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Non-coronavirus genome sequences identified from metagenomic analysis of clinical samples from COVID-19 infected patients: An evidence for Co-infection

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Abstract

In December 2019, pneumonia caused by severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) infection emerged in Wuhan City, Hubei Province, China. Early in 2020, the World Health Organization (WHO) announced a new name for the 2019-nCoV-caused epidemic disease: coronavirus disease 2019 (COVID-19) and declared COVID-19 to be the sixth international public health emergency. Cellular co-infection is a critical determinant of both viral fitness and infection outcome and plays a crucial role in shaping the host immune response to infections. In this study, sixty-eight public next-generation sequencing libraries from SARS-CoV-2 infected patients were retrieved from the NCBI Sequence Read Archive database using SRA-Toolkit. Using an alignment-free method based on K-mer mapping and extension, SARS-CoV-2 was identified in all except three patients. Influenza A H7N9 (3/68), Human immunodeficiency virus 1 (1/68), Spodoptera frugiperda rhabdovirus isolate (3/68), Human metapneumovirus (1/68), coronaviruses NL63 (1/68), Sri Lankan cassava mosaic virus (1/68), Indian cassava mosaic virus (1/68), Parvovirus (1/68), Simian virus 40 (1/68), Woodchuck hepatitis virus (1/68), Saccharomyces 20S RNA narnavirus (2/68), and Autographa californica nucleopolyhedrovirus (2/68) genome sequences were detected in SARS-CoV-2 infected patients.

Keywords: COVID-19, Viral Co-infection, SARS-CoV-2, Influenza A virus, Human Immunodeficiency virus

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Introduction

In December 2019, the first cases of coronavirus disease 2019 (COVID-19) were possibly due to a zoonotic transmission in China, tied to a large seafood market which also traded in live wild animals (*1*). The causative virus, severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), is capable of human-to-human transmission and rapidly spread to other regions of China, and then to other countries (*2*). It is now a global pandemic and is a considerable concern for public health. So far, more than 5,637,367 confirmed cases were diagnosed in nearly 213 countries and territories around the world and two international conveyances, causing globally over 349,000 deaths (*3*).

Coronaviruses in humans and animals are known to cause disease. Of these, four (human coronaviruses 229E, NL63, OC43, and HKU1) typically only infect the upper respiratory tract and cause relatively minor symptoms (*4*). However, there are three coronaviruses (severe acute respiratory syndrome coronavirus (SARS-CoV), Middle East respiratory syndrome coronavirus (MERS-CoV), and SARS-CoV-2) that can replicate in the lower respiratory tract and cause pneumonia which can be fatal. With 79% genome sequence similarity, SARS-CoV is the closest relative to SARS-CoV-2 among human coronaviruses (*5*). However, of all known coronavirus sequences, SARS-CoV-2 is most similar to bat coronavirus RaTG13, with a similarity of 98 percent (*6*), and coronavirus sequences in pangolin (a scaly anteater) also have high similarity (*7*). SARS-CoV-2 pathophysiology closely parallels that of SARS-CoV infection, with active inflammatory responses strongly implicated in the resulting airway damage (*8*). Hence

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the extent of the disease in patients is attributed not only to the viral infection but also to the host 's response (*1*).

Underlying co-infections in primary infectious disease are an important variable that needs to be considered but is often undetected. A better understanding of the prevalence of co-infection is urgently required, partly because co-infecting pathogens can interact with each other either directly or indirectly via the host 's resources or immune system (*9, 10*). These interactions within co-infected hosts can alter the transmission, clinical progression and control of multiple infectious diseases as compared to single pathogen species infection (*9, 11, 12*). Recent studies appear to indicate that the adverse effects of co-infection are more common than no-effects or positive impact on human health (*13*).

The underdiagnosis of co-infections is attributed, among other factors, to a lack of clinical suspicion, similar symptoms and or the fact that in the absence of a priori knowledge, conventional methods have little capacity to detect co-infections. Exploring new diagnostic approaches is, therefore, essential to advance understanding of co-infection contribution to disease manifestations and treatment responses (*14*).

