Since January 2020 Elsevier has created a COVID-19 resource centre with free information in English and Mandarin on the novel coronavirus COVID-19. The COVID-19 resource centre is hosted on Elsevier Connect, the company's public news and information website.

Pathology (June 2022) **54(4)**, pp. 466–471

VIROLOGY

Evaluation of the influenza and respiratory syncytial virus (RSV) targets in the AusDiagnostics SARS-CoV-2, Influenza and RSV 8-well assay: sample pooling increases testing throughput

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Summary

During the COVID-19 pandemic, sample pooling has proven an effective strategy to overcome the limitations of reagent shortages and expand laboratory testing capacity. The inclusion of influenza and respiratory syncytial virus (RSV) in a multiplex tandem PCR platform with SARS-CoV-2 provides useful diagnostic and infection control information. This study aimed to evaluate the performance of the influenza and RSV targets in the AusDiagnostics SARS-CoV-2, Influenza and RSV 8-well assay, including the effect of pooling samples on target detection.

RSV target detection in clinical samples was compared to the Cepheid Xpert Xpress Flu/RSV assay as a reference standard. Samples were then tested in pools of four and detection rates were compared. Owing to the unavailability of clinical samples for influenza, only the effect of sample pooling on simulated samples was evaluated for these targets.

RSV was detected in neat clinical samples with a positive percent agreement (PPA) of 100% and negative percent agreement (NPA) of 99.5% compared to the reference standard, demonstrating 99.7% agreement. This study demonstrates that sample pooling by four increases the average Ct value by 2.24, 2.29, 2.20 and 1.91 cycles for the target's influenza A, influenza A typing, influenza B and RSV, respectively. The commercial AusDiagnostics SARS-CoV-2, Influenza and RSV 8-well assay was able to detect influenza and RSV at an intermediate concentration within the limit of detection of the assay. Further studies to explore the applicability of sample pooling at the lower limit of detection of the assay is needed. Nevertheless, sample pooling has shown to be a viable strategy to increase testing throughput and reduce reagent usage. In addition, the multiplexed platform targeting various respiratory viruses assists with public health and infection control responses, clinical care, and patient management.

Key words: Influenza; respiratory syncytial virus; sample pooling; laboratory diagnosis; MT-PCR.

Received 6 July 2021, revised 9 January, accepted 22 February 2022
Available online 21 April 2022

INTRODUCTION

During the COVID-19 pandemic, demand has greatly increased for high throughput testing of large numbers of patients with symptoms of viral upper respiratory tract infection (URTI). To improve patient care and assist with infection control management, SARS-CoV-2 has been included in multiplex polymerase chain reaction (PCR) assays with other respiratory viruses, such as influenza A, B, and respiratory syncytial virus (RSV).[1,2] These are highly infectious respiratory viruses which are a common cause of viral URTI symptoms in community outbreaks and are pathogenic and transmissible in hospital environments.[3–9] This study evaluated the influenza and RSV targets of the commercially available multiplex tandem PCR assay, the AusDiagnostics SARS-CoV-2, Influenza and RSV 8-well assay.

Real-time reverse transcription-PCR has proven the most robust method of detection for respiratory viruses, with high analytical sensitivity, and is able to exploit existing platforms and infrastructure. However, it can be time consuming and costly. During the COVID-19 pandemic, it has been necessary to optimise laboratory resources, including equipment and reagents, to maintain diagnostic capability. Sample pooling has proven an effective strategy to meet these ends.[10]

In the context of the COVID-19 outbreak, high throughput testing turnaround times contribute to monitoring of community transmission, and effective contact tracing.[11] In circumstances of surges in testing demand, especially seen in pandemics and outbreaks, laboratories may experience various challenges, such as reagent shortages and increased turnaround times due to limited sample capacity of an instrument. Sample pooling, initially introduced by Dorfman in 1943, may be considered to overcome these limitations.[12] Several patient samples are combined and tested together. Negative results can be released without further testing, but a pool that tests positive requires individual testing. Therefore, it is more useful in a low prevalence setting, where fewer pools will require individual testing.[10] Urgent samples are not pooled. Sample pooling has been implemented to increase testing capacity, conserve resources, limit reagent usages and reduce costs, in settings which require enhanced surveillance of diseases with low prevalence but high public health

