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Integrated characterization of SARS-CoV-2 genome, microbiome, antibiotic resistance and host response from single throat swabs

Bo Lu[1,2,11], Yi Yan[3,4,5,6,11], Liting Dong[1,2,11], Lingling Han[7,11], Yawei Liu[8,11], Junping Yu[3], Jianjun Chen[3,4,5], Danyang Yi[1], Meiling Zhang[1], Chao Wang[7], Runkun Wang[7], Dengpeng Wang[7*], Hongping Wei[3*], Di Liu[3,4,5,6,9*] and Chengqi Yi[1,2,10*]

[1]*State Key Laboratory of Protein and Plant Gene Research, School of Life Sciences, Peking University, Beijing 100871, China*

[2]*Peking-Tsinghua Center for Life Sciences, Peking University, Beijing 100871, China*

[3]*CAS Key Laboratory of Special Pathogens, Wuhan Institute of Virology, Center for Biosafety Mega-Science, Chinese Academy of Sciences, Wuhan 430071, China*

[4]*National Virus Resource Center, Wuhan Institute of Virology, Chinese Academy of Sciences, Wuhan 430071, China*

[5]*Computational Virology Group, Center for Bacteria and Viruses Resources and Bioinformation, Wuhan Institute of Virology, Chinese Academy of Sciences, Wuhan 430071, China*

[6]*University of Chinese Academy of Sciences, Beijing 101409, China*

[7]*GrandOmics Biosciences, Wuhan, Hubei 430000, China*

[8]*Department of Gastroenterology, The First Medical Center of PLA General Hospital/Chinese PLA Postgraduate Military Medical School, Beijing 100853, China*

[9]*First Affiliated Hospital of Xinjiang Medical University, Urumqi 830054, China*

[10]*Department of Chemical Biology and Synthetic and Functional Biomolecules Center, College of Chemistry and Molecular Engineering, Peking University, Beijing 100871, China*

[11]*These authors contributed equally*

[*]*Correspondence to: chengqi.yi@pku.edu.cn (C.Y.), liud@wh.iov.cn (D.L.), hpwei@wh.iov.cn (H.W.) and wangdp@grandomics.com (D.W.)*

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Abstract

The ongoing coronavirus disease 2019 (COVID-19) pandemic, caused by severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) infection, poses a severe threat to humanity. Rapid and comprehensive analysis of both pathogen and host sequencing data is critical to track infection and inform therapies. In this study, we performed unbiased metatranscriptomic analysis of clinical samples from COVID-19 patients using a newly-developed RNA-seq library construction method (TRACE-seq), which utilizes tagmentation activity of Tn5 on RNA/DNA hybrids. This approach avoids the laborious and time-consuming steps in traditional RNA-seq procedure, and hence is fast, sensitive and convenient. We demonstrated that TRACE-seq allowed integrated characterization of full genome information of SARS-CoV-2, putative pathogens causing coinfection, antibiotic resistance and host response from single throat swabs. We believe that the integrated information will deepen our understanding of pathogenesis and improve diagnostic accuracy for infectious diseases.

Keywords: SARS-CoV-2, COVID-19, metatranscriptomics, coinfection, antibiotic resistance, host response, TRACE-seq, Tn5.

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Introduction

Longstanding, emerging, and re-emerging infectious diseases continuously threaten human health across centuries(1). Precise and rapid identification of pathogens from clinical samples is important for both guiding infection treatment strategies and monitoring novel infectious disease outbreaks, e.g. the outbreak of SARS-CoV-2, in the community. While most nucleic acid amplification-based and pathogen specific antibody detection-based molecular techniques only detect a limited number of pathogens and need their prior knowledge, metagenomic or meta-transcriptomic approaches allow for comprehensive and unbiased identification and characterization of microbiome directly from clinical specimens(2).

Compared to meta-genomic sequencing, meta-transcriptomic sequencing has several distinct advantages: it permits detection of RNA viruses that would not be interpreted in metagenomic data, reveals transcriptionally active organism(s) which are more etiologically important, and indicates host immune response which is essential to distinguish true pathogens from colonizers(3-5). However, the laborious and time-consuming steps in traditional RNA-seq experiments hinder the development of meta-transcriptomic based clinical diagnostics for rapid pathogen identification.

