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Detection of respiratory viruses in adults with suspected COVID-19 in Kuala Lumpur, Malaysia

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ABSTRACT

Background: Reports of co-circulation of respiratory viruses during the COVID-19 pandemic and co-infections with SARS-CoV-2 vary. However, limited information is available from developing countries.

Objectives: We aimed to investigate the incidence of respiratory viruses in adult patients with suspected COVID-19 in Kuala Lumpur, Malaysia.

Study Design: We collected 198 respiratory samples from adult patients hospitalized with suspected COVID-19 in a single teaching hospital in Kuala Lumpur in February-May 2020 and tested combined oro-nasopharyngeal swabs with the NxTAG Respiratory Pathogen Panel (Luminex) and Allplex RV Essential (Seegene) assays. Forty-five negative samples further underwent viral metagenomics analysis.

Results: Of the 198 samples, 74 (37.4%) had respiratory pathogens, including 56 (28.3%) with SARS-CoV-2 and 18 (9.1%) positive for other respiratory pathogens. There were five (2.5%) SARS-CoV-2 co-infections, all with rhinovirus/enterovirus. Three samples (6.7%; 3/45) had viruses identified by metagenomics, including one case of enterovirus D68 and one of Saffold virus genotype 6 in a patient requiring ICU care. Most of the COVID-19 patients (91.1%; 51/56) had mild symptoms but 5.4% (3/56) died.

Conclusion: During the early COVID-19 period, common respiratory viruses other than SARS-CoV-2 only accounted for 9.1% of hospitalization cases with ARI and co-infections with SARS-CoV-2 were rare. Continued surveillance is important to understand the impact of COVID-19 and its associated public health control measures on circulation of other respiratory viruses. Metagenomics can identify unexpected or rare pathogens, such as Saffold virus, which is rarely described in adults.

1. Introduction

Since the emergence of severe acute respiratory syndrome coronavirus-2 (SARS-CoV-2) in December 2019, the coronavirus disease 2019 (COVID-19) pandemic has posed a major public health threat and economic burden worldwide. As of September 2021, Malaysia has had more than 2.1 million confirmed cases and 23,000 deaths [1]. A few studies have reported decreases in common respiratory viral infection incidence and low rates of SARS-CoV-2 co-infections with common respiratory viruses during the COVID-19 pandemic period [2–4]. However, in developing tropical countries such as Malaysia, rapid testing for SARS-CoV-2 has been the main focus among persons with respiratory symptoms and broad testing for other respiratory viruses is performed inconsistently. Hence, there is little information on co-circulating respiratory pathogens during COVID-19 in these countries. Furthermore, while Burkholderia pseudomallei is endemic in Malaysia, other select agents causing respiratory infections such as MERS-CoV, SARS-CoV, Bacillus anthracis, Yersinia pestis, Francisella tularensis and Coxiella

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burdetii are not routinely tested for and their prevalence remains unknown.

Over the last decade, viral metagenomics analysis has emerged as a sensitive pan-pathogen assay for diagnosis and identification of unknown or rare viral pathogens. Metagenomics analysis can be used to detect undiagnosed pathogens in clinical samples that are negative for or not assessed with routine diagnostic tests, [5–7] and would be of particular interest for countries in Southeast Asia such as Malaysia, which are potential hotspots for emerging infections.

Here we report the detection rate of SARS-CoV-2 co-infections and other respiratory infections in suspected COVID-19 adult patients during the early wave of COVID-19 (February to May 2020) in Malaysia. We used multiplex molecular respiratory panels including assays for select agents and viral metagenomics analysis to comprehensively capture potential respiratory pathogens that might have been missed out by conventional diagnostic approaches.

2. Materials and Methods

2.1. Study approval

This study was approved by the University Malaya Medical Centre ethics committee (no. 202002-8337). Our institution does not require informed consent for retrospective studies of archived and anonymised samples.