Print ISSN 0031-3025/Online ISSN 1465-3931
DOI: https://doi.org/10.1016/j.pathol.2022.02.002

significance.[12–15] This strategy has been previously employed for the mass detection of seasonal and avian influenza, bacteria, and parasites.[16–20] Key principles for successful application of sample pooling requires assessing the performance parameters of the assay.[21]

This study aimed to evaluate the effects of sample pooling on detection of the influenza and RSV targets in the commercial multiplex tandem PCR (MT-PCR) AusDiagnostics SARS-CoV-2, Influenza and RSV 8-well assay, and an evaluation of the performance of the RSV target. Samples were tested in pools of four, and detection rates and cycle threshold (Ct) values were compared to those of the samples tested neat.

METHODS

Study setting and study design

The study was conducted in the Department of Microbiology, Concord Repatriation General Hospital, NSW Health Pathology, during March to October 2020. The laboratory serves a tertiary hospital, and during the study period, it also served its two attached COVID-19 community testing centres. Samples from healthcare workers, hospital inpatients and outpatients, symptomatic or SARS-CoV-2 exposed patients were included in the analysis. A convenience sample of 294 patient specimens was selected based on results of routine diagnostic testing with the AusDiagnostics SARS-CoV-2, Influenza and RSV 8-well assay (AusDiagnostics, Australia), and these were re-tested on the Cepheid assay (Cepheid, USA). Overall agreement, positive and negative agreement were calculated to determine assay performance. This work was done as part of validation of the assay.

Specimen collection and storage

Specimen types used in this study included nasal swabs, throat swabs and nasopharyngeal swabs (NP). Specimens were collected with flocked swabs and transported in viral transport medium (VTM). Samples were stored at 4°C prior to testing.

Nucleic acid extraction

Viral RNA was extracted from samples with either the EZ1 DSP Viral extraction kit (Qiagen, Germany) and the QIAGEN EZ1 Advanced (Qiagen) or the MT-Prep kit (AusDiagnostics, Australia) and the MT-Prep 24 Extractor (AusDiagnostics), as per manufacturer's instructions. Both platforms have been validated and are compatible with the AusDiagnostics SARS-CoV-2, Influenza and RSV 8-well assay.[22] Pooled or neat samples of 200 µL were extracted to elute volumes of 60 µL or 50 µL (QIAGEN EZ1 Advanced, and AusDiagnostics MT-Prep, respectively). AusDiagnostics Extraction control and carrier RNA (AusDiagnostics MT-Prep carrier RNA and QIAGEN EZ1 Viral carrier RNA, corresponding with the extraction method) was included in all sample testings prior to extraction.

Multiplex-tandem PCR and analysis

Ten µL of eluted nucleic acid extracts were added to the AusDiagnostics assay tubes. These were run as per the manufacturer's instructions on the AusDiagnostics High-Plex 24 System, a commercial *in vitro* diagnostic (IVD) nucleic acid testing platform based on multiplex tandem polymerase chain reaction (MT-PCR) technology.[23] The method involves a reverse transcriptase and pre-amplification step (15–18 cycles) to enrich all targets, a subsequent dilution step, then a final step 2 PCR reaction: 95°C for 10 min (1 cycle); 95°C for 10 s (30 cycles); 60°C for 20 s (30 cycles); 72°C for 10 s (30 cycles); 75°C for 5 s (50 cycles). The step 2 PCR reaction amplifies specific gene targets, using nested primers to increase specificity and sensitivity, and reduce competition and non-specific amplification.

The AusDiagnostics SARS-CoV-2, Influenza and RSV 8-well assay qualitatively detects SARS-CoV-2 *(orf1a and orf8)*, influenza A haemagglutinin surface protein (H1, H3, H5 and H7), influenza A typing (pdH1N1, H3 and H3N2), influenza B (Yamagata and Victoria lineages) and RSV A and B. It also includes a human reference gene (NONO) and an artificial sequence (SPIKE) to detect human DNA as a check for sample adequacy, and inhibition, respectively. Positive controls (AusDiagnostics) and negative controls (DNase free water; no template control) were included in each run.