Very recently, we and others have independently developed a rapid and cost-effective RNA-seq method, based on Tn5 tagmentation activity towards RNA/DNA hybrids(6, 7). Our method, termed “TRACE-seq”, enables rapid one-tube library construction for RNA-seq experiments and shows excellent

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72 performance in compsrison to traditional RNA-seq methods. We thus
73 envisioned that this convenient and sensitive method could be applied to
74 clinical specimens for unbiased meta-transcriptomic analysis. In this study, we
75 modified the TRACE-seq procedure, shorten the total time and optimized
76 analytical pipeline to meet the needs for clinical meta-transcriptomic diagnosis
77 and analysis. We then applyed TRACE-seq to meta-transcriptomic sequencing
78 of single throat swabs specimens from COVID-19 patients and healthy
79 individuals. We found library construction of specimens could be accomplished
80 in ~2h with high quality. Analysis of TRACE-seq meta-transcriptomic data of 13
81 SARS-CoV-2 positive samples and 2 negative samples demonstrated the
82 success of this method to sensitively detect SARS-CoV-2 with high coverage
83 even for samples with relatively high Ct values, or to assemble unknown
84 microbe genome *de novo* (using SARS-CoV-2 as an example). Moreover,
85 TRACE-seq sensitively detected the microbiome and simultaneously allowed
86 for interrogating antibiotic resistance and host responses. Taken together,
87 TRACE-seq enables unbiased pathogen detection and could have broad
88 applications in meta-transcriptomic study and clinical diagnosis.
89
90 **Results**
91 **TRACE-seq enables metatranscriptomic analysis**
92 To perform metatranscriptomic analysis on clinical samples, such as throat
93 swabs in this study, we made several modifications to TRACE-seq. First, to
94 achieve unbiased sequencing of microbiome, we used both random hexamer
95 and oligo $d(T)_{23}VN$ primers for reverse transcription, using approximately 1/10
96 total RNA extracted from a single throat swab as input. Secondly, we reduced

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the total time of library construction to around 2 hours (Figure 1a), which enables TRACE-seq to be more compatible for clinical use, especially when substantial numbers of specimens require investigation. Third, we developed a tailored analytical pipeline of TRACE-seq to simultaneously identify known and unknown pathogens and at the meanwhile to characterize host transcriptional response in a single metatranscriptomic profiling reaction (Figure 1b). This new pipeline allowed us to obtain rich information from the metatranscriptomic data generated by the modified TRACE-seq.

Sensitive detection of SARS-CoV-2 genome

Since the samples were positive or suspected positive throat swabs from COVID-19 patients, we asked whether the untargeted meta-transcriptomic sequencing could yield a full genome sequence of SARS-CoV-2 virus. After removing low quality reads and human reads, the remaining reads were mapped to the SARS-CoV-2 reference genome Wuhan-Hu-1 (accession number: NC_045512). Sequencing covered the reference genome from 171 bp to 29,903 bp (0.57%-100%), with an average sequencing depth from 2.56× to 44,737× (Supplementary table 1). Four samples (B101, A193, B13, C1) with low Ct values showed poor coverage and sequencing depth; the isolated RNA samples from the throat swabs were repeatedly frozen and thawed, probably compromising the integrity of RNA and hence were excluded. The other nine samples were used for subsequent correlation analysis. Among the remaining nine samples, the proportion of obtained reads of SARS-CoV-2, the coverage to the reference genome, the average sequencing depth and the median sequencing depth all showed a negative correlation with the Ct value of the

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samples (Spearman test, $p < 0.05$) (Figure 2a). In addition, the whole genome sequence could be acquired from mapping-based approach when the Ct value is as high as 32 (n=4, 44.44% of samples), with the average sequencing depth of 131×. Even in samples with Ct values up to 35 (n=7, 77.78% of samples), more than 94% of genome can be covered by TRACE-seq (Figure 2b, Supplementary table 1, Supplementary figure 1).

Reconstruction of full-length genome of SARS-CoV-2

Of the 452,865 contigs (average 34,836, from 18,160 to 53,415) assembled *de-novo* from non-human reads, 3,500 contigs (average 269, from 0 to 2,976) were determined to be SARS-CoV-2 genome fragments. There were no SARS-CoV-2 contigs in sample C31, B101 and B13. Most of contigs (n=3,461, 98.89%) were less than 1,000 bp (Figure 2c). To determine the accuracy of this method in acquisition of pathogen genome, all SARS-CoV-2 contigs were searched against genomes of each sample. In contigs with matched length over 1,000 bp, most contigs (22/39, 56.41%) were completely consistent with their corresponding genome (Figure 2d), while the other contigs had error bases from 1 to 7. In samples C14 and C13 with excellent coverage and depth, almost full-length genome (29,793 bp and 29,825 bp) were obtained just from *de-novo* assembly. Thus, TRACE-seq could enable the *de-novo* assembly of the complete genome of unknown pathogens and be readily utilized to identify emerging pathogens in patients with unknown etiology of infection and efficiently complement routine diagnostics.