2.2. Sample collection and respiratory pathogens detection

This study was conducted in University Malaya Medical Centre (UMMC), a 1600-bed tertiary referral teaching hospital serving the populations of Kuala Lumpur and Selangor in Malaysia. From February to May 2020, a single combined oro-nasopharyngeal swab was obtained from each adult patient (≥18 years old) admitted with suspected COVID-19. The inclusion criteria for suspected COVID-19 used by the hospital at that time is described in Supplementary Material. Nucleic acids in clinical samples were extracted using IndiSpin Pathogen kit (Indical Bioscience, Germany). Common respiratory pathogens were tested for using NxTAG Respiratory Pathogen Panel (NxTAG RPP) assay (Luminex Corporation, USA) and Allplex RV Essential assay (Seegene, Korea). The NxTAG assay detects influenza virus (A/H1, A/H3 and B), adenovirus, parainfluenza (types 1-4), respiratory syncytial virus (A and B), metapneumovirus, rhinovirus/enterovirus, coronavirus (HKU1, 229E, NL63 and OC43), bocavirus, Chlamydophila pneumoniae, Mycoplasma pneumoniae and Legionella pneumophila. The Allplex assay detects influenza virus (A and B), adenovirus, parainfluenza virus, metapneumovirus, respiratory syncytial virus and rhinovirus. SARS-CoV-2 was detected using the Allplex SARS-CoV-2 assay (Seegene, Korea). Additionally, MERS-CoV [8], SARS-CoV [9] and bacterial select agents (B. pseudomallei [10], C. burnetii [11], B. anthracis [12], F. tularensis [13], and Y. pestis [14]) were also tested using published real-time PCR assays with minor modifications and the primer and probes sequences as described in Supplementary Material. Where collected, 5-10 ml of venous blood was cultured in aerobic and anaerobic bottles for up to 5 days using the BacT/ALERT Virtuo system (bioMérieux, France).

2.3. Viral metagenomics analysis

Of the 124 samples which were negative for all tested pathogens, forty-five cases which either required ICU admission or mechanical ventilation, and/or had C-reactive protein ≥ 10mg/L (which is associated with viral respiratory infection) [15] were randomly selected for viral metagenomics analysis. These samples were subjected to a sequence-independent, single-primer amplification (SISPA) method as previously described with minor modifications [16]. Briefly, second DNase treatment using DNase I, amplification grade (Invitrogen, USA) was added after extraction using QIAamp MinElute Virus Spin kit (QIAGEN, Germany). Reverse transcription was performed using SuperScript IV First-Strand Synthesis System (Invitrogen, USA). The SISPA amplification was performed and the amplicon was purified using Zymo DNA clean & concentrator (Zymo Research, USA). The purified amplifications were subjected to library preparation using Illumina DNA Prep kit and sequenced on an Illumina NextSeq 500 platform using a NextSeq 500/550 High Output kit v2.5 (300 cycles) (Illumina, USA). The sequencing runs included molecular grade water as a non-template control (NTC).

2.4. Bioinformatic analysis

Raw sequencing reads were trimmed to remove sequencing adapters and low quality sequencing reads. Host sequences and NTC reads were filtered out before identifying potential viral pathogens using IDseq portal (https://idseq.net) [17]. Detection of a virus was reported if non-overlapping reads from ≥3 distinct genomic regions were identified [18]. The reference-based mapping approach was employed to assess the level of identity and genome coverage of the corresponding viruses. PCR confirmatory testing of viral respiratory hits was performed. The primers used are listed in Supplementary Table 2. The raw reads for all 45 samples have been submitted to the Sequence Read Archive (SRA) (accession numbers SRR15039589 – 15039633).

