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Coronavirus Pandemic

Low-cost high-throughput targeted sequencing for the accurate detection of respiratory tract pathogens

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Abstract

Introduction: The current gold standard for severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) diagnosis by real-time reverse transcriptase polymerase chain reaction (RT-PCR) is limited by the number of genes that can be detected. In this study, we developed a low-cost and high-throughput next-generation sequencing technology that can overcome the limitations of real time RT-PCR.

Methodology: A targeted sequencing panel (TSP) consisting of approximately 500 amplicons was designed. This panel could simultaneously detect a broad range of gene loci of SARS-CoV-2, and genes for the most common infectious viruses that affect the respiratory tract, in a single run and could include up to 96 samples. Four hundred and forty-eight samples and 31 control samples were analyzed independently with both TSP and RT-PCR, and the results were compared for accuracy and other indicators.

Results: TSP identified 50 SARS-CoV-2 positive samples with a 99.33% match to RT-PCR results. It is not surprising that TSP also identified multiple infections from the 96 samples, whereas RT-PCR could not. Thus, TSP was able to accurately diagnose the samples which could not be identified based on single RT-PCR test.

Conclusions: Our data demonstrated that TSP is a fast and accurate testing method for identifying multiple pathogen infections of the respiratory tract.

Key words: targeted sequencing; respiratory tract pathogens; SARS-CoV-2; real-time PCR.

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Introduction

The severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) outbreak, known as coronavirus disease 2019 (COVID-19), posed a massive threat to public health worldwide [1]. According to the World Health Organization (WHO), over 760 million cases and 6.9 million deaths were recorded globally in the period from December 2019 to August 2023, and it is believed that the actual number could be higher. The coronavirus genome contains at least ten open reading frames (ORFs), among which the first ORF (*ORF1ab*) constitutes about two-thirds of the viral RNA. Four major structural proteins including spike protein (S), membrane protein (M), envelope protein (E), and nucleocapsid protein (N) are encoded by the additional SARS-CoV-2 ORFs, which make up one-third of the genome [2,3]. The current gold standard for SARS-CoV-2 diagnosis is the detection of viral RNA via real-time reverse transcriptase polymerase chain reaction (RT-PCR) assay with primers specifically designed to target a few genes, mostly ORFs and *N* genes. The limitations of this technology may negatively affect detection rates and result in false negative reports [4]. For example, a number of deletions were observed in *ORF8* and further deletion variants may emerge due to immune-driven selection [5]. Phan *et al.* performed a genetic analysis of 86 complete or near-complete SARS-CoV-2 genomes, revealing many mutations and deletions in coding and non-coding regions [6]. Khailany *et al.* identified 116 mutations in the *ORF1ab* gene [7]. It has been widely proven that high mutation rate drives genome variation and the evolution of viruses, thus enabling the virus to evade host immunity and develop drug resistance [8].

Detection of other common viral pathogens that typically cause seasonal respiratory tract infection has mainly been neglected over the years. Studies have shown that there may be synergistic effects of coinfection compared to SARS-CoV-2 infection alone, resulting in a higher risk of death in patients [9]. Patients with SARS-CoV-2 coinfected with other respiratory viruses are more likely to be admitted to the intensive care unit (ICU) [10]. Most respiratory infections, however, have similar clinical symptoms and are indistinguishable from SARS-CoV-2, and current routine laboratory tests alone have failed to distinguish SARS-CoV-2 from other respiratory viral infections [11]. Therefore, there is urgent need for high-throughput and low-cost methods for precision diagnosis of respiratory viral infections. Here, we developed a high-throughput targeted sequencing panel (TSP) of approximately 500 amplicons that simultaneously detects a wide range of genetic loci for both SARS-CoV-2 and other common respiratory viruses. This diagnostic method is low cost and has a faster turnaround time.

Methodology

Samples

This study was approved by the Institutional Review Board of the Nanshan Center for Disease Control and Prevention and conducted at Guangdong Ardent BioMed Co., Ltd. Written informed consent was obtained from all subjects. Four hundred and forty-eight nasopharyngeal swabs, 15 synthetic positive controls, and 16 non-template controls (NTCs) were evaluated in this study. Four hundred and nineteen samples were collected from the SARS-CoV-2 screening population in the Nanshan Center for Disease Control and Prevention, and 29 from the fever clinic of Guangzhou Eighth People's Hospital. Nasopharyngeal swabs were immersed in 3 mL of Hank's solution and transferred to the laboratory within 2 hours.

