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Evaluation of diagnostic performance of comprehensive respiratory virus panel: comparison of next-generation-sequencing to real-time polymerase chain reaction for detection of respiratory viruses

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Abstract

Background Respiratory viral infections remain a global health concern, particularly among children, the elderly, and immunocompromised individuals. Although real-time polymerase chain reaction (RT-PCR) is the diagnostic gold standard, its limitations in strain-level typing and mutation tracking highlight the need for complementary approaches such as next-generation sequencing (NGS).

Methods We compared a hybridization-based NGS respiratory virus panel (RVP) in comparison with RT-PCR using 81 nasopharyngeal swab (NPS) specimens. The performance metrics included concordance rates, cycle threshold (Ct)-based stratification, co-infection detection, and strain-level classification.

Results Among the 81 NPS specimens, RT-PCR identified respiratory viruses in 56 cases, including eight co-infections. Excluding co-infections, RVP showed 74.5% positive percent agreement, 92.3% negative percent agreement, and 80.8% overall accuracy. The detection and positive concordance rates declined with higher Ct values, and the sequencing depth also decreased. In co-infections, RVP failed to detect low-titer viruses. Strain-level classification was achieved in 65.5% of the positive samples, by subtyping rhinovirus A and C, respiratory syncytial virus A and B, and influenza A (H1N1 and H3N2).

Conclusions NGS panel tests complement RT-PCR by enabling viral detection and strain typing, thereby offering added value to genomic surveillance and outbreak investigations.

Keywords Next-generation sequencing, Multiplex real-time PCR, Respiratory tract infections, Genomic surveillance

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Background

Respiratory viral diseases continue to exhibit high global incidence and mortality rates, posing a substantial burden particularly in children, the elderly, and immunocompromised individuals [1]. Since the recent COVID-19 pandemic caused by SARS-CoV-2, resurgences of respiratory syncytial virus (RSV) and influenza have been reported, and the phenomenon of co-circulating epidemics becoming more pronounced [2]. These shifts highlight the growing need for precision diagnostic technologies that integrate early detection, pathogen identification, and epidemiological surveillance.

The current standard clinical method for respiratory viral detection is the real-time polymerase chain reaction (RT-PCR). RT-PCR has the advantages of high sensitivity and specificity, fast analysis speed, cost-effectiveness, and the ability to detect multiple pathogens [3]. However, because targets are set based on prior genetic information, virus sequences that are not included in the test probes cannot be detected, and false negatives may occur if the primer-binding site changes due to mutation [4]. In addition, this method can only determine the presence or absence of a virus, and is limited to genetic information or mutation analysis.

Target-based next-generation (NGS) sequencing panels have recently been used as diagnostic tools to overcome these limitations. These respiratory virus panels (RVPs) utilize hybridization-based capture methods to enrich viral genomic regions, thereby enabling comprehensive sequence-based analysis. In addition, they can support full genome reconstruction, mutation detection, co-infection confirmation, and phylogenetic analysis, making them applicable not only to clinical diagnostics but also to molecular epidemiology and public health fields [5, 6].

In this study, we compared the detection performances of RT-PCR and RVP using nasopharyngeal swab (NPS) specimens from patients with confirmed respiratory viral infections. This study aimed to determine the clinical usefulness of RVP as a platform complementary to conventional molecular diagnostic methods for respiratory viral detection.

Methods

Clinical specimens

A total of 81 clinical NPS specimens were obtained from patients with suspected respiratory viral infections at Gangnam Severance Hospital. The samples were stored at −80 °C in universal transport medium (Becton Dickinson, Sparks, MD, USA) until analysis.

RT PCR assay

Nucleic acids were extracted from 200 µl specimens using TANBead Nucleic Acid Extraction Kit (Taiwan Advanced Nanotech Inc., Taoyuan city, Taiwan), with a final elution volume of 100 µl. Extracted nucleic acids were then amplified and probed for respiratory viruses with AdvanSure™ RV-plus real-time RT-PCR (LG chemical Inc, Cheong-ju, Korea) using the SLAN-96P program.