The AusDiagnostics MT-Analyser Software provides automated curve interpretation. Amplification of the target gene is reported as 'present', whereas a target gene is reported 'not detected' when there is no amplification that falls within predetermined parameters. In cases where cycling curve acceleration is slower than these parameters, it is reported as a 'check' result. This indicates that an operator's involvement is required to investigate and interpret the result. The software also provides calculated Ct values and concentration of the target molecule expressed as arbitrary units, relative to the internal control (SPIKE) set at 10,000.

Evaluation of RSV target detection

A total of 294 patient samples were tested for the RSV target on the AusDiagnostics SARS-CoV-2 Influenza and RSV 8-well assay. These samples were also tested on the Cepheid GeneXpert Xpress Flu/RSV, according to the manufacturer's instructions, as a comparator standard.

Evaluation of sample pooling on RSV target detection

To determine the effect of 1:4 sample pooling on RSV target detection, 50 µL of an RSV-positive sample was pooled with 50 µL of three RSV-negative samples, to a total sample volume of 200 µL (n=43). The sensitivity of RSV detection and the difference in Ct values were compared to neat samples.

Evaluation of sample pooling on influenza A and B target detection

There was limited influenza disease activity in 2020, meaning no influenza positive patient samples were available for evaluation.[24] Therefore, positive samples were simulated by spiking negative patient samples with influenza A and influenza B viral cultures [supplied by the Centre for Infectious Diseases and Microbiology Laboratory Services (CIDMLS), NSW Health Pathology, Westmead]. These viral stock solutions were provided by a reference laboratory at CIDMLS, with a concentration of 1,000,000 copies/mL and 200,000 copies/mL respectively. They were tested on the Cepheid GeneXpert Rapid Flu assay at CIDMLS to confirm viral detection. To approximate viral concentrations typically found in clinical infection, 20µL of 1:10 dilutions of the Influenza A and Influenza B viral stock solutions were added to 240 µL aliquots of influenza negative patient specimen in VTM.[25–28]

To determine the effect of sample pooling, simulated samples were tested neat compared to pools of four (n=95). To create a pooled sample, 50 µL of the simulated samples was added to 150 µL VTM. Sensitivity of detection, and the difference in Ct values were compared.

Limit of detection and precision

The limit of detection (LOD) was determined by conducting serial dilutions of the AusDiagnostics Synthetic Respiratory positive control to find the lowest relative concentration of the targets (RSV, influenza A and influenza B) detected by the AusDiagnostics SARS-CoV-2, Influenza and RSV 8-well assay. The AusDiagnostics Synthetic Respiratory positive control was tested neat to establish initial concentration and serially diluted by factors of 0.1, 0.01, 0.001, 0.0005, and 0.00025. The precision and reproducibility of the assay was assessed by testing the AusDiagnostics Synthetic Respiratory positive control three times a day over the span of 16 days (n=44). Ct values obtained by different technical operators and three AusDiagnostics High plex systems were recorded.

Statistical analysis

Overall percent agreement (OPA), positive percent agreement (PPA) and negative percent agreement (NPA), Cohen kappa statistics for the proportion agreement expected by chance (P_e) and proportion of agreement observed (P_o) to obtain κ-value were calculated with Microsoft Excel software (Microsoft, USA). The formula below was used to calculate 99% confidence intervals (CI):

$$CI\ (\%) = \left(p \pm z_{\frac{\alpha}{2}} \sqrt{\frac{p(1-p)}{n}}\right) \times 100$$

where $z = 2.576$.

The effect of sample pooling on Ct values was analysed by calculating the delta Ct (ΔCt), its average and standard deviation (SD), between the neat and

pooled samples. Coefficient of variation (CV%) was calculated to determine variability and assay reproducibility. All statistics were calculated using Microsoft Excel.