Unbiased identification of putative pathogens in addition to SARS-CoV-2

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147 It is widely reported that coinfection (multi-species infection) contributes to
148 enhanced morbidity and mortality, especially in elderly and immunosuppressed
149 influenza patients(8, 9). Thus, we were curious to see if our metatranscriptomic
150 sequencing approach could capture other pathogens in addition to SARS-CoV-
151 2. Indeed, alignment of TRACE-seq data to microbe reference databases
152 identified many bacteria, fungi and viruses in both patient and healthy samples
153 (Figure 3a). To assess whether COVID-19 patients and healthy individuals have
154 different microbe community in their throat, principal coordinates analysis
155 (PCoA) was conducted using relative abundance of the microbiome. We
156 observed that COVID-19 patients harbored a throat microbiome that is quite
157 different from healthy individuals (Figure 3b). In addition, sample C31 differed
158 significantly from other SARS-CoV-2 positive samples. Further investigation of
159 the relative abundance of probable respiratory pathogens revealed that patient
160 C31 contained the most abundant *Klebsiella pneumoniae* and Human
161 gammaherpesvirus 4, compared to other samples (Figure 3c), which might be
162 a cause of the separation between sample C31 and the rest of the SARS-CoV-
163 2 positive samples.
164
165 Among the probable respiratory pathogens listed in Figure 3c,
166 *Stenotrophomonas maltophilia*, *Haemophilus parainfluenzae, Staphylococcus*
167 *aureus, Streptococcus pneumoniae, Haemophilus influenzae* and
168 *Acinetobacter baumannii* are common commensal organism of the normal
169 oropharynx, however, they can also become opportunistic pathogens and
170 cause infectious disease, such as endocarditis, bacteremia and pneumonia(10-
171 13). *Klebsiella pneumoniae, Stenotrophomonas maltophilia, Pseudomonas*

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aeruginosa, Neisseria meningitidis and *Legionella pneumophila* cause disease infrequently in normal hosts but can be a major cause of infection in patients with underlying or immunocompromising conditions(14-18). *Mycoplasma pneumoniae* is a type of “atypical” bacteria that commonly causes mild infections of the respiratory system(19). As for identified fungi, *Candida dubliniensis* and *Candida albicans* are both opportunistic yeast and can be detected in the gastrointestinal tract in healthy adults; they were also known to cause respiratory diseases (20-22). Human respirovirus 1, also known as Human parainfluenza virus 1, is the most common cause of croup and also associated with pneumonia. Human gammaherpesvirus 4 is one of the most common viruses in human; it is best known as the cause of infectious mononucleosis (23, 24), and is also constantly detected in lungs of patients with idiopathic pulmonary fibrosis (25). In our results, a relatively high abundance of *Haemophilus parainfluenzae, Streptococcus pneumoniae, Acinetobacter baumannii*, *Pseudomonas aeruginosa* and *Neisseria meningitidis* were identified in several SARS-CoV-2 positive samples compared with negative samples, which indicated potential coinfection. Nevertheless, these data by itself could not prove that COVID-19 patients were coinfected by these identified microorganism; these data have to be carefully interpreted in the clinical context.

Profiles of antibiotic resistance genes

Antimicrobial resistance has become a global issue. Pathogens with antibiotic resistance are increasing and many pathogens are becoming multidrug-resistant (26, 27). To characterize antibiotic resistance gene expression profiles,

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we aligned metatranscriptomic reads against the Comprehensive Antibiotic Resistance Database (CARD) (28). On average, around 84 antibiotic resistance genes were identified in SARS-CoV-2 positive samples, while only around 23 genes were identified in negative samples. According to the CARD, the identified antibiotic resistance genes confer resistance to 23 classes of antibiotics. Almost all resistance gene classes were more abundant in COVID-19 patients compared to healthy individuals. Genes conferring resistance to beta-lactam, aminoglycoside, tetracycline, phenicol, rifamycin, fluoroquinolone and macrolide were the most abundant (Figure 3d). Overall, the distinct microbiome, emergence of potential coinfection, and the elevated abundance of antibiotic resistance genes provide new data for establishing clinical therapeutic scheme during the treatment for COVID-19 patients.

