



Extensive microbiological respiratory tract specimen characterization in critically ill COVID-19 patients

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Thomsen K, Pedersen HP, Iversen S, Wiese L, Fuursted K, Nielsen HV, Christensen JJE, Nielsen XC. Extensive microbiological respiratory tract specimen characterization in critically ill COVID-19 patients. *APMIS*. 2021; 129: 431–437.

Microbial co-infections may contribute to the pulmonary deterioration in COVID-19 patients needing intensive care treatment. The present study portrays the extent of co-infections in COVID-19 ICU patients. Conventional culture, molecular detections for atypical aetiologies, QiaStat-Dx® respiratory panel V2 detecting 21 respiratory pathogens and ribosomal DNA genes 16S/18S amplicon-based microbiome analyses were performed on respiratory samples from 34 COVID-19 patients admitted to the ICU. Potential pathogens were detected in seven patients (21%) by culturing, in four patients (12%) by microbiome analysis and in one patient (3%) by respiratory panel. Among 20 patients receiving antibiotics prior to ICU admission, fungi (3 *Candida albicans*, 1 *C. tropicalis*, 1 *C. dubliniensis*) were cultured in 5 (15%) endotracheal aspirates. Among 14 patients who were antibiotic-naïve at ICU admission, two patients (6%) had bacterial respiratory pathogens (*Staphylococcus aureus*, *Streptococcus pseudopneumoniae*) cultured in their endotracheal aspirates. Microbiome analysis recognized four potential respiratory pathogens (3 *Haemophilus influenzae*, 1 *Fusobacterium necrophorum*) isolated in samples from four other patients (12%). QiaStat-Dx® respiratory panel V2 detected adenovirus in one patient (3%). The prevalence of pulmonary microbial co-infections is modest among COVID-19 patients upon admission to ICU. Microbiome analysis complements conventional microbial diagnostics in characterization of respiratory co-infections.

Key words: COVID-19; co-infections; microbiome; microbiological characterization; SARS-CoV-2.

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Coronavirus disease-2019 (COVID-19), a respiratory tract disease with relatively high case fatality rate, is caused by a novel coronavirus, the severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) [1]. SARS-CoV-2 can lead to a broad spectrum of disease, ranging from very mild symptoms of upper respiratory tract infection to life-threatening pneumonia. The pneumonia caused by SARS-CoV-2 is characterized by a resilient inflammatory response triggering a potential deleterious pulmonary disease [2], and the presence of respiratory co-infections might contribute to the injurious process. However,

critical COVID-19 disease is associated with high inflammation marker levels upon admission, which challenge the wariness of respiratory co-infection as it is difficult to distinguish between severe COVID-19 and bacterial or fungal co-infection. Consequently, critically ill COVID-19 patients are prone to receive broad-spectrum antibiotics upon hospitalization [3]. As COVID-19 patients often need prolonged hospitalization and intensive care, redundant antibiotics upon hospitalization may increase the risk of subsequent hospital-acquired infections and other adverse events [4, 5]. Thus, prompt sampling of respiratory secretions and subsequent microbial diagnostics are demanded in order to validate the

likelihood of bacterial co-infections and the consequent requirement for antibiotic treatment. Identification of respiratory pathogens by conventional culture is challenged by the comprehensive use of antimicrobial therapy prior to ICU admission and the fastidious nature of some respiratory tract microbes