 %	95.07 % - 100 %
					PPV	100.0 %	85.18 % - 100 %
					NPV	100.0 %	92.89 % - 100 %
	23	50	Allplex SCV2FabR	Reference Diagnosis	PPA	100.0 %	85.18 % - 100 %
					NPA	100.0 %	92.89 % - 100 %
					OPA	100.0 %	95.0 % - 100 %
	23	50	Xpert CoV2FR+	Reference Diagnosis	PPA	100.0 %	85.18 % - 100%
					NPA	100.0 %	89.42 % - 100%
					OPA	100.0 %	95.0 % - 100 %
HRV	53	53	Allplex RP 3	Reference Diagnosis	PPA	100.0 %	93.28 % - 100%
					NPA	100.0 %	93.28 % - 100 %
					OPA	100.0 %	96.5 % - 100 %
MPV	19	50	Allplex RP 2	Reference Diagnosis	PPA	100.0 %	82.35 % - 100 %
					NPA	100.0 %	92.89 % - 100 %
					OPA	100.0 %	94.7 % - 100 %
PIV	11	51	Allplex RP 2	Definite Diagnosis	Sensitivity	100.0 %	71.51 % - 100 %
					Specificity	100.0 %	93.02 % - 100 %
					Accuracy	100.0 %	94.22 % - 100 %
					PPV	100.0 %	71.51 % - 100 %
					NPV	100.0 %	93.02 % - 100 %
					OPA	100.0 %	94.1 % - 100 %
AdV	10	51	Allplex RP 2	Definite Diagnosis	PPA	100.0 %	69.15 % - 100 %
					NPA	100.0 %	93.02 % - 100 %
					OPA	100.0 %	94.1 % - 100 %

assay on prospective clinical diagnostic samples and positive-control spiked saline samples. Of note, all retrospective positive samples used in this study had Ct values <35 (Supplemental Dataset Link 1). This specific Ct value was selected due to observations from our previous study, which showed that samples with Ct values >35 are not readily genotyped (Umunnakwe et al., 2022).

We observed high sensitivity, specificity, accuracy, PPV, NPV, PPA, NPA, and OPA for all RVM targets tested compared to definite diagnosis and reference assays (>96 %). Out of 102 total positive and negative SARS-CoV-2 samples analysed on the RVM assay in comparison with definite diagnosis and reference assays, we observed two discrepant samples (Table 1 and Supplemental Dataset Link 1, SCV2). Out of 89 total positive and negative Flu A samples analysed on the RVM assay in comparison to definite diagnosis and reference assays, we observed one discrepant sample (Table 1 and Supplemental Dataset 1, FluA). For all other targets (AdV, Flu B, HRV, MPV, PIV, and RSV) analysed on the RVM assay in comparison with definite diagnosis and/or reference assays we observed no discrepant results yielding, where applicable, 100 % sensitivity, specificity, accuracy, PPV, NPV, PPA, NPA, and OPA for these targets (Table 1 and Supplemental Dataset 1). Both SARS-CoV-2 discrepant samples were negative on the RVM assay but positive by definite and reference diagnosis assays. One of the samples was of

Table 2
Percent Agreement (Positive, Negative, Overall) of the RVM assay for Reproducibility and Diagnostic Accuracy.

	pos_n	neg_n	Comparator Methods	Comparison Type	Parameter	Observed Value	95 % C.I
Reproducibility	45	48	Nimbus	Inter-Instrument	PPA	100 %	92 % - 100 %
					NPA	98.9 %	89.3 % - 99.6 %
			SEEPREP		OPA	97.9 %	94.2 % - 99.8 %
	24	24	Operator 1	Inter-Operator	PPA	100 %	
					NPA	100 %	
			Operator 2		OPA	100 %	92.6 % - 100 %
Diagnostic Accuracy	25	26	Nimbus	Spiked-samples	PPA	100 %	86.3 % –100 %
					NPA	100 %	86.8 % –100 %
					OPA	100 %	93.0 % - 100 %,
	n=15 (unknown		Allplex™ RVM	Real-Time	PPA	100 %	63.1 % - 100 %
				Clinical Diagnosis	NPA	98.1 %	89.7 % - 100 %
	pos, unknown		Allplex™ SCV2FabR		OPA	98.3 %	91.1 % - 99.7 %
	neg)						
TOTAL	**207**						

Omicron lineage (21 K) and the other was a Delta variant (Supplemental Dataset 1, SCV2). We speculate that these discrepancies are not due to mismatched probes or high Ct values because the Ct values of both were well below 35 and the RVM assay successfully identified similar Omicron and Delta variants in other samples. Additionally, since the samples were extracted and set up for PCR using an automated system, the chances of human error causing the discrepancy is low, but this cannot be completely ruled out. We hypothesize that the discrepancy might be due to a potential slight loss in sensitivity of the RVM assay as a result of the added diagnostic burden of having to simultaneously detect 8 targets compared to the 4 targets of either the GeneXpert or Allplex™ SARS-CoV-2/FluA/FluB/RSV previous generation assays. Nonetheless, this hypothetical trade-off in sensitivity is very minimal and is vastly outweighed by its high sensitivity and specificity in detecting all 8 targets.