Characterization of host response to SARS-CoV-2

Distinguishing infection from colonization remains challenging. Because host transcriptional profiling has emerged as a promising diagnostic tool for infectious diseases (29, 30), we next tested whether the host response to SARS-CoV-2 could be simultaneously characterized by TRACE-seq mediated metatranscriptomic analysis from throat swabs. As shown in Figure 3a, a substantial percentage of the reads are derived from human, and an average of 14,766 human genes with FPKM > 1 were detected per sample (Figure 4a, Figure S2a and b). Based on the gene expression profiles, the relationships between samples were inspected using a multidimensional scaling (MDS) plot (Figure 4b). As expected, SARS-CoV-2 positive samples were clearly separated from negative samples. In addition, sample C31 differed significantly

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in host gene expression from other SARS-CoV-2 positive samples, which might be caused by the relatively high abundance of Klebsiella pneumoniae and Human gammaherpesvirus 4 identified in sample C31. To characterize the common host response to SARS-CoV-2, we excluded sample C31 when performing differential gene expression analysis between SARS-CoV-2 positive and negative samples. We identified 153 differentially expressed genes, 149 of which were up-regulated (Figure 4c, Figure S2c). Gene Ontology enrichment analysis identified the top up-regulated biological processes to be immune response, defense response, viral process and response to cytokine (Figure 4d). Further investigation revealed that a subset of up-regulated genes involve in IL1B-associated inflammatory response (IL1B, IL8, IL36A, CXCR2, FOS, ANXA1, CASP4, KRT16, S100A8, S100A9). Moreover, another subset of up-regulated genes (ISG15, EGR1, IFI27, IFIT2, IFIT3, IFITM1, IFITM2, IFITM3, HLA-B, HLA-C) were enriched in type I interferon signaling pathway (Figure 4e). These results were highly consistent with previously reported host response to SARS-CoV-2 (31-33). Overall, metatranscriptomic data via TRACE-seq of throat swab samples demonstrates reliable performance in characterization of host transcriptional response to the infection of SARS-CoV-2.

Discussion

Although next generation sequencing holds a great potential to directly detect known and unknown pathogens including viruses, bacteria, fungi and parasites in a single application, the laborious and time-consuming steps in traditional RNA library construction procedure hinders its clinical application. As a rapid and convenient one-tube RNA-seq library construction method, TRACE-seq

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247 significantly lower the barrier for extensive application of unbiased RNA-seq in
248 clinical diagnosis. In addition, multiplexing libraries by utilizing Tn5 transposase
249 containing barcoded adaptors could enable sample investigation in a high-
250 throughput manner, particularly when comprehensive surveillance for emerging
251 pathogens is needed during a sudden disease outbreak.
252
253 It is very challenging to discriminate pathogens from background commensal
254 microbiota, since substantive bacteria or fungi can colonize multiple body sites
255 of healthy individuals. The microbe present at a relatively higher abundance in
256 patients compared to healthy individuals are often considered as a pathogen,
257 yet the abundance thresholds indicating infection is difficult to define based
258 solely on microbiome information. On the other hand, host transcriptional
259 profiling has been reported to distinguish infectious and noninfectious diseases
260 (30) and to further discriminate between virial and bacterial infections (29). A
261 previous study integrates host response and unbiased microbe detection for
262 lower respiratory tract infection diagnosis in critically ill adults, using both RNA-
263 seq and DNA-seq but yet lacking antibiotic resistance analysis (3). Another
264 study characterized microbial gene expression profiles (including antibiotic
265 resistance genes) using nasal and throat swab samples, and host response
266 using blood samples during influenza infection (34). To our knowledge, this is
267 the first study integrating unbiased pathogens detection, antibiotic resistance
268 and host response in a single approach with throat swabs from COVID-19
269 patients. In our results, SARS-CoV-2 positive and negative samples differed
270 significantly in both microbiome composition and host response. Among SARS-
271 CoV-2 positive samples, sample C31 harbored a throat microbiome and host

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response notably distinct from others, indicating sufficiently different pathogens present in patient C31 compared to other samples. Moreover, TRACE-seq hold the potential to construct a network of microbiome composition, antibiotic resistance and host response for characterizing the complex host-microbiome interactions. Ideally, TRACE-seq data can be utilized to develop a model combining pathogens metric, antibiotic resistance and host transcriptional classifier for infectious diseases diagnosis. We believe that the integrated information acquired from a TRACE-seq library will deepen our understanding of pathogenesis, improve diagnostic accuracy and more precisely inform optimal antimicrobial treatment for infectious diseases caused by not only SARS-CoV-2 but also other pathogens and eventually facilitate the utility of metatranscriptomic profiling as a routine diagnostic method.