According to the manufacturer's instruction, 5 µl of extracted nucleic acid was added to 5 µl of primer-probe mix and 10 µl of RT-PCR premix. Reverse transcription was performed at 50 °C for 10 min, followed by initial denaturation at 95 °C for 30 s. Amplification was carried out in two stages: 10 cycles (15 s at 95 °C and 30 s at 53 °C, and 30 s at 60 °C), followed by 30 cycles (15 s at 95 °C, 30 s at 53 °C, and 30 s at 60 °C) for fluorescence detection.

The AdvanSure assay enables simultaneous reverse transcription and multiplex amplification using internal controls that target human RNase P. The panel detected 17 respiratory viruses: adenovirus, bocavirus, coronavirus 229E, coronavirus NL63, coronavirus OC43, enterovirus, human metapneumovirus, influenza A, influenza B, parainfluenza 1, parainfluenza 2, parainfluenza 3, RSV-A, RSV-B, and rhinovirus A/B/C.

In addition, SARS-CoV-2 was detected using the PowerChek™ SARS-CoV-2 Real-time PCR Kit (Kogene Biotech, Seoul, Korea), targeting the E gene and the ORF1ab. The reactions were performed using a CFX 96 Touch Real-Time PCR Detection System (Bio-Rad, Hercules, CA, USA) under the conditions specified by the manufacturer. According to the manufacturer's instructions, a result was interpreted as positive if the cycle threshold (Ct) value was <35 for the AdvanSure™ RV-plus assay, and ≤ 38 for both target genes (E and ORF1ab) in the PowerChek™ SARS-CoV-2 Real-time PCR assay. Samples with higher Ct values or failures in internal control detection were considered invalid and were retested.

NGS-based RVP

The NGS assay was performed using the Comprehensive RVP (CRVP, Celemics, Inc., Seoul, Korea) according to the manufacturer's instructions. The CRVP was designed to target nine major respiratory virus types- Human Adenovirus, Human Bocavirus (type 1–4), Human Rhinovirus (A/B/C), Coronavirus, Human Enterovirus, Influenza A, Influenza B, Parainfluenza virus, and Respiratory Syncytial Virus-comprising 39 reference from the NCBI RefSeq database (Additional file1).

Libraries were prepared using a Celemics Library Prep Kit, followed by target enrichment using a Celemics Target Enrichment Kit. Sequencing was performed on an Illumina NextSeq 500 System using the Mid/High Output Kit v2.5 (300 cycles) with a 2 × 150 bp paired-end read length.

Bioinformatics analysis

The Celemics Virus Verifier was used for FASTQ data processing. The sequences were trimmed and filtered to remove adaptors and low-quality reads. The reads of human DNA sequences were computationally removed, and the remaining reads were aligned to the CRVP reference database to detect viral genomes and reconstruct consensus sequences. Genome coverage was calculated at a 1× depth threshold, which corresponded to the default reporting output of the Celemics Virus verifier pipeline used in this study.

Analytical sensitivity

Analytical sensitivity was assessed using three reference materials: SARS-CoV-2 (Twist Bioscience, South San Francisco, CA, USA), Human Bocavirus 1 (Twist Bioscience), and Human Rhinovirus A (ATCC, Manassas, VA, USA). Each sample was serially diluted in 2-fold steps across eight to 11 concentration levels. The dilution range was based on expected viral copy numbers per reaction : for SARS-CoV-2, from 100,000 to 156 copies; for Human Bocavirus 1, from 100,000 to 195 copies; and for Human Rhinovirus A, from 100,000 to 98 copies. The detection limit was determined as the mean of duplicate measurements across dilutions (Additional file2).