RESULTS

RSV detection from clinical samples

A total of 294 clinical patient samples were tested for the RSV target on the AusDiagnostics SARS-CoV2, Influenza and RSV 8-well assay and were compared to the Cepheid Xpert Xpress Flu/RSV assay (Table 1). The overall agreement between platforms was 99.7% (99% CI 99–100%), with 100% PPA and 99.5% NPA (99% CI 98–100%) (Table 1). Cohen kappa (κ) was calculated to measure the degree of agreement between the AusDiagnostics SARS-CoV2, Influenza and RSV 8-well assay and the Cepheid Xpert Xpress Flu/RSV assay. P_e=0.528, P_o=0.997 and the calculated κ=0.993. As the $0.81 < \kappa < 1.00$, according to Cohen kappa's range, results demonstrated an excellent level of agreement between the two assays.

Evaluation of sample pooling

The detection rates were compared to samples tested in pools of four versus neat. The average Ct values for neat and pooled samples was recorded for RSV, influenza A, influenza A typing, and influenza B (Table 2, Fig. 1). For the RSV target the average difference was −1.91±1.18 cycles. Four samples of the tested 43 samples demonstrated lower Ct values for the pooled sample compared to the neat sample (Supplementary Table 1, Appendix A). The influenza A target had an average Ct of 22.70±0.50 for neat samples which increased by 2.24 cycles when pooled to average 24.94±0.58 cycles (Supplementary Table 2, Appendix A). The influenza A typing H1 target had Ct values of 23.10±0.42 and 25.39±0.55 for neat and pooled samples, respectively, and a difference of 2.29 cycles. Neat samples spiked with influenza B had an average Ct of 25.02±0.84 for that target, compared to the pooled samples' average Ct of 27.22±0.91, with a difference of 2.20 cycles. For a comparison of the Ct values and concentrations of the influenza stock solutions on the assay vs the Cepheid Flu/RSV assay, see Supplementary Table 3, Appendix A.

Limit of detection (LOD)

The LOD was determined by conducting serial dilutions of the positive control to find the lowest relative concentration and Ct value of the targets detected by the assay. A dilution series by factors of 0 (neat), 0.1, 0.01, 0.001, 0.0005, and 0.00025 of the AusDiagnostics Synthetic Positive Control was tested. Table 3 displays Ct values and concentration for the target's influenza A, influenza A typing, influenza B and RSV determined by the analysis software. The lowest relative concentration detected for influenza A was 13 (Ct=32.1), influenza A typing 8 (Ct=32.83), influenza B 6 (Ct=33.4) and RSV 10 (Ct=33.4).

Precision – reproducibility

Reproducibility was assessed by testing the AusDiagnostics Synthetic Respiratory positive control three times a day over the span of 16 days (44 replicates). The average Ct value for influenza A was 18.28±0.43 (CV=2.33%, 99% CI=18.12–18.45), influenza A Typing 20.71±0.37 (CV=1.79%, 99% CI=20.57–20.85), influenza B 19.23±0.46 (CV=2.41%, 99% CI=19.05–19.41) and RSV 18.67±0.38 (CV=2.02%, 99% CI=18.53–18.82). The low CV suggests that the assay is highly precise and reproducible.

Analysis of discrepant results

Initially, a pooled sample of four was positive for RSV on the AusDiagnostics assay with a pooled Ct of 32.53. The sample was re-tested on the Cepheid Xpert RSV/Flu Assay and the result was negative. To rule out contamination, the patients in the pool were tested individually on the AusDiagnostics, with one patient positive for RSV with a neat Ct of 30.22. The sample was collected from an 8-year-old boy with a clinical history of respiratory symptoms. The sample was referred on to another laboratory for testing using the Roche Cobas Influenza A/B and RSV assay, which was positive, so this result is likely to be a false negative on the Cepheid Xpert Xpress Flu/RSV assay.

DISCUSSION

During the COVID-19 pandemic, many studies have focused on employing sample pooling in the detection of SARS-CoV-2. However, there is a lack of research on applying this strategy in detecting other highly infectious respiratory viruses. This is important to identify in patients as some respiratory viruses such as influenza and RSV are associated with outbreaks and severe mortality.[3–9] Therefore, it is advantageous to use the multiplexed AusDiagnostics SARS-CoV-2, Influenza and RSV 8-well assay for simultaneous detection of these viruses.[1,2] The major aim of this study is to determine the suitability of sample pooling in detecting influenza A, influenza B and RSV gene targets on the commercial assay.