Materials and methods

Ethics statement

The study and use of all samples were approved by the Ethics Committee of Wuhan Institue of Virology (No. WIVH17202001).

Sample collection and nucleotide extraction

Respiratory specimens (swabs) collected from patients admitted to various Wuhan health care facilities were immediately placed into sterile tubes containing 3 ml of viral transport media (VTM). The swabs were deactivated by heating at 56℃ for 30 minutes in a biosafety level 2 (BSL 2) laboratory at the Wuhan Institute of Virology in Zhengdian Park with personal protection equipment for biosafety level 3 (BSL 3) laboratory. Total nucleic acids were

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297 extracted using QIAamp 96 virus Qiacube HT kit on QIAxtractor Automated
298 extraction (Qiagen, US) following the manufacturer's instructions.
299
300 **TRACE-seq library preparation and sequencing**
301 TRACE-seq libraries were constructed using TruePrep® RNA Library Prep Kit
302 for Illumina (Vazyme, TR502-01) according to the manufacturer's instructions
303 with several modifications. 1/10 volume of total nucleic acids extracted from
304 each swab was used for each library. After 18 PCR cycles, the library was
305 purified using 0.8X Agencourt AMPure XP beads (Beckman Coulter) and eluted
306 in 20 μl nuclease-free water. The concentration of resulting libraries was
307 determined by Qubit 3.0 fluorometer with the Qubit dsDNA HS Assay kit
308 (Invitrogen) and the size distribution of libraries was assessed by Agilent 2100
309 Bioanalyzer. Finally, libraries were sequenced on the Illumina Hiseq X10
310 platform which generated 2 x 150 bp of paired-end raw reads.
311
312 **Data preprocessing**
313 Raw reads from sequencing were firstly subjected to Trim Galore (v0.6.4_dev)
314 (http://www.bioinformatics.babraham.ac.uk/projects/trim_galore/) for quality
315 control and adaptor trimming. The minimal threshold of quality was 20, and the
316 minimal length of reads to remain was set as 20 nt.
317
318 **Host transcriptional profiling analysis**
319 Filtered reads were mapped to human genome (hg19) and transcriptome using
320 STAR (v2.7.1a) (35). The FPKM value for annotated genes was calculated by
321 cuffnorm (v2.2.1) (36), and genes with FPKM > 1 were considered to be

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expressed. Multidimensional scaling and differential gene expression analysis were conducted using EdgeR (v3.28.1) (37) with gene count data generated by HTSeq (v0.11.2) (38). Gene Ontology Enrichment Analysis for biological processes was performed by DAVID (v6.8) (39) with all significantly up-regulated genes as input. Due to the redundancy of enriched GO terms, GO terms and their p values were further summarized using REViGO (40). The top 10 enriched representative GO terms were plotted.

Discrimination and de-novo assembly of SARS-CoV-2

After removal of human reads, the remaining data were aligned to the reference genome of Wuhan-Hu-1 (GenBank accession number: NC_045512) using Bowtie2 (v2.2.9) (41) for SARS-CoV-2 identification. The coverage and sequencing depth of SARS-CoV-2 genome were calculated by Samtools (v1.9) (42). On the other hand, to verify the method could screen for aetiologic agents and obtain pathogen genome, all non-human reads were processed for de-novo assembly using MEGAHIT (v1.2.9) with default parameters (43), and then all contigs were searched against NCBI nt database using blastn for classification(44). As for accuracy of assembly sequences, contigs determined to come from SARS-CoV-2 were performed blastn (with the parameter “-outfmt 3”) to display the differences with corresponding genome.

Microbiome analysis

After removing human reads, the remaining reads were subjected to microbial taxonomic classification using Kraken2 (v2.0.8-beta) (45) with a custom database. To build the custom database, standard RefSeq complete bacterial

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genomes were downloaded through “kraken2-build --download-library bacteria” and complete genomes of human viruses and genome assemblies of fungi were downloaded from NCBI’s RefSeq and added to the custom database’s genomic library using the “--add-to-library” switch. Principal coordinate analysis (PCoA) of relative abundances of microbial taxa at the genus level was done using cmdscale command in R. Distances between samples were calculated using Morisita-horn dissimilarity index by vegdist command from vegan package version 2.5-6 (https://CRAN.R-project.org/package=vegan). The antibiotic resistance genes were annotated by aligning the filtered metatranscriptomic reads to the Comprehensive Antibiotic Resistance Database (CARD). Antibiotic resistance genes with more than 10 completely matching reads were considered. The relative expression of antibiotic resistance genes were determined as RPM (reads per million non-host reads). All corresponding graphs were plotted using R scripts by RStudio (v1.2.5033) (https://rstudio.com/).