Statistical analysis

Concordant results between RT-PCR and NGS were defined as cases in which the same viral species detected by RT-PCR was also identified RVP, or in which both assays yielded negative results for that virus. These concordant cases, comprising both true positives and true negatives, were regarded as true results without further verification. In cases of discordance, RVP-positive/RT-PCR-negative results were interpreted as false-positives, and RT-PCR-positive/RVP-negative results as false negatives. Using RT-PCR as the reference, we calculated positive percent agreement. The positive percent agreement (PPA) (true positive / [true positive + false negative]), negative percent agreement (NPA) (true negative / [true negative + false positive]), and overall accuracy ([true positive + true negative] / total) were calculated to compare the performances of the two assays. The positive concordance rate was calculated as the (true positives / [toal RT-PCR positive cases]). Differences in sequencing depth and genome coverage across Ct-based subgroups were analyzed using the Kruskal-Wallis test, followed by pairwise Mann-Whitney U tests with Bonferroni correction where applicable. Statistical analyses were performed using Microsoft Excel 2010 (Microsoft, Seattle, WA, USA) and Analyse-it for Microsoft Excel Method Evaluation Edition version 3.70.1 (Analyse-it, UK). Statistical significance was set at $p < 0.05$.

Results

Clinical sample overview

Of the 81 NPS specimens analyzed, 56 (69.1%) tested positive for at least one respiratory virus by RT-PCR, whereas the remaining 25 (30.9%) yielded negative results. Among the positive cases, eight samples (9.9%) were co-infected with two different viruses. Nine viral strains were included in the analysis: adenovirus, bocavirus, coronavirus 229E, coronavirus NL63, human metapneumovirus, influenza A virus, respiratory syncytial virus, rhinovirus, and SARS-CoV-2. Among them, rhinovirus was the most common pathogen (34%), followed by bocavirus (21%).

Sequencing performance

A total of 54 viral targets were detected across all samples. Sequencing depth per virus ranged from 319,518 to 53,411,946 reads, with an average of 12.3 million reads per virus. The breadth of genome coverage at 1× depth varied considerably by viral type, with SARS-CoV-2 and coronavirus showing the highest coverage (99.7% and 98.4%, respectively), followed by influenza A (98.8%) and human bocavirus 1 (97.3%). In contrast, human rhinovirus C and A yield markedly lower coverage values (3.7% and 12.0%, respectively), suggesting reduced hybridization efficiency for these targets.

Among the frequently detected pathogens, respiratory syncytial virus ($N = 6$) and human adenovirus type 2 ($N = 4$) showed moderate-to-high genome coverage levels (86.3% and 81.6%, respectively). The average 1× genome coverage across all detected viruses was 56.6%, reflecting substantial variability in capture efficiency depending on viral genome structure and probe design.

Concordance between RT-PCR and RVP

To evaluate the agreement between RT-PCR and RVP, a total of 73 samples were included, excluding eight cases with co-infections. As summarized in Table 1, the overall PPA was 74.5% (95% CI, 59.7–86.1), the NPA was 92.3% (95% CI, 74.9–99.1), and the overall accuracy was 80.8% (95% CI, 69.9–89.1). The positive concordance rate between the two methods was 72.9%.

Most virus types showed high PPA values (> 95%), except for Rhinovirus, bocavirus and RSV, which exhibited a reduced PPA of 40.0%. In terms of NPA, most viruses achieved values exceeding 95%, except for bocavirus, which showed 94.7%, and RT-PCR-negative samples, which showed an NPA of 47.8%. Twelve viruses were detected exclusively by RT-PCR, including bocavirus, RSV, and rhinovirus, with bocavirus accounting for half of the discordant detections.

To further investigate the discordance between RVP and RT-PCR results, Ct values were compared between concordant and discordant samples. For SARS-CoV-2,