Design of TSP

The primers were designed based on the information from a database of 38 pathogens and 528 loci that are listed in Table 1. These 38 pathogens include the most prevalent respiratory viruses.

DNA/RNA extraction and RT-PCR

The total DNA/RNA was extracted using the virus DNA/RNA extraction kit (Tianlong Technology, Xian, China). Briefly, samples were vortexed for 15 seconds, and 200 µL of each sample was used for DNA/RNA extraction. RT-PCR amplification was then performed on the PCR system (ABI 7500, Thermo Fisher Scientific, Waltham, MA, U.S.A.) using a commercial SARS-CoV-2 detection kit (Daan Gene, Guangzhou, China) following the instructions provided with the kit. This method targets specific genomic regions of SARS-CoV-2, O*RF1ab,* and *nucleocapsid* (*N*) genes. Samples were considered positive if the Ct value of FAM and VIC channels were ≤ 37.0, or, the Ct value was between 37 and 40 for duplicated tests.

Library preparation and sequencing

A DNA AmpliSeq library was established by multiplex RT-PCR. Briefly, 1 µL extracted DNA was mixed with 5 µL 2× primer panel, 2 µL AMPure XP containing 5× amplification enzyme mixture, Master Mix, sequence beads, and 2 µL DNase-free water. PCR primers that were complementary to the adapters of the library were linked to the surface of the beads. The target fragment amplification was carried out through PCR reaction with 1 cycle at 99 ℃ for 2 mins; 1 cycle at 99 ℃ for 15s, 20 cycles at 60 ℃ for 4 mins, followed by another cycle of synergy by adding 2 µL synergist

Table 1. Genes or pathogens for targeted sequencing panel.

Pathogens	Types	Locus number	Gene or pathegens
SARS-CoV-2	1	66	N, E, S of *Orf1ab*
Other coronaviruses	7	23	SARS-CoV, MERS-CoV, HCoV-229E, HCoV-HKU1, HCoV-NL63, HCoV-OC43 and EboV
Common respiratory RNA viruses	12	48	H9N2, H5N1, H3N2, H2N2, H7N9, H1N1, Flu-B, HPIV, HRSV-A, HRSV-B, HMPV-A and HMPV-B
Common respiratory DNA viruses	16	389	HAdV-5, HAdV-A, HAdV-B, HAdV-C, HAdV-D, HAdV-E, HHV-1, HHV-2, HHV-3, HHV-4, HHV-5, HHV-7, HPV-B19, JCPyV, SV40 and BKV
C. glabrata and *C. parapsilosis*	2	2	

BKV: bk virus; EboV: ebola virus; H9N2: influenza A (H9N2); ORF: open reading frame; Flu-B: influenza B; H1N1: influenza A (H1N1); H2N2: influenza A (H2N2); H3N2: influenza A (H3N2); H5N1: influenza A (H5N1); H7N9: influenza A (H7N9); HAdV: human adenovirus; HCoV-229E: human Coronavirus 229E; HCoV-HKU1: human coronavirus HKU1; HCoV-NL63: human coronavirus NL63; HCoV-OC43: human coronavirus OC43; HHV: human herpesvirus; HMPV: human metapneumovirus; HPIV: human parainfluenza virus; HPV-B19: human parvovirus B19; HRSV: human respiratory syncytial virus; JCPyV: John Cunningham polyomavirus; MERS-CoV: Middle East respiratory syndrome coronavirus; SARS-CoV: severe acute respiratory syndrome coronavirus; SARS-CoV-2: severe acute respiratory syndrome coronavirus 2; SV40: simian vacuolating virus 40. *C. glabrata: Candida glabrata*; *C. parapsilosis*: *Candida parapsilosis*.

Table 2. Standard read numbers for the limit of detection of the SARS-CoV-2 virus.

Samples	SARS-CoV-2 standard			SARS-CoV standard	Result
	ORF1ab	S	E		
human RNA	0	0	0	0	Negative
human RNA + 1 pg/mL standard	70118	0	0	95887	Positive
human RNA + 0.1 pg/mL standard	5310	0	0	2341	Positive
human RNA + 0.01 pg/mL standard	240	0	0	327	Positive
human RNA + 0.001 pg/mL standard	1	0	0	0	Negative
water (blank)	0	0	0	0	Negative

SARS-CoV-2: severe acute respiratory syndrome coronavirus 2; ORF: open reading frame.