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Conflict of interests

The authors have filed patents related to TRACE-seq applications.

Contributions

C.Q.Y. and D.L. conceived the project; C.Q.Y., D.L., H.P.W., and D.P.W. supervised the project; B.L., Y.Y., and L.T.D. designed the experiments together and wrote the manuscript; L.L.H. performed experiments with the help of C.W. and R.W.; B.L. and Y.Y. performed the bioinformatics analysis; J.P.Y. collected the clinical samples; J.J.C., D.Y.Y., M.L.Z., and Y.W.L. participated in discussion.

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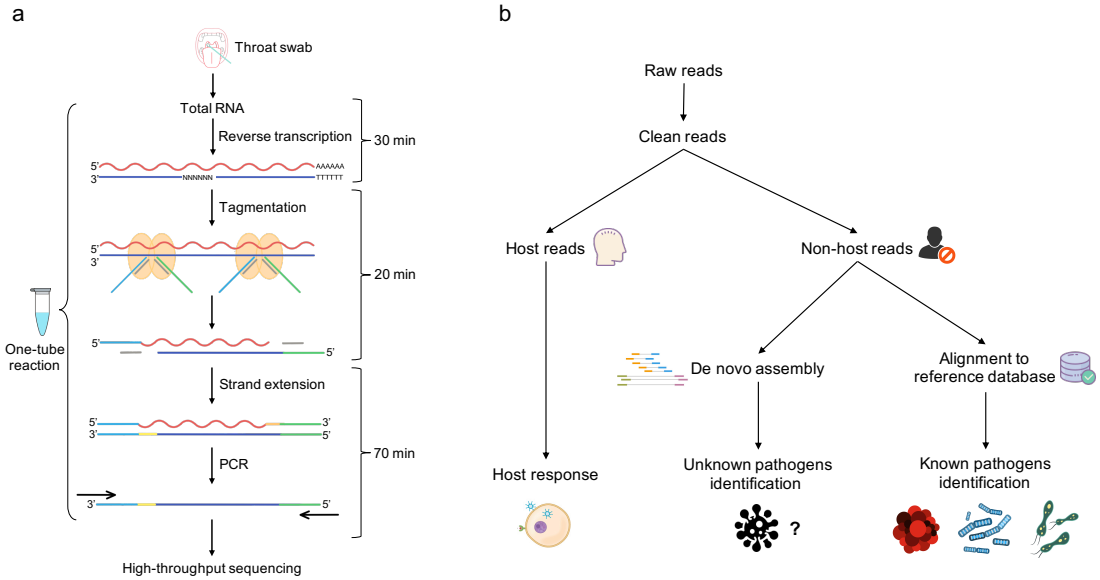

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493 **Figure 1.** Workflow of TRACE-seq enabled metatranscriptomic sequencing for
494 clinical diagnosis. **a.** A wet lab protocol of TRACE-seq starting with total RNA
495 extracted from throat swabs of COVID-19 patients. **b.** A dry lab pipeline
496 including known and unknown pathogens identification and host response
497 characterization.