The assay demonstrated good agreement level for the RSV gene target when compared to the Cepheid Xpert Xpress Flu/RSV assay. Sample pooling showed an average increase of Ct

Table 1 Agreement, positive agreement (sensitivity) and negative agreement (specificity) of the RSV target in neat clinical samples tested on the AusDiagnostics SARS CoV-2, Influenza and RSV 8-well assay compared to the Cepheid Xpert Xpress Flu/RSV assay

AusDiagnostics SARS CoV2, Influenza and RSV 8-well assay	Agreement *n*=294	Positive agreement (sensitivity) *n*=112	Negative agreement (specificity) *n*=182
RSV gene target, Number (%) [99% CI]	293 (99.7%) [99–100%]	112 (100%) [NA]	181 (99.5%) [98–100%]

n, sample number; CI, confidence interval; NA, not available as confidence intervals cannot be calculated at 100%.

Table 2 Comparative analysis of Ct values from neat vs pooled for RSV positive patient sample (n=43), samples spiked with influenza A and influenza B viral cultures (n=95) tested on the AusDiagnostics SARS-CoV-2, Influenza and RSV 8-well assay

Gene target (n)	Neat	Pooled	ΔCt
	Average Ct ± SD	Average Ct ± SD	Average Ct ± SD
RSV (43)	20.37 ± 4.5	22.28 ± 4.5	−1.91 ± 1.18
Influenza A (95)	22.70 ± 0.50	24.94 ± 0.58	−2.24 ± 0.47
Influenza A typing (95)	23.10 ± 0.42	25.39 ± 0.55	−2.29 ± 0.51
Influenza B (95)	25.02 ± 0.84	27.22 ± 0.91	−2.20 ± 0.51

Ct, cycle threshold; SD, standard deviation.

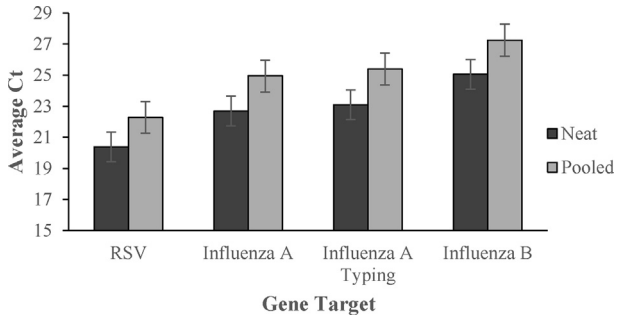

Fig. 1 Comparative analysis of neat and pooled samples for influenza A, influenza B and respiratory syncytial virus (RSV) gene targets tested on the AusDiagnostics SARS-CoV-2, Influenza and RSV 8-well assay by assessing the average Ct (± standard error). RSV-positive patient samples were tested neat and pooled with three RSV-negative patient samples (n=43). Influenza A and influenza B viral cultures were used to spike influenza-negative samples and tested neat and pooled 1:4 (n=95).

by 2.16 in a pool of four compared to neat samples and the assay was able to detect reliably at a high to intermediate viral concentration. This is consistent with other pooling studies performed on avian influenza (1:5) and SARS-CoV-2 (1:4), which results in an average increase in Ct of 2.00–2.30 cycles.[10,29] Other sample pooling studies on nucleic acid amplification test detection of respiratory viruses similarly observed that small pooling ratios (1:3 to 1:10) minimally affected the sensitivity.[13,14,30] However, this is taking into consideration the pool ratio, and the assay's performance. Patients with low viral loads, especially viral concentrations that are close to the LOD of the assay, risk a false negative test result. This type of error can have severe clinical and epidemiological impact.[31,32]

The LOD of the influenza and RSV targets was assessed by conducting serial dilutions of the AusDiagnostics Synthetic Respiratory positive control. The assay detected low relative concentrations of 13, 8, 6 and 10 (Ct ranging from 32.1–33.4) for the influenza A, influenza A typing, influenza B and RSV targets, respectively, at dilution factor of 0.0005 and 0.00025. However, the serial dilution used did not go low enough to definitively establish the true LOD and lie somewhere below these concentration values.