Remarkable developments in next-generation sequencing have recently made metagenomics, an unbiased shotgun method of analysis, a widely used tool in just about every field of biology, including diagnosis of infectious diseases (*15, 16*).

Metagenomics is powerful because it is capable of diagnosing unsuspected microbial agents (*17*). It directly analyzes samples in their entirety, eliminating the need for prior knowledge to obtain comprehensive information. In this capacity, metagenomics exceeds traditional diagnostic limitations.

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With the viral genomes in hand, we can now explore the possibility of using metagenomic and metatranscriptomic next-generation sequencing (mNGS) directly as a screening method of other viruses in a sample.

In theory, a simple and straightforward approach would be to first map sequencing reads from the sample to the viral genome. Such an alignment-based method is vulnerable to problems stemming from both false positives and false negatives. Some viruses have genomes very similar to SARS-CoV-2, which can lead to false-positive results (*18*). On the other hand, in some cases, the virus-specific reads obtained may not be abundant enough for unambiguous detection, which can lead to false-negative results. Such results can occur when the viral RNA is highly degraded, or when the sequencing library has been incompletely target enriched by multiple-PCR (*19*) or hybrid capture (*20*).

Fastv is an ultra-fast tool for detecting the microbial sequences in sequence data. It can identify target microorganisms using unique k-mers. It has a 100% sensitivity and 100% specificity for detecting SARS and other coronaviruses from sequencing data and can distinguish SARS from MERS.

In this study, identification experiments were conducted on public next-generation sequencing libraries from SARS-CoV-2 infected patients using fastv, along with the pre-computed unique k-mer resources (*18*). The findings of the present study have confirmed the actual existence of genome sequences of other viruses in SARS-CoV-2 infected patients.

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Material and Methods

SRA Database Mining

Next-generation sequencing technologies have enabled large-scale genomic surveillance of SARS-CoV-2 as thousands of isolates are being sequenced around the world and deposited in public data repositories. SRA files were fetched with the NCBI SRA toolkit using fastq-dump from the following bioprojects (PRJNA631042 (44 samples), PRJNA608742 (12 samples), PRJNA632678 (1 sample), PRJNA605983 (9 samples), PRJNA633241 (1 sample) and PRJNA603194 (1 sample)) **(Table.1)**.

Read Pre-processing, Analysis Using Fastv

Fastv, along with the pre-computed unique k-mer resources, was used as previously described (*18*). Briefly, Fastv performed data quality control (QC) and quality filtering on FASTQ input files. Then, Fastv collect sequences that contain any unique k-mer and output results to downstream tools. To pay particular attention to SARS-CoV-2 while scanning for all viruses, we used SARS-CoV-2 Genomes/k-mer files from fastv data directory (https://github.com/OpenGene/fastv/tree/master/data), and k-mer collection file for viral genomes was downloaded from (http://opengene.org/viral.kc.fasta.gz). The k-mer scanning results of different inputs were visualized in a figure on a single HTML page by fastv. The Krona tool (https://github.com/marbl/Krona/wiki) was used to visualize the co-infecting viruses in clinical samples (*21*).

Results

SARS-CoV-2 Identification

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We conducted identification experiments on samples sequenced from COVID-19 infected patients **(Table.1)**. SARS-CoV-2 was detected in all tested samples with three out of 68 clinical samples were considered negative for SARS-CoV-2 by fastv. These three samples belong to one bio-project, PRJNA631042, where the research group used different sequencing technologies on the same sample to find the cost-effective and highly scalable method for SARS-CoV-2 sequencing. Because sequence technologies vary in reading depth and coverage thresholds, fastv was unable to detect SARS-CoV-2 in sequenced samples with lower coverage metrics. The output for targeted k-mer hits and the result for genome coverage were visualized by fastv. Statistics on genome coverage indicate that SARS-CoV-2 fits the Wuhan seafood market pneumonia virus isolate Wuhan-Hu-1 strain (NC_045512.2) most closely **(Figure.1)**.