Table 1 Performance comparison between RT-PCR and RVP for each respiratory virus

Virus (*n*)	**RVP detected**	**PPA (%) [95% CI]**	**NPA (%) [95% CI]**	**Accuracy (%) [95% CI]**	**Positive concordance rate (%)**
Positive (48)	37	74.5 [59.7–86.1]	0.0 [0.0–97.5]	72.9 [58.2–84.7]	72.9
Rhinovirus (16)	12	80.0 [51.9–95.7]	97.0 [84.2–99.9]	91.7 [80.0–97.7]	75.0
Bocavirus (10)	6	40.0 [12.2–73.8]	94.7 [82.3–99.4]	83.3 [69.8–92.5]	40.0
Respiratory syncytial virus (6)	3	50.0 [11.8–88.2]	100.0 [91.6–100.0]	93.8 [82.8–98.7]	50.0
Adenovirus (4)	4	100.0 [39.8–100.0]	100.0 [92.0–100.0]	100 [92.6–100.0]	100.0
SARS-CoV-2 (4)	4	100.0 [39.8–100.0]	100.0 [92.0–100.0]	100 [92.6–100.0]	100.0
Coronavirus (3)	3	100.0 [29.2–100.0]	100.0 [92.1–100.0]	100 [92.6–100.0]	100.0
Influenza A virus (3)	3	100.0 [29.2–100.0]	100.0 [92.1–100.0]	100 [92.6–100.0]	100.0
Human metapneumovirus (2)	2	100.0 [15.8–100.0]	100.0 [92.3–100.0]	100 [92.6–100.0]	100.0
Negative (25)	36	96.0 [79.7–99.9]	47.8 [26.8–69.4]	70.9 [58.2–84.7]	–
Total (73)	73	74.5 [59.7–86.1]	92.3 [74.9–99.1]	80.8 [69.9–89.1]	–

This table summarizes the diagnostic performance of RVP in comparison with RT-PCR across 73 clinical samples. Metrics include PPA, NPA, overall accuracy and positive concordance rate for each viral type. Co-infection cases ($n=8$) were excluded from this analysis. PPA and NPA were calculated with 95% confidence intervals

the Ct value of the E gene (the lowest of the two target regions) was used for the analysis.

The mean Ct value for the concordant group ($n=45$) was 15.04, with a median of 14.31 and a range of 3.23–28.30. In contrast, the discordant group ($n=13$) exhibited a relatively higher mean Ct (21.15), a median of 22.44, and a range of 13.77–26.67 ($p<0.05$). These findings suggest that lower viral loads, as reflected by higher Ct values, may contribute to failure of RVP detection. The differences in Ct distributions between the groups are illustrated in Fig. 1.

Effect of Ct value on RVP detection and sequencing

To assess the impact of viral load on detection sensitivity, RT-PCR-positive samples ($n=48$) were stratified into three groups according to Ct values: high (<15, $n=19$), intermediate (15–22, $n=15$), and low (≥ 22, $n=14$). As shown in Fig. 2, both RVP detection rate and positive concordance rate declined progressively with increasing Ct values. The high-viral load group exhibited a detection and concordance rate of 94.74%, while the intermediate and low groups showed rates of 73.33% and 50.00%, respectively. In this analysis, the detection rate refers to the proportion of RT-PCR-positive samples in which viral sequences were detected by RVP.

To further understand this trend, NGS sequencing depth and genome coverage were compared across groups. The mean read depth decreased from 15.99 million reads in the high group to 10.07 million in the low group, and genome coverage declined from 63.43 to 60.93%. Although the overall differences were not statistically significant when all 48 samples were analyzed, viral type-specific outliers were identified. To minimize bias from these outliers-namely, Rhinovirus and SARS-CoV-2, which showed extremely low and near-complete coverage, respectively-these samples were excluded from a subgroup analysis. After exclusion, sequencing depth differed significantly among Ct groups ($p=0.03$), while coverage remained non-significant ($p=0.11$). These findings suggest that reduced template concentration in high-Ct value samples may contribute to insufficient hybridization or incomplete genome capture, thereby lowering the detection sensitivity of RVP.

Co-infection analysis

Among the clinical samples, eight cases (9.9%) were identified as co-infections involving two respiratory viruses based on the RT-PCR results. The most frequently observed pathogens were rhinovirus ($n=6$) and bocavirus ($n=5$), followed by adenovirus and RSV ($n=3$). In two of the eight co-infection cases, RVP successfully detected both viruses, demonstrating complete agreement with the RT-PCR results. In the remaining six cases, only one of the two viruses was identified using RVP, resulting in partial concordance.