reagent and continued with 50 ℃ for 10 mins, 55 ℃ for 10 mins, 60 ℃ for 20 mins, and 10 ℃ hold. The end product was mixed with 2 μL linker mixture, 1 μL DNA ligase, and 1 μL sequence linker; and linker ligation was performed at 22 ℃ for 30 mins, 68 ℃ for 5 mins, 72 ℃ for 5 mins, and hold at 16 ℃ prior to purification. The final product was eluted with DNase-free water and mixed with 1 μL library enrichment primers, and 5 μL library enrichment enzyme mixture, to enrich the amplified cDNA strand. Purified magnetic beads were then added to the enriched library for the second round of purification, and 25 μL TE buffer was used for elution. The product was subjected to sequencing on Ion Torrent PGM Sequencing System (Life Technologies, Shanghai, China) after qualification with Agilent 2100 Bioanalyzer (Agilent, California, U.S.A).

Data processing and quality control

Initial quality control was performed to retain the reads with double-ended length > 60 bp. In order to generate high-quality data, a secondary quality control was performed to retain the reads with Q30 > 85%. Sequencing depth, uniformity, and on-target rate were also evaluated.

Bioinformatic analysis

Sequencing data were submitted to the Partek analysis pipeline for pathogen detection. Each virus's pathogen detection results and genome coverage were obtained in base space sequence analysis. A sample was considered positive for SARS-CoV-2 when there were more than three distinct regions' amplicons in this panel. The results of other common viruses were considered positive if there were more than three reads found at any locus. The heatmap of the reads number of SARS-CoV-2 was generated using online bioinformatic tools [12].

Determination of accuracy

Performance indicators of samples were calculated by comparing the sequencing results with RT-PCR results. Five performance indicators, including positive percent agreement (PPA), negative percent agreement (NPA), accuracy, false negative rate (FNR), and false positive rate (FPR), were evaluated.

Determination of assay efficiency

To determine the limit of detection (LOD) of SARS-CoV-2 virus, we used titers of healthy human RNA mixed with SARS-CoV-2 and SARS-CoV virus standards (1 pg, 0.1 pg, 0.01 pg, and 0.001 pg). These titers of isolated virus standards were measured in SI derived units (pg/mL). The sequencing process was conducted according to the next generation sequencing (NGS) technique described above.

Results

Limit of detection for SARS-CoV-2

Analytical sensitivity was assessed by determining the LOD of healthy human RNA containing SARS-CoV-2 and SARS-CoV standards. The SARS-CoV-2 standard (1, 0.1, 0.01, and 0.001 pg) was mixed in four copies of human RNA as positive control, and human RNA with water as negative control. The LOD was determined to be 0.01 pg/mL standard (Table 2).

Metric performance evaluation

All 448 samples were subjected to TSP on the Ion Torrent Platform (Life Technologies, China) independent of RT-PCR assay. One TSP run included 96 samples, and 11 batches of 448 samples were

Table 3. Sequencing results: concordance with reverse transcriptase polymerase chain reaction (RT-PCR).

		RT-PCR		
		Positive	Negative	Total
SARS-CoV-2 targeted sequencing panel	Positive	49	1	50
	Negative	2	396	398
	Total	51	397	448

SARS-CoV-2: severe acute respiratory syndrome coronavirus 2.

sequenced with 15 positive controls and 16 negative controls in each run. The overall accuracy of SARS-CoV-2 detection with RT-PCR was 99.33%. The PPA and NPA of the targeted sequencing were 96.08% and 99.75%, respectively (Table 3). We calculated PPA, NPA, FPR, FNR, accuracy from the true positive (TP), true negative (TN), false positive (FP), false negative (FN), as below.

$$\text{PPA} = \frac{\text{TP}}{\text{TP} + \text{FN}} = \frac{49}{49 + 2} = 96.08\%$$

$$\text{NPA} = \frac{\text{TN}}{\text{TN} + \text{FP}} = \frac{396}{396 + 1} = 99.75\%$$

$$\text{Accuracy} = \frac{\text{TP} + \text{TN}}{\text{All results}} = \frac{49 + 396}{448} = 99.33\%$$

$$\text{FPR} = \frac{\text{FP}}{\text{FP} + \text{TN}} = \frac{1}{1 + 396} = 0.25\%$$

$$\text{FNR} = \frac{\text{FN}}{\text{FN} + \text{TP}} = \frac{2}{2 + 49} = 3.92\%$$