We also observed high reproducibility of the RVM assay when using fully-automated extraction systems compared to semi-automated workflows as well as between two independent operators (>97 %). Only one discrepancy was observed in our reproducibility evaluation (Flu B sample; Ct 30.99). This sample was positive on the Nimbus system but negative on the SEEPREP32 workflow. We speculate that this discrepancy is due to either increased sensitivity of the Nimbus automated system and/or human error during manual setup of the SEEPREP32 workflow.

Taken together, our results demonstrate the diagnostic robustness of the RVM assay in detecting SARS-CoV-2, Flu A, Flu B, RSV, HRV, MPV, PIV, and AdV from NP specimens. Our study, which is among the first performance evaluations of the RVM assay, demonstrates a high level of agreement and comparable performance of the assay with older generation commercial reference assays in detecting all eight RVM targets from NP specimens. Altogether, this study provides relevant clinical and diagnostic data for a next-generation MDx multiplex RT-PCR assay. Such an assay can be incredibly useful for clinical and diagnostic use in areas with seasonal viruses that cause overlapping symptoms as well as epidemiological surveys and pandemic preparedness initiatives.

Real-time PCR molecular assays have become the *de-facto* gold standard for diagnosis of infectious diseases in clinical settings and recent advances in technology now allow for one-step simultaneous detection of multiple targets from one tube in a single reaction. The ability to distinguish between different possible causes of respiratory infections is useful for clinical management of patients as well as for epidemiological efforts during infectious disease outbreaks, pandemics and routine surveillance. In the context of the recent COVID-19 pandemic, the ability to identify other pathogens that cause symptoms overlapping with that of SARS-CoV-2, or to identify coinfections of SARS-CoV-2 with other respiratory pathogens in patient samples in a single experiment can provide added value in clinical management. Furthermore, the ability of the RVM real-time RT-PCR assay to detect eight respiratory pathogens, including SARS-CoV-2, from a single specimen in a one-step reaction makes it well suited for such purposes. Lastly, the flexibility of the RVM assay in being implemented on either fully-automated high-throughput, semi-automated medium-throughput or manual low-throughput workflows, makes it a viable option for a wide range of clinical laboratory settings (Brouwer et al., 2024).

5.1. Limitations, strengths, weaknesses and future directions

Our study contains several inherent limitations. First, although the RVM assay is intended for use on multiple specimen types, only NP swabs were used in the present study; therefore, results from this evaluation may not be generalizable to other specimen types. Nonetheless, the Nimbus automated liquid handling system used in this study is compatible with all specimen types, therefore, the RVM assay can be easily implemented on it for multiple specimen types. Next, the sample size of some pathogens tested was limited (especially Flu B, MPV, PIV), and a more comprehensive analysis may be warranted in follow-up evaluations for more robust diagnostic metrics.

A significant strength of this study is the inclusion of diverse variants/subtypes for several pathogens (SARS-CoV-2, Flu A, RSV and PIV). For SARS-CoV-2 in particular, all major variants were included in the evaluation, and the RVM assay was shown to detect each successfully. Similarly, for Flu A, RSV and PIV, our results showed that the RVM assay is capable of detecting all the major subtypes (Flu A H1/H3, RSV A/B, and PIV 1–4). These observations underscore the robustness and utility of the RVM assay for clinical and epidemiological use.

Potential future studies on the RVM assay could focus on more detailed clinical evaluations as well as quantitative analyses of linearity and limit of detection of the assay. Altogether, our study provides strong support for the use of the Allplex™ RVM assay for reliable detection of eight respiratory viruses in diagnostic and clinical settings.

Author statement

We have completed revision of our manuscript according to the editor and reviewers' comments and all issues raised have been addressed to the best of our ability. No generative artificial intelligence (AI) or AI-assisted technology was used in the writing process.

CRediT authorship contribution statement

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Declaration of Competing Interest

None of the authors of this manuscript have any conflicts of interest to declare.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.jviromet.2024.115042.

Data availability

All raw data used in the study has been shared as a Supplemental Dataset link.

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