To investigate the cause of detection failure, Ct values were compared between viruses that were successfully detected by RVP and those that were not. The mean Ct value of viruses detected by RVP in the co-infection cases was 12.74, whereas that of undetected viruses was significantly higher at 21.47 ($p<0.05$), indicating that lower viral loads may have contributed to missed detection.

A similar trend was observed when analyzed according to viral type. For rhinovirus, the mean Ct of detected cases was 10.72, compared to 19.12 for undetected cases. For bocaviruses, the Ct values were 7.91 (detected) versus 22.35 (undetected). These findings highlight the limited sensitivity of RVP under co-infection conditions, especially in the presence of low-titer viral targets.

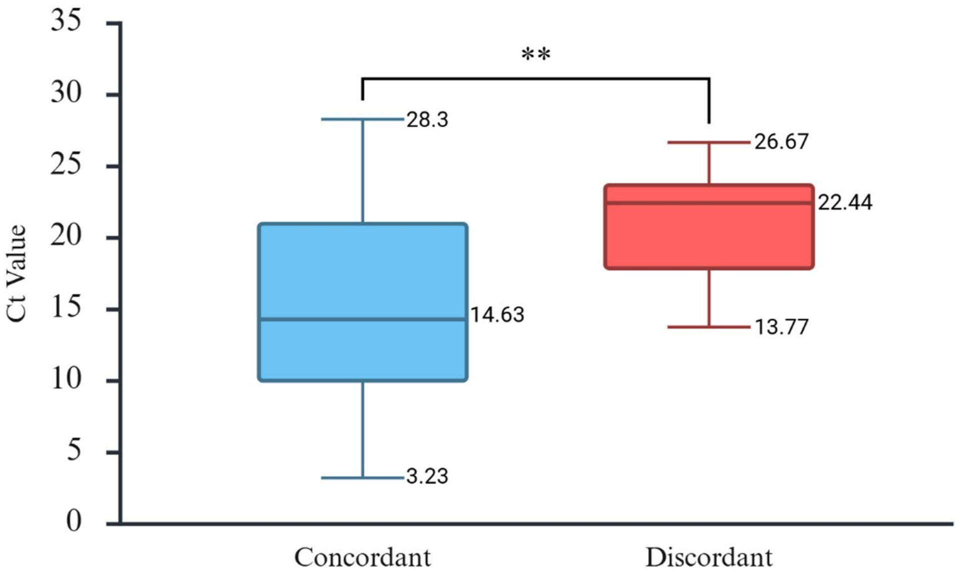

Fig. 1 Distribution of Ct values in concordant and discordant cases between RT-PCR and RVP. Cycle threshold (Ct) values were compared between samples with concordant RVP results ($n = 35$) and those with discordant results ($n = 13$). The discordant group exhibited significantly higher Ct values, with a median of 22.44 and a mean of 21.71, compared to the concordant group (median 14.63, mean 15.38). These findings suggest that lower viral loads are associated with discordant detection by RVP ($p < 0.05$)