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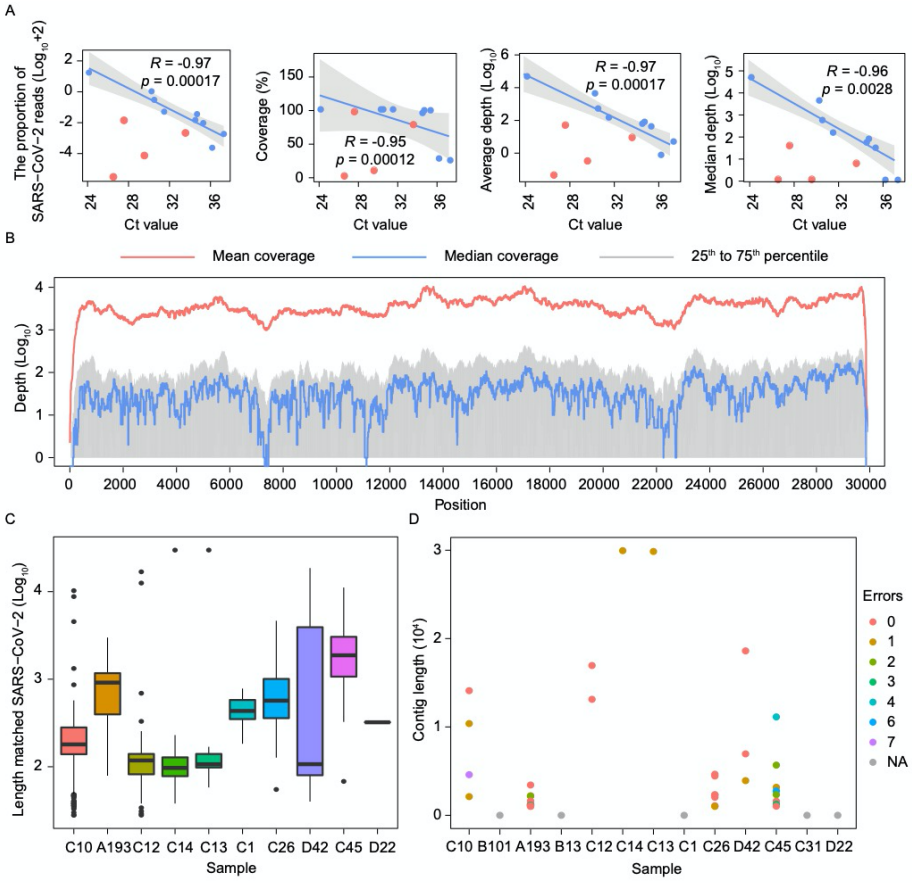

Figure 2. Genome coverage of SARS-CoV-2. **a.** Correlation between SARS-CoV-2 sequencing reads and Ct value in 13 positive samples. From the left to the right: the correlation of the ratio of SARS-CoV-2 reads, the coverage of SARS-CoV-2 genome, the average sequencing depth, the median sequencing depth and the Ct value of each sample are shown in order. The red dots represent samples with abnormal sequencing results, and linear regression indicates the relationship between the sequencing data and the Ct value of samples with normal sequencing results (blue dots). **b**. Genome coverage of sequenced samples across the SARS-CoV-2 genome. The x axis represents the virus genome position, y axis represents the log10 depth of each site. Lines in red represent the mean sequencing depth, lines in blue represent the median sequencing depth, and areas in grey represent 25th to

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512 75^{th} percentile of sequencing depth. **c.** Length distribution of contigs matched
513 SARS-CoV-2. The x axis represents each sample, and the y axis represents
514 log_{10} lengths of contigs matched SARS-CoV-2. **d.** De novo assembly results
515 of SARS-CoV-2. The graph shows contigs only when the length of matched
516 to the SARS-COV-2 genome over 1,000 bp. The y axis represents length of
517 contigs of each sample (the x axis). Different colors represent the number of
518 error bases (shown in legends) in each contig relative to previously known
519 genome sequences.