A limitation of this study is that positive patient samples infected with influenza viruses were not able to be tested in this study, and due to the inaccessibility to positive clinical samples, a spiking approach was conducted. A spike of a high to intermediate viral concentration (Ct 20–e30) was chosen to reflect patient populations which were at peak infection, symptomatic, hospitalised, unvaccinated or with a comorbidity that typically presents with a high viral load.[33,34] In this study, we have not included patient populations with low viral loads (Ct >30), and this is a key limitation, as sample pooling may impair the assay's sensitivity. This is because pooling may increase the Ct, exceeding the LOD threshold and may result in a false negative error.[31,32] Another limitation to consider is that only one influenza A (containing the H1 surface haemagglutinin) and influenza B strain was used. Therefore, it is considered in future studies to test the assay's sensitivity and specificity to identify or detect different influenza A strains (e.g., H1N1, H5N1o or H3N2) and influenza B variants (Yamagata or Victoria lineages).

Sample pooling (1:4) is beneficial to laboratories challenged with high testing rates, by increasing the capacity by 4-fold. This is because performing rt-PCR is costly and time-consuming. Another factor to consider is that data entry, sample tracking, staff coordination and handling are more

Table 3 Serial dilution of the AusDiagnostics Synthetic Respiratory Positive Control to determine the limit of detection (LOD) for the AusDiagnostics SARS-CoV-2, Influenza and RSV 8-well assay

Dilution factor	Influenza A		Influenza A typing		Influenza B		RSV	
	Ct	RC	Ct	RC	Ct	RC	Ct	RC
Neat	18.2	133345	20.81	25048	19.1	76856	18.6	103275
0.1	22	8552	24.69	1543	22.9	4888	22.9	6324
0.01	26.4	503	28.61	122	26.7	408	26.7	447
0.001	30.1	48	C	C	30.5	34	30.2	41
0.0005	30.7	33	32.83	8	32.1	13	32.1	13
0.00025	32.1	13	ND	ND	33.4	6	33.4	10

C, check; ND, not detected; RC, relative concentration (in arbitrary units) is calculated relative to the internal control ('SPIKE') set at 10,000.

burdensome, so a systematic approach must be taken for efficiency, traceability, and accountability.[35–37] Additionally, sample pooling is most effective at low positivity rates. At higher positivity rates, the likelihood of a pool containing a positive patient is greater. Re-testing more positive samples individually would result in an increase in turnaround time and incur more reagent usage to de-pool the samples to identify the positive patient(s) within the pool.[12,37] Therefore, for laboratories considering employing sample pooling, it is suggested to monitor the positivity rate, thoroughly evaluate the assay's performance to determine the LOD, dynamic range and sensitivity of the assay, and consider the limitation of pooling of patients with low viral load.[31,32] Moreover, laboratories must outline a robust operational procedure in data and sample tracking, staff coordination and risk mitigation.[35–37] Although sample pooling to detect SARS-CoV-2 has been well reported, this study provides insight on the applicability of sample pooling to detect RSV and influenza A/B.

CONCLUSION

The AusDiagnostics SARS-CoV-2, Influenza and RSV 8-well assay demonstrated excellent agreement with the Cepheid Xpert Xpress Flu/RSV assay and high precision. The sample pooling analysis showed acceptable performance parameters for pools of four at high intermediate viral concentration, and this technique may be used to increase sample throughput and reduce reagent usage for this assay at low positivity rates.

Acknowledgements: We thank the staff members of the Concord Hospital Microbiology and Infectious Diseases Department for providing support to the team.

Conflicts of interest and sources of funding: The authors state that there are no conflicts of interest to disclose.

APPENDIX A. SUPPLEMENTARY DATA

Supplementary data to this article can be found online at https://doi.org/10.1016/j.pathol.2022.02.002.

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