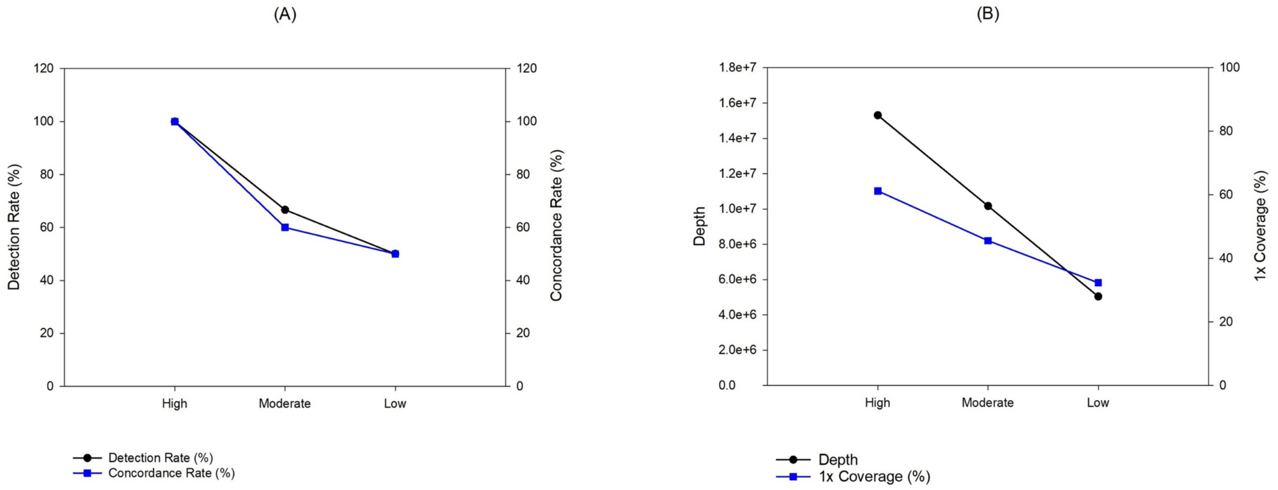

Fig. 2 RVP detection performance and sequencing depth by Ct value group. **A** Detection and positive concordance rate (%) between RVP and RT-PCR across Ct categories: High (<15), Intermediate (15–22), and Low (≥22). Both performances decline with increasing Ct value. **B** NGS read depth and genome coverage across Ct groups. Lower Ct groups had higher sequencing depth and broader genome coverage

Strain-level typing performance

In addition to detecting the presence of respiratory viruses, RVP enables strain or subtype-level classification of a substantial proportion of RT-PCR-positive cases. A total of 55 RT-PCR-positive samples from the four major virus types were evaluated for subtype resolution (Table 2). Rhinovirus, the most frequently detected virus, was subtyped in 16 of 22 RT-PCR-positive cases (72.7%). Among them, nine were classified as type A and one as type C, with six cases showing co-detection of both subtypes in the same specimen. The influenza A virus was successfully subtyped in all three RT-PCR-positive samples, with the identification of H3N2 ($n = 1$) and H1N1 ($n = 2$). For adenoviruses and bocaviruses, RVP identified Human Adenovirus type 2 ($n = 4$) and co-detection of type 2 and 5 ($n = 1$) and Human Bocavirus 1 ($n = 7$), respectively.

Discussion

Respiratory viruses are genetically diverse pathogens that are responsible for global health crises. Early detection and genomic surveillance are crucial for managing both endemic and emerging respiratory viruses. In this study, we evaluated the performance of a hybridization-based

Table 2 Strain-level classification of respiratory viruses by RVP in RT-PCR-positive samples

RT-PCR	NGS subtype	Case
Rhinovirus (22)	Human Rhinovirus A	9
	Human Rhinovirus C	1
	Human Rhinovirus A and C (co-detected)	6
Bocavirus	Human Bocavirus 1	7
Adenovirus	Human adenovirus type 2	4
	Human adenovirus type 2 and 5 (co-detected)	1
Influenza A Virus	Influenza A-H1N1	2
	Influenza A-H3N2	1

NGS enabled precise subtyping of viruses initially identified by RT-PCR, including differentiation of human rhinovirus A and C, as well as co-detection of multiple subtypes within the same sample. Additionally, NGS distinguished adenovirus and influenza A virus into specific genotypes (e.g., HAdV-C2, H1N1, H3N2), which are not discernible by RT-PCR. This highlights the enhanced resolution and epidemiological utility of NGS-based diagnostics. Case counts represent the number of samples in which each subtype was detected. Subtyping was based on ≥ 1× genome coverage thresholds

NGS panel (RVP) in comparison with conventional RT-PCR using 81 clinical NPS samples.

The overall accuracy of RVP was 80.8%, with a high agreement observed in samples with high viral loads (Ct < 15). These findings affirm that viral load is a major determinant of NGS-based respiratory viral detection, which is consistent with previous reports [7–9]. The detection sensitivity of RVP decreased markedly in samples with higher Ct values, with a detection rate of 50% in the Ct ≥ 22 group.