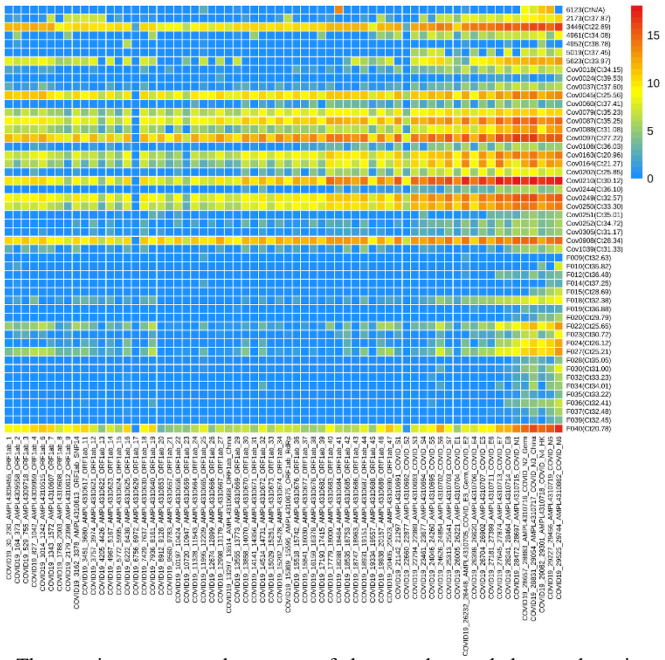
TSP of SARS-CoV-2

Sixty-six loci of SARS-CoV-2 were screened through targeted sequencing and 50 samples were found positive. The numbers of various loci in the reads were significantly different ranging from 0 to 432,369. A heatmap of SARS-CoV-2 was generated with logarithmic value of reads numbers with a base of 2 from 50 positive samples (Figure 1). According to Figure 1, Cov0018-Cov1039 had more reads compared to F009-F039. One sample was confirmed positive based on TSP data, while it had negative RT-PCR results. In addition, four samples had undecided results in a single RT-PCR test, out of which two were confirmed positive through TSP.

TSP of other respiratory pathogens

Of the 448 samples, n = 5 tested concurrent infections of SARS-CoV-2 with other pathogens; n = 96 tested positive for at least one pathogen except SARS-CoV-2, among which 21 detected as coinfections of at least two pathogens. Among the other respiratory viral pathogens detected, human herpesvirus (HHV, n = 68), human parainfluenza virus (HPIV, n = 7), and H1N1 influenza virus (n = 6) were the most detected pathogens. 37.9% (11/29) of patients at the fever clinic had HHV infections, while 13.6% (57/419) of the SARS-CoV-2 screening population carried HHV. There was a statistical difference in the prevalence of HHV between the patients and the asymptomatic

Figure 1. Heatmap of numbers of reads of SARS-CoV-2 by targeted sequencing.



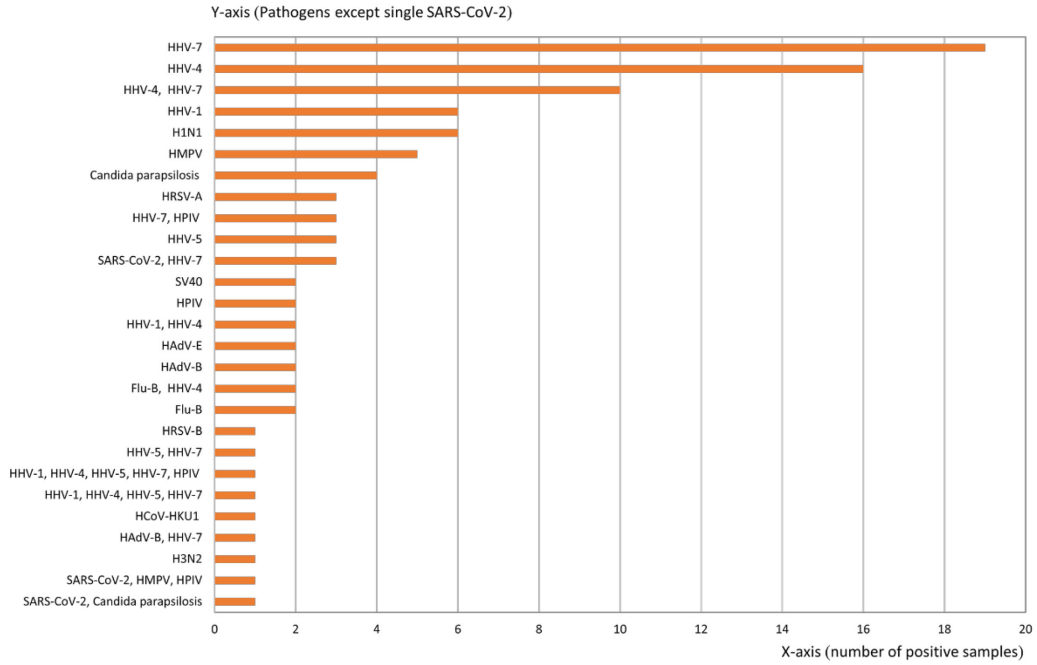
The y-axis represents the names of the samples, and the numbers in brackets are the RT-PCR Ct values of the *orf1ab* gene. The x-axis represents the names of the loci detected. The various colors denote the read numbers, expressed as a logarithmic value with a base of 2. RT-PCR, reverse transcriptase polymerase chain reaction; SARS-CoV-2, severe acute respiratory syndrome coronavirus 2.