2.5. Whole genome sequencing and phylogenetic analysis

The Saffold virus reads obtained from viral metagenomics analysis were subsequently mapped to the closest full reference genome SA6V-6, Pak1570 (GenBank accession number AB747252). The leftover gaps were sequenced separately by conventional Sanger sequencing using the primers described in Supplementary Table 1 and the consensus sequence was generated and deposited in GenBank (accession number MZ384007). All 51 Saffold virus complete genomes available on GenBank as of 31 March 2021 were included in a multiple sequence alignment using MAFFT implemented in Geneious Prime 2020 (Biomatters, New Zealand) with default parameters. A phylogenetic tree was generated with IQ-TREE v2.1.2 [19], using the GTR+F+I model with 1000 ultrafast bootstrap replicates, and visualized with FigTree v1.4.4 [20]. A partial VP1 sequence of enterovirus D68 was also generated and deposited in GenBank (accession number MZ384008). Phylogenetic analysis of EV-D68 strains using partial VP1 gene sequences (810bp) including Malaysian sequences was performed using the same model as for Saffold virus.

2.6. Indirect immunofluorescence assay for Saffold virus antibodies

We retrieved archived serum samples from the Saffold virus-positive patient collected during the second day of hospital admission and 10 months after infection. Vero cells (CCL-81) (ATCC, USA) infected with Saffold virus strain Penang (GenBank accession number HQ162476) were used as antigen. Infected cells were harvested when 50% cytopathic effect was observed. Infected cells were washed with sterile phosphate buffered saline (PBS) (Oxoid, France) and spotted onto 8-well microscope glass slides. The slides were allowed to air-dry and then fixed with 100% pre-chilled acetone. To detect IgM antibody, serum samples were pre-treated with rheumatoid factor (RF) absorbent to remove IgG and incubated at 37°C for 20 min. The serum samples without treatment (IgG) and serum samples treated with RF absorbent (IgM) were added to a coated well and incubated at 37°C for 30 min and 45 min respectively, in a moist chamber. After washing with PBS, diluted fluorescein isothiocyanate-conjugated rabbit anti-human IgG and goat anti-human IgM antibody (Thermo Scientific, USA) were added followed by incubation at 37°C for 30 min in a moist chamber. After PBS washing, slides were examined under a fluorescence microscope (Leica, Germany).
which are not available in the Allplex RV assay. The NxTAG assay also
detection of enterovirus, bocavirus, coronavirus and
commonly detected virus, followed by parainfluenza virus (1.5%; 3/
198). The NxTAG assay had a higher detection rate (10.6%; 21/198)
compared to the Allplex RV assay (6.1%; 12/198), mainly because of
SARS-CoV-2, rhinovirus/enterovirus (5.6%; 11/198) was the most
identified in three samples (6.7%; 3/45), of which two were res-
piratory pathogens (enterovirus D68 (EV-D68) and Saffold virus), and
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3. Statistical analysis

Univariable analysis using chi-square and Mann-Whitney U (for age)
tests was performed to compare features of patients with SARS-CoV-2 and
patients with other respiratory pathogens. Variables with a p-
value of <0.2 in univariable comparison were included in a multivari-
able regression analysis using the same outcome. The standard
regression analysis with default variable selection (enter) was used.
Results were expressed as odd ratios (OR) and adjusted OR (aOR) with
95% confidence intervals (CI). A two-sided p-value of
0.05 was
regarded as statistically significant. All analyses were performed with
SPSS version 23.0 (IBM, USA).

4. Results

Out of 198 patients, 74 (37.4%) were positive for any respiratory
pathogens, including 56 (28.3%) with SARS-CoV-2 and 18 (9.1%) pos-
itive for other respiratory pathogens. Five (2.5%) SARS-CoV-2 patients
were co-infected with rhinovirus/enterovirus (Table 1). No co-infections
were found in patients positive for other respiratory viruses. Other than
SARS-CoV-2, rhinovirus/enterovirus (5.6%; 11/198) was the most
commonly detected virus, followed by parainfluenza virus (1.5%; 3/
198). The NxTAG assay had a higher detection rate (10.6%; 21/198)
compared to the Allplex RV assay (6.1%; 12/198), mainly because of
detection of enterovirus, bocavirus, coronavirus and M. pneumoniae
which are not available in the Allplex RV assay. The NxTAG assay also
detected an additional parainfluenza virus case missed by the Allplex
assay. Additionally, no samples were positive for SARS-CoV-MERS-CoV
and bacterial select agents.