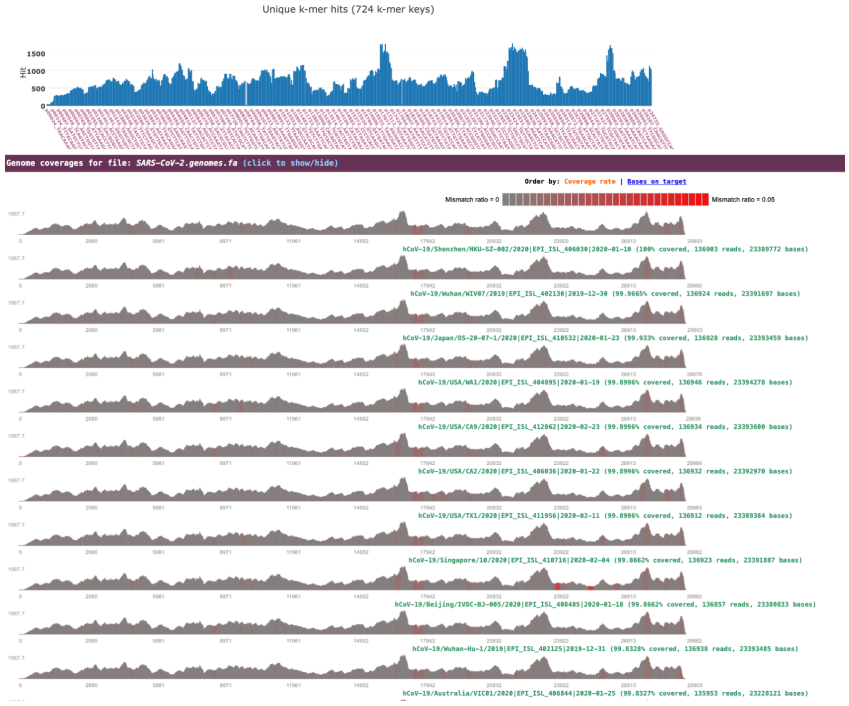

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Figure.1, a: SARS-CoV-2 detection using fastv. Eleven SARS-CoV-2 strains are included in the genome list ordered by genome coverage rate, with the k-mer coverage varying from 100% to 99.83%. Mismatches were highlighted in red.