population ($\chi^2 = 12.468$, $p<0.05$). Other viral pathogens detected were human metapneumovirus (HMPV, n = 5), human adenovirus (HAdV, n = 5), human respiratory syncytial virus (HRSV, n = 4), and influenza B (Flu-B, n = 4) (Figure 2). Among the five samples with *Candida parapsilosis* infection, the reads numbers were 5, 10, 173, 431, and 110885; and one of them was a coinfection with SARS-CoV-2.

Discussion

The spread of COVID-19 worldwide brought huge pressure to public health services [13,14]. RT-PCR has become the standard method for screening and diagnosis for virus infection. Nonetheless, while it demonstrated huge advantage in large population screening, the limitation with the targeted microorganism, as well as high rate of false positivity hinders the use of this technology for diagnosis of multiple co-infections. NGS technology has been used not only to demonstrate the origin of the novel coronavirus, but also for virus detection, transmission, and mutation monitoring [15-17]. In this study, we used a TSP by combining specific biotinylated probes and hybrid capture enrichment that targeted 36 viruses and two fungi in one panel. The data demonstrated that TSP

Figure 2. Respiratory pathogens detected by the targeted sequencing panel



The x-axis represents the number of positive samples, and the y-axis represents the respiratory pathogens detected by targeted sequencing. H1N1, influenza A (H1N1); HAdV, human adenovirus; HCoV-HKU1, human coronavirus HKU1; HHV, human herpesvirus; HMPV, human metapneumovirus; HPIV, human parainfluenza virus; HRSV, human respiratory syncytial virus; flu, influenza; SARS-CoV-2, severe acute respiratory syndrome coronavirus 2; SV40, simian vacuolating virus 40.

can identify multiple co-infections simultaneously, develop precision diagnosis of questionable samples from RT-PCR, and lower cost that might have been incurred from repeated testing with RT-PCR.

The performance evaluation showed that our panel had a SARS-CoV-2 LOD of 0.01 pg/mL, making the analysis highly sensitive and accurate for detecting the SARS-CoV-2 genome. The consistency between the TSP and RT-PCR was 99.33%. Performance evaluation showed that PPA, NPA, FPR, and FNR were 96.08%, 99.75%, 0.25%, and 3.92%, respectively.

Our sequencing results showed that one sample that was negative for *ORF1ab* when tested with RT-PCR, had a high copy fragment of the *ORF1ab* gene. The TSP also helped to confirm another two samples that were questionable based on RT-PCR data, highlighting the advantage of TSP in assisting RT-PCR in improving the true positive detection rate of the diagnosis.

NGS includes PCR amplicon sequencing, target enrichment sequencing, and metagenomic sequencing [18]. The advantage of targeted sequencing over the amplicon-based method is that it is based on many fragments and probes. It can also significantly reduce the sequencing depth, lower the detection cost, and has more accessible analytical performance compared to metagenomic sequencing [19-21].

There are no standard criteria for targeted sequencing results interpretation. Thorburn *et al.* deemed samples with fewer than 10 unique viral reads to be negative by NGS [22]. However, in the study by Gaston *et al.*, raw read counts for viruses of ≥1 were taken forward for result interpretation [20]. In our study, we considered a sample as positive for SARS-CoV-2 or other virus when there were amplicons of more than three distinct regions in the panel. The results of other common viruses were considered positive if there were more than three reads found at any locus.

This targeted sequencing relies on educated microbial prediction that may limit it from identifying unknown pathogens. Moreover, we detected only viruses, but not bacteria in this study. The limited sample size may affect the geographical representation.

Conclusions

We developed a custom-designed TSP with high sensitivity and specificity for detecting SARS-CoV-2 and other respiratory viruses using Ion Torrent's Enrichment Workflow, hybrid capture method, and bioinformatics pipeline. The low cost and high sample throughput analysis make it suitable for large-scale accurate detection of respiratory viruses.

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