A subset of 45 cases which gave negative results for all respiratory
molecular assays underwent viral metagenomics analysis. The average
raw reads per sample was 15,228,085, ranging from 10,943,252 to
20,744,776 reads. After human reads (average 47.6%) and contamina-
tion reads (44.1%) were trimmed, the clean reads were assigned to
taxonomic distribution and only 0.7% belonged to viral reads. Viruses
were identified in three samples (6.7%; 3/45), of which two were res-
piratory pathogens (enterovirus D68 (EV-D68) and Saffold virus), and
the third was human papillomavirus type 4, usually found on skin
(Table 2). Phylogenetic analysis of the partial VP1 of EV-D68 sequences
revealed that our EV-D68 sequence was grouped into clade A1 and
closely related to strains from Philippines, USA and New Zealand
(Figure 1). Notably, the full genome of Saffold virus was successfully
obtained and this is the first time this virus has been identified in our
lab. Phylogenetic analysis showed that this newly identified
virus belongs to genotype 6 (Figure 2). The patient’s serum samples from
day 2 were negative for Saffold virus IgM and IgG using indirect
immunofluorescence assay, while the sample from 10 months was pos-
lative for Saffold virus IgG (Supplementary Figure 1).

The median age of 198 adult patients suspected of COVID-19 was
48.5 years (range 19–93 years old), with 44.9% male and 55.1% female
(Table 3). Among the patients, 20.7% (41/198) had severe clinical
outcomes, including death (10.6%; 21/198), ICU admission (15.2%; 30/
Fig. 1. Phylogenetic tree of EV-D68 virus partial VP1 gene sequences. Strain names are in the format: accession number_strain name_country of isolation_year of isolation. The numbers refer to percentage of bootstrap support at key nodes. Malaysian sequences are colored red. The newly identified sequence from this study is denoted with the red circle. The tree is rooted with prototype Fermon strain.
Fig. 2. Phylogenetic tree of Saffold virus full genome sequences. Strain names are in the format: accession number_strain name_country of isolation_year of isolation. The numbers refer to percentage of bootstrap support at key nodes. Malaysian sequences are colored red. The newly identified sequence from this study is denoted with the red circle. The tree is rooted with the earliest Saffold virus whole genome sequence (NC_009448).
198 or requiring mechanical ventilation (11.1%; 22/198). Most of the COVID-19 patients (91.1%; 51/56) had mild symptoms. Univariable analysis revealed that asthma, chronic lung disease, severity of illness, hospitalization, and ventilation requirement were significantly associated with patients with other respiratory pathogens compared with SARS-CoV-2 patients (Table 3). However, none of these were found to be statistically significant predictors following multivariable analysis.  

5. Discussion

After a few early sporadic cases, the SARS-CoV-2 pandemic escalated in Malaysia at the end of February 2020, after a large mass religious gathering held in Kuala Lumpur acted as a superspreading event and led to lineage B.6 viruses spreading nationally and regionally [21]. During this early pandemic period, other than SARS-CoV-2, rhinovirus/enterovirus (5.6%) was the most common virus for respiratory tract infection and was detected in all 5/56 (8.9%) of SARS-CoV-2 cases with co-infections. This low overall co-infection rate is similar to previously reported rates ranging from 1.8 – 9.7% [4, 22-24]. The low rate of other respiratory pathogens of 9.1% (18/198) we detected during this pandemic has been described elsewhere [25-27], and contrasts with pre-pandemic papers using multiplex PCR testing reporting respiratory infection rates ranging from 22-63% [28-30]. Notably, influenza virus, which usually accounts for 5-14% of respiratory infections in Malaysia [31, 32], was much reduced to only 0.5% (1/198) in this study. These low rates of other respiratory viruses likely resulted from public health measures including face mask wearing, handwashing, social distancing, school closures, and movement restrictions implemented during the pandemic. For example, surgical face masks significantly reduced the detection of influenza virus in droplets and coronavirus in aerosols, but were less effective for rhinovirus [33]. This supports our data showing rhinovirus/enterovirus as the most frequently detected respiratory virus other than SARS-CoV-2.