The observed decline in read depth and genome coverage in low-viral-load samples suggests that hybridization-based enrichment is less effective when the viral nucleic acid abundance is limited [10]. This limitation also affected the detection of co-infections; RVP failed to identify one of the pathogens in 75% of dual-positive cases, typically when the undetected virus had a higher Ct value. These results highlight the importance of considering the viral load and potential competition during enrichment when interpreting NGS results [9].

Beyond its detection performance, RVP demonstrated added value in strain-level typing. In this study, the panel successfully provided subtype information for rhinovirus (types A and C), RSV (types A and B), and influenza A viruses (H1N1 and H3N2). However, for RSV and rhinoviruses, the clinical or epidemiological usefulness of strain-level typing remains to be established, as these subtypes rarely influence disease management or clinical outcomes. Nevertheless, such genomic resolution cannot be achieved using conventional RT-PCR and may offer important advantages in epidemiological surveillance, outbreak investigations, and public health responses.

However, the assay has virus-specific limitations. For instance, bocavirus, which has a single-stranded DNA genome, demonstrated the lowest agreement between RVP and RT-PCR (PPA: 40.4%). Its genomic structure and poor compatibility with RNA-targeted enrichment protocols may explain its low detection performance. Rhinovirus also showed inconsistent typing results, likely due to their extensive genetic heterogeneity and high mutation rates, which reduce capture probe efficiency and lead to uneven sequencing coverage. These findings suggest the need to improve the probe design or sequencing depth to enhance detection accuracy [2, 11]. RSV subtyping using RVP revealed notable discrepancies. RT-PCR identified six RSV A and three RSV B cases; however, RVP misclassified two RSV-A samples as RSV-B, and failed to detect two high-Ct RSV-A samples (Ct 25.81 and 26.67). Follow-up Sanger sequencing with external primers confirmed one sample as RSV-A, and the remaining sample was unresolved owing to low template availability. These cases underscore the need to improve sequencing depth and analytical stringency to achieve reliable strain-level discrimination of RSV and other genetically diverse viruses [12].

To validate the performance of the assay, we tested 25 RT-PCR-negative clinical samples and three synthetic control materials (SARS-CoV-2, rhinovirus A, and bocavirus 1). These results aided in defining the performance thresholds and validating detection specificity. However, the absence of standardized cut-offs for genome coverage and alignment metrics may affect reproducibility across laboratories.

Although the traditional barriers to clinical NGS adoption include high costs, slow turnaround time, and interpretive complexity, they are being addressed through technical advancements. Notably, RVP analysis, which requires up to seven days, can now be completed within two days, making it more feasible for clinical use [7].

Although the detection sensitivity of RVP in low-titer samples remains inferior to that of RT-PCR, its ability to resolve strain-level information, identify coinfections, and track variant emergence makes it a valuable complementary tool in molecular virology [13–16]. These advantages are particularly beneficial in outbreak scenarios, unexplained infections, and situations involving novel or vaccine escape variants.

Conclusions

In summary, NGS panels, such as RVP, are not intended to replace RT-PCR in routine diagnostics, but should instead be integrated as adjunct tools that extend clinical and epidemiological insights. Continued improvements in sequencing protocols, capture efficiency, and software pipelines, along with better standardization, will further enhance their clinical utility.

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s12941-026-00851-w.

Supplementary Material 1

Supplementary Material 2

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Author contributions

SL and YK contributed equally to this work and are co-first authors. SL conceptualized the study protocol, coordinated sample processing, and drafted the initial manuscript. YK performed bioinformatic analysis, interpreted the results, and contributed to manuscript revision. KL supervised the entire study, provided clinical expertise, and finalized the manuscript. All authors read and approved the final manuscript.

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Data availability

All data generated or analysed during this study are included in this published article and its supplementary information files.

Declarations

Ethics approval and consent to participate

This study was approved by the Institutional Review Board of the Gangnam Severance Hospital (IRB number: 3-2022-0348).

Consent for publication

Not applicable.

Competing interests

The authors declare no competing interests.

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