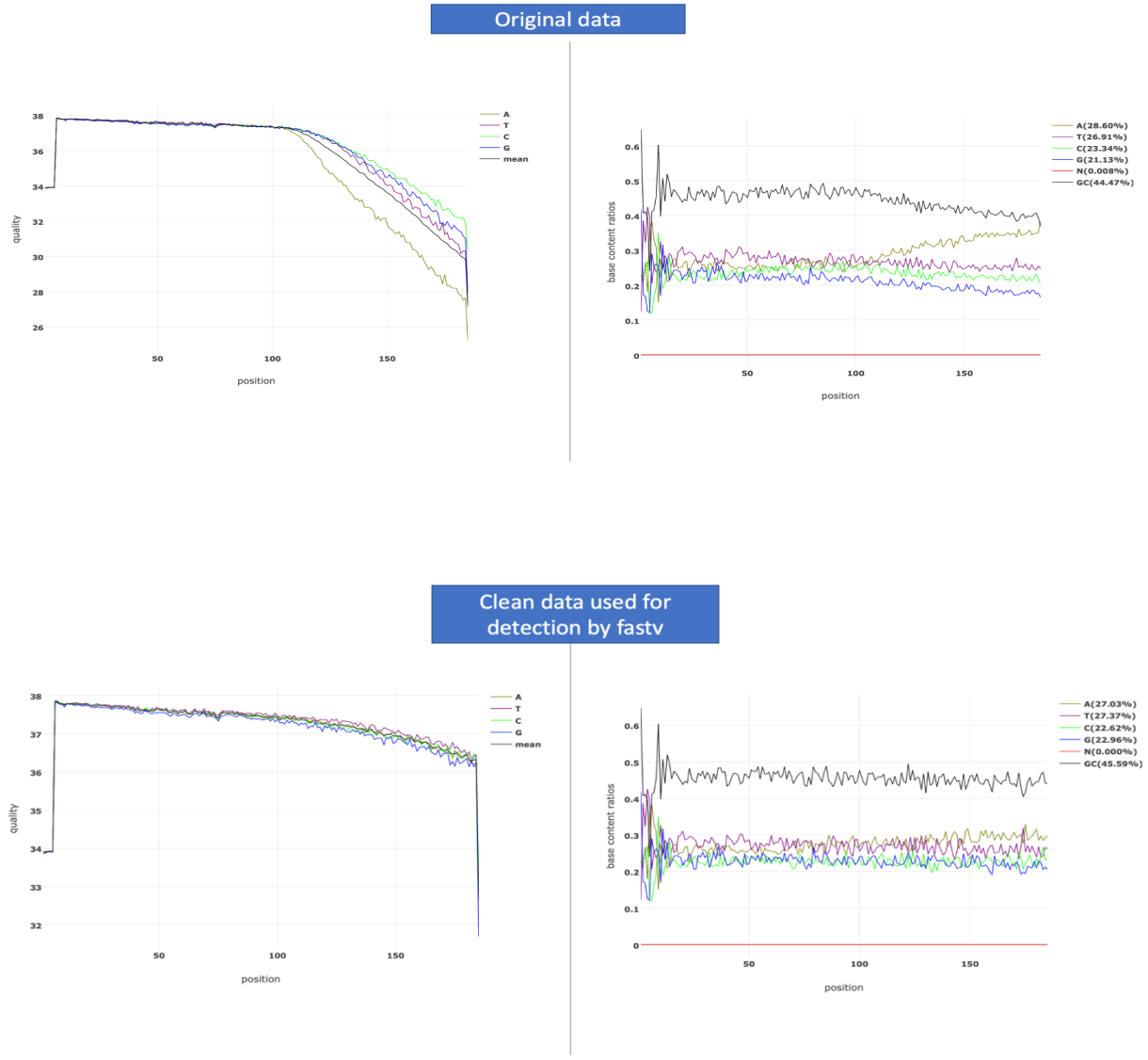

Figure.1, b: FASTQ file after adapter trimming, quality pruning and base correction for accurate k-mer analysis.

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Viral Metagenomic Analysis identified non-coronavirus genome sequences in COVID-19 infected patients

Influenza type A (A/Shanghai/02/2013(H7N9) (3/68), Human immunodeficiency virus 1 (1/68), Spodoptera frugiperda rhabdovirus isolate Sf (3/68), Simian virus 40 (1/68), Woodchuck hepatitis virus (1/68), Saccharomyces 20S RNA narnavirus (2/68), and Autographa californica nucleopolyhedrovirus (2/68) were detected in SARS-CoV-2 infected patients in China **(Figure.2)**.

Sri Lankan cassava mosaic virus (1/68), Indian cassava mosaic virus (1/68) were detected in one patient with symptoms to COVID-19 in Colombia.

Human metapneumovirus (accession No: NC_039199.1) and Human Coronavirus NL63, complete genome (accession No: NC_005831.2) were detected in SRR11772648 (Bioproject: PRJNA631042) with low confidence while SARS-CoV-2 could not be detected by fastv.

Parvovirus NIH-CQV genes coding for putative replication-associated protein (rep), and putative capsid protein (cap) were detected in SRR10971381 (Bioproject: PRJNA603194) with low confidence, however, SARS-CoV-2 was detected in the same sample with a 100% coverage.