Although B. pseudomallei is endemic in Malaysia, and there have been sporadic imported cases of MERS-CoV and SARS-CoV [34, 35], none of the samples were positive for select agents. Continued surveillance for these agents is important in Malaysia.

With the use of viral metagenomics analysis, three samples had
unique viral reads and two were respiratory viruses (Saffold virus and EV-D68). The sample with EV-D68 positive was not identified by NxTag assay initially and a repeat PCR using specific primers was positive, suggesting lower sensitivity or primer mismatch in the initial testing. A partial VP1 sequence was obtained and this strain was from the A1 clade, which has been previously reported in Malaysia in 2012 [36]. Additionally, human papillomavirus type 4 was detected in one sample. This virus is known to cause skin warts but the pathogenic relevance in the respiratory tract is unknown.

Saffold virus is an emerging human cardiovascular and most of the reported infections have been in children with respiratory and gastrointestinal infections [37–39]. Saffold virus causing respiratory tract infection in adult patients has been rarely reported. In this study, Saffold virus was detected from a 34-year-old male with fever, myalgia, and hypoxia. He required ICU care for a day and discharged after 4 days in hospital. We obtained a full-length genome of Saffold virus genotype 6, which clustered with Cameroon and Pakistan strains (87.1% covered). A Saffold virus strain Penang (genotype 3) was reported in Malaysia in 2009, and more than 70% of 400 schoolchildren aged 10-12 years had serological evidence of exposure to this virus [40], indicating that it is widespread. We did not detect Saffold virus IgM and IgG in a very early serum sample from the patient at 2 days illness, but did detect IgG in a sample after 10 months (no other serum samples were available). The high number of Saffold viral reads, absence of other respiratory pathogens despite respiratory symptoms, and development of IgG supports the likely pathogenic role of Saffold virus in this adult patient, which merits further research into this potentially emerging virus in adults.

In our study, 82% (46/56) of COVID-19 patients were aged below 65 years old and 91.1% had mild symptoms, similar to a large Malaysian study [41]. In univariable analysis, COVID-19 patients were less likely to have severe disease compared to patients with other respiratory pathogens. During the early pandemic in Malaysia, when case numbers were low, all COVID-19 patients were admitted regardless of severity, mainly for isolation. Many of these patients had very mild symptoms which could have been managed at home. Otherwise, after multivariable analysis, there were no significantly different factors between patients with COVID-19 and those with other respiratory pathogens.

Our study has some limitations. The study was limited to a single hospital and only 45/126 negative samples (35.7%) were subjected to viral metagenomics analysis. Viral detection in the remaining negative samples might have been missed. In addition, we only focused on viruses and may have missed bacterial and fungal agents in the remaining negative samples. Multiple variables tested in a small sample size could increase the likelihood of false positive results. Further studies with larger sample sizes and multiple hospital sites are needed.

In summary, the detection rate of respiratory viruses other than SARS-CoV-2 declined during the pandemic, likely with the implementation of public health measures in Malaysia. Co-infections between SARS-CoV-2 were rare and only occurred with rhinovirus/enterovirus, resulting in mild symptoms. Metagenomics analysis is useful to identify not only novel viruses but also emerging viruses likely to be missed by routine diagnostic tests. The emerging cardiovasc Saffold virus, rarely reported in adults, was detected in a single case using viral metagenomics. Continued surveillance of respiratory viruses is important to detect changes in circulating viruses, including emerging pathogens.

Declaration of Competing Interests

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Supplementary materials

Supplementary material associated with this article can be found in the online version, at doi:10.1016/j.jcv.2021.105000.

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