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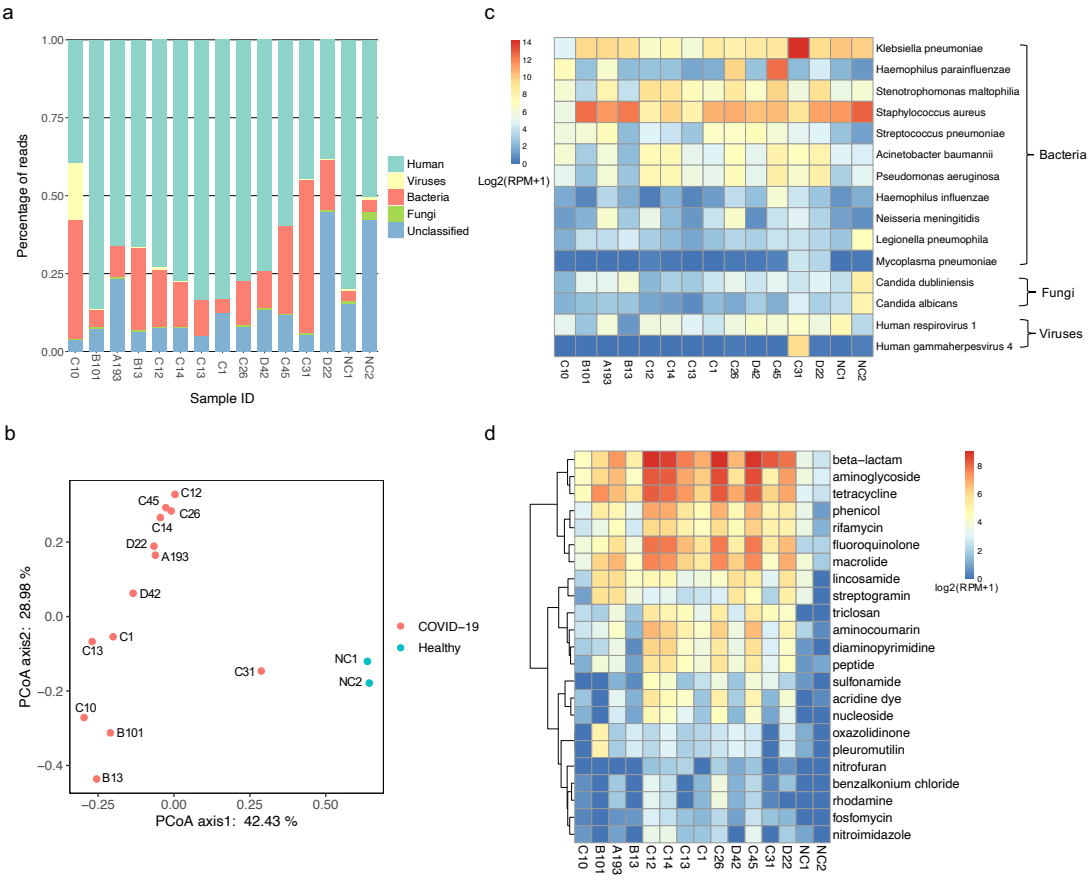

Figure 3. Microbiome profiles in COVID-19 patients and healthy individuals. **a.** Histogram showing percentage of reads mapping to human, viruses, bacteria and fungi for the individual samples. **b.** PCoA of microbiome using relative abundance at the genus level. **c.** Heatmap showing relative abundance of potential respiratory pathogens identified in SARS-CoV-2 positive and negative samples. RPM: reads per million non-host reads. **d.** Heatmap displaying relative abundance of antibiotic resistance genes in SARS-CoV-2 positive and negative samples.

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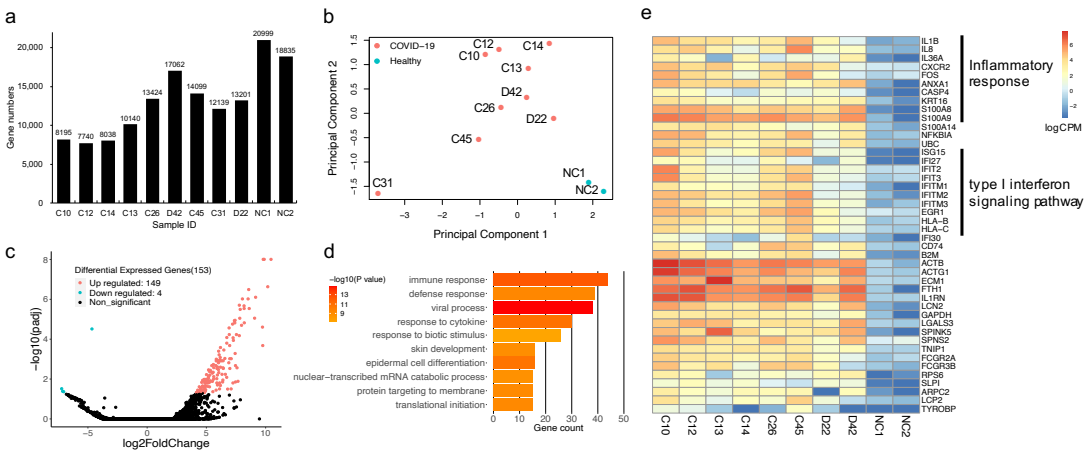

Figure 4. Profiling of host transcriptional response. **a.** Bar plot showing gene numbers detected in each sample. **b.** MDS plot showing variation among samples based on host transcriptional profiles. **c.** Volcano plot showing differentially expressed genes between SARS-CoV-2 positive and negative samples. Significantly up- and down-regulated genes (padj < 0.05, |log2FoldChage| > 1) are highlighted in red and blue, respectively. **d.** Bar plot of the most enriched Gene Ontology terms. **e.** Heatmap presenting the differentially expressed immune response related genes between SARS-CoV-2 positive and negative samples.