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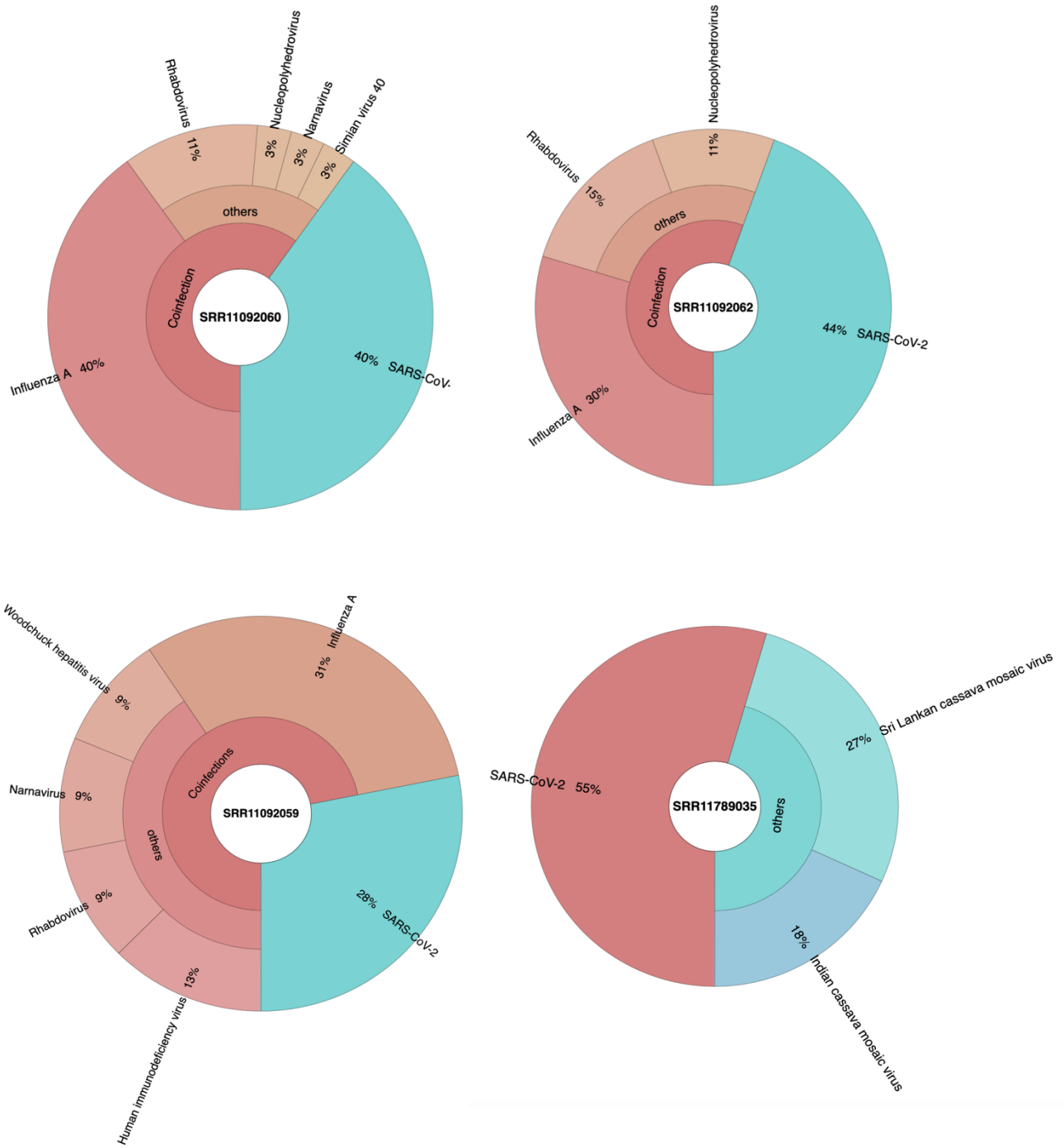

184 **Figure.2:** Viruses identified from metagenomic analysis of samples collected from

185 COVID-19 patients were visualized using krona tool.

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Discussion

The value of identifying underlying co-infection(s) is gaining greater appreciation (*9, 22*), but it remains challenging to get such information. The source of clinical samples and the sequencing technology can be inferior in co-infection detection (*23*). Using the viral metagenomics analysis, we were able to identify various viruses, including SARS-CoV-2 virus.

Previous studies reported co-infection of SARS-CoV-2 with influenza type A (*24-29*). In this study, we detected influenza A virus in three COVID-19 infected patients in China which suggests that COVID-19 might be underdiagnosed, especially during the influenza season, since typical clinical symptoms of COVID-19, including fever, cough, and dyspnea, resemble those of influenza (*28, 30*).

Understanding the nature and consequences of co-infection is essential for accurate estimates of infectious disease burden. More holistic data on infectious diseases, in particular, will indeed help to quantify the magnitude of co-infection effects on human health. Improved knowledge of the factors influencing an individual's risk of co-infection, circumstances in which co-infecting pathogens interact, and the mechanisms behind these pathogen-pathogen interactions, especially from experimental studies, will also help design and evaluate programs for the management of infectious diseases. Up to now, most disease control programs typically adopt a vertical intervention approach that addresses every pathogen infection in isolation. If co-infecting pathogens typically interact to worsen human health, control strategies may need to be more integrated, and specialist therapies developed for clinical cases of co-infection.

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Future studies are urgently needed not only to genetically characterize these viruses and conduct screening studies for different viruses in larger sample sets but also to research the function of these viruses alone and during co-infection situations with the aims of elucidating how these viruses interact with the host immune system to confirm their role in the pathogenesis of diseases and secondary infections.

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287 **Table.1: SRA sequences used in this study with the detection result for SARS-CoV-2 K-mer**

No.	Run	BioSample	source	Platform	BioProject	Center Name	Detection result for SARS-CoV-2 k-mer
1	**SRR11181954**	**SAMN14207961**	BALF	BGISEQ	**PRJNA608742**	Shenzhen 3rd People's Hospital	**Positive**
2	**SRR11181955**	**SAMN14207960**	BALF	BGISEQ	**PRJNA608742**	Shenzhen 3rd People's Hospital	**Positive**
3	**SRR11181956**	**SAMN14207959**	BALF	BGISEQ	**PRJNA608742**	Shenzhen 3rd People's Hospital	**Positive**
4	**SRR11181957**	**SAMN14207958**	BALF	BGISEQ	**PRJNA608742**	Shenzhen 3rd People's Hospital	**Positive**
5	**SRR11181958**	**SAMN14207957**	BALF	BGISEQ	**PRJNA608742**	Shenzhen 3rd People's Hospital	**Positive**
6	**SRR11181959**	**SAMN14207956**	BALF	BGISEQ	**PRJNA608742**	Shenzhen 3rd People's Hospital	**Positive**
7	**SRR11537949**	**SAMN14594848**	BALF	BGISEQ	**PRJNA608742**	Shenzhen 3rd People's Hospital	**Positive**
8	**SRR11537950**	**SAMN14594847**	BALF	BGISEQ	**PRJNA608742**	Shenzhen 3rd People's Hospital	**Positive**

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9	**SRR11537951**	**SAMN14594846**	BALF	BGISEQ	**PRJNA608742**	Shenzhen 3rd People's Hospital	**Positive**
10	**SRR11537952**	**SAMN14594845**	BALF	BGISEQ	**PRJNA608742**	Shenzhen 3rd People's Hospital	**Positive**
11	**SRR11537953**	**SAMN14594844**	BALF	BGISEQ	**PRJNA608742**	Shenzhen 3rd People's Hospital	**Positive**
12	**SRR11537954**	**SAMN14594843**	BALF	BGISEQ	**PRJNA608742**	Shenzhen 3rd People's Hospital	**Positive**
13	**SRR11245351**	**SAMN14306710**	BALF	BGISEQ	**PRJNA608742**	Shenzhen 3rd People's Hospital	**Positive**
14	**SRR11245352**	**SAMN14306709**	BALF	BGISEQ	**PRJNA608742**	Shenzhen 3rd People's Hospital	**Positive**
15	**SRR11245353**	**SAMN14306708**	BALF	BGISEQ	**PRJNA608742**	Shenzhen 3rd People's Hospital	**Positive**
16	**SRR11245354**	**SAMN14306707**	BALF	BGISEQ	**PRJNA608742**	Shenzhen 3rd People's Hospital	**Positive**
17	**SRR11245355**	**SAMN14306706**	BALF	BGISEQ	**PRJNA608742**	Shenzhen 3rd People's Hospital	**Positive**
18	**SRR11245356**	**SAMN14306705**	BALF	BGISEQ	**PRJNA608742**	Shenzhen 3rd People's Hospital	**Positive**
19	**SRR11772640**	**SAMN14891483**	clinical biospecimens	Illumina MiSeq	**PRJNA631042**	UNIVERSITY OF MINNESOTA	**Positive**

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20	**SRR11772641**	**SAMN14891483**	clinical biospecimens	Illumina MiSeq	**PRJNA631042**	UNIVERSITY OF MINNESOTA	**Positive**
21	**SRR11772642**	**SAMN14891483**	clinical biospecimens	Illumina MiSeq	**PRJNA631042**	UNIVERSITY OF MINNESOTA	**Positive**
22	**SRR11772643**	**SAMN14891483**	clinical biospecimens	Illumina MiSeq	**PRJNA631042**	UNIVERSITY OF MINNESOTA	**Positive**
23	**SRR11772644**	**SAMN14891483**	clinical biospecimens	Illumina MiSeq	**PRJNA631042**	UNIVERSITY OF MINNESOTA	**Positive**
24	**SRR11772654**	**SAMN14891482**	clinical biospecimens	Illumina MiSeq	**PRJNA631042**	UNIVERSITY OF MINNESOTA	**Positive**
25	**SRR11772656**	**SAMN14891484**	clinical biospecimens	Illumina MiSeq	**PRJNA631042**	UNIVERSITY OF MINNESOTA	**Positive**
26	**SRR11772657**	**SAMN14891482**	clinical biospecimens	Illumina MiSeq	**PRJNA631042**	UNIVERSITY OF MINNESOTA	**Positive**
27	**SRR11772658**	**SAMN14891482**	clinical biospecimens	Illumina MiSeq	**PRJNA631042**	UNIVERSITY OF MINNESOTA	**Positive**
28	**SRR11772660**	**SAMN14891490**	clinical biospecimens	Illumina MiSeq	**PRJNA631042**	UNIVERSITY OF MINNESOTA	**Positive**
29	**SRR11772661**	**SAMN14891490**	clinical biospecimens	Illumina MiSeq	**PRJNA631042**	UNIVERSITY OF MINNESOTA	**Positive**
30	**SRR11772662**	**SAMN14891490**	clinical biospecimens	Illumina MiSeq	**PRJNA631042**	UNIVERSITY OF MINNESOTA	**negative**

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31	**SRR11772663**	**SAMN14891489**	clinical biospecimens	Illumina MiSeq	**PRJNA631042**	UNIVERSITY OF MINNESOTA	**negative**
32	**SRR11772664**	**SAMN14891489**	clinical biospecimens	Illumina MiSeq	**PRJNA631042**	UNIVERSITY OF MINNESOTA	**negative**
33	**SRR11772665**	**SAMN14891489**	clinical biospecimens	Illumina MiSeq	**PRJNA631042**	UNIVERSITY OF MINNESOTA	**Positive**
34	**SRR11772666**	**SAMN14891488**	clinical biospecimens	Illumina MiSeq	**PRJNA631042**	UNIVERSITY OF MINNESOTA	**Positive**
35	**SRR11772667**	**SAMN14891488**	clinical biospecimens	Illumina MiSeq	**PRJNA631042**	UNIVERSITY OF MINNESOTA	**Positive**
36	**SRR11772668**	**SAMN14891482**	clinical biospecimens	Illumina MiSeq	**PRJNA631042**	UNIVERSITY OF MINNESOTA	**Positive**
37	**SRR11772669**	**SAMN14891488**	clinical biospecimens	Illumina MiSeq	**PRJNA631042**	UNIVERSITY OF MINNESOTA	**Positive**
38	**SRR11772670**	**SAMN14891488**	clinical biospecimens	Illumina MiSeq	**PRJNA631042**	UNIVERSITY OF MINNESOTA	**Positive**
39	**SRR11772671**	**SAMN14891488**	clinical biospecimens	Illumina MiSeq	**PRJNA631042**	UNIVERSITY OF MINNESOTA	**Positive**
40	**SRR11772672**	**SAMN14891487**	clinical biospecimens	Illumina MiSeq	**PRJNA631042**	UNIVERSITY OF MINNESOTA	**Positive**
41	**SRR11772673**	**SAMN14891487**	clinical biospecimens	Illumina MiSeq	**PRJNA631042**	UNIVERSITY OF MINNESOTA	**Positive**

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42	**SRR11772674**	**SAMN14891487**	clinical biospecimens	Illumina MiSeq	**PRJNA631042**	UNIVERSITY OF MINNESOTA	**Positive**
43	**SRR11772675**	**SAMN14891486**	clinical biospecimens	Illumina MiSeq	**PRJNA631042**	UNIVERSITY OF MINNESOTA	**Positive**
44	**SRR11772676**	**SAMN14891486**	clinical biospecimens	Illumina MiSeq	**PRJNA631042**	UNIVERSITY OF MINNESOTA	**Positive**
45	**SRR11772677**	**SAMN14891486**	clinical biospecimens	Illumina MiSeq	**PRJNA631042**	UNIVERSITY OF MINNESOTA	**Positive**
46	**SRR11772678**	**SAMN14891486**	clinical biospecimens	Illumina MiSeq	**PRJNA631042**	UNIVERSITY OF MINNESOTA	**Positive**
47	**SRR11772679**	**SAMN14891482**	clinical biospecimens	Illumina MiSeq	**PRJNA631042**	UNIVERSITY OF MINNESOTA	**Positive**
48	**SRR11772680**	**SAMN14891485**	clinical biospecimens	Illumina MiSeq	**PRJNA631042**	UNIVERSITY OF MINNESOTA	**Positive**
49	**SRR11772681**	**SAMN14891485**	clinical biospecimens	Illumina MiSeq	**PRJNA631042**	UNIVERSITY OF MINNESOTA	**Positive**
50	**SRR11772682**	**SAMN14891485**	clinical biospecimens	Illumina MiSeq	**PRJNA631042**	UNIVERSITY OF MINNESOTA	**Positive**
51	**SRR11772683**	**SAMN14891485**	clinical biospecimens	Illumina MiSeq	**PRJNA631042**	UNIVERSITY OF MINNESOTA	**Positive**
52	**SRR11772684**	**SAMN14891485**	clinical biospecimens	Illumina MiSeq	**PRJNA631042**	UNIVERSITY OF MINNESOTA	**Positive**

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53	**SRR11772685**	**SAMN14891484**	clinical biospecimens	Illumina MiSeq	**PRJNA631042**	UNIVERSITY OF MINNESOTA	**Positive**
54	**SRR11772686**	**SAMN14891484**	clinical biospecimens	Illumina MiSeq	**PRJNA631042**	UNIVERSITY OF MINNESOTA	**Positive**
55	**SRR11772687**	**SAMN14891484**	clinical biospecimens	Illumina MiSeq	**PRJNA631042**	UNIVERSITY OF MINNESOTA	**Positive**
56	**SRR11772688**	**SAMN14891484**	clinical biospecimens	Illumina MiSeq	**PRJNA631042**	UNIVERSITY OF MINNESOTA	**Positive**
57	**SRR11789035**	**SAMN14917563**	clinical biospecimens	OXFORD_NANOPORE	**PRJNA632678**	Colombia	**Positive**
58	**SRR11092056**	**SAMN14082199**	BALF	Illumina MiSeq	**PRJNA605983**	China	**Positive**
59	**SRR11092057**	**SAMN14082197**	BALF	Illumina MiSeq	**PRJNA605983**	China	**Positive**
60	**SRR11092058**	**SAMN14082196**	BALF	Illumina MiSeq	**PRJNA605983**	China	**Positive**
61	**SRR11092059**	**SAMN14082200**	BALF	Illumina HiSeq 3000	**PRJNA605983**	China	**Positive**
62	**SRR11092060**	**SAMN14082199**	BALF	Illumina HiSeq 3000	**PRJNA605983**	China	**Positive**
63	**SRR11092061**	**SAMN14082198**	BALF	Illumina HiSeq 3000	**PRJNA605983**	China	**Positive**
64	**SRR11092062**	**SAMN14082197**	BALF	Illumina HiSeq 1000	**PRJNA605983**	China	**Positive**
65	**SRR11092063**	**SAMN14082196**	BALF	Illumina HiSeq 3000	**PRJNA605983**	China	**Positive**
66	**SRR11092064**	**SAMN14082200**	BALF	Illumina MiSeq	**PRJNA605983**	China	**Positive**

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67	**SRR11801823**	**SAMN14938301**	nasopharynx	Illumina ISeq 100	**PRJNA633241**	Bangladesh	**Positive**
68	**SRR10971381**	**SAMN13922059**	BALF	Illumina MiniSeq	**PRJNA603194**	China: Wuhan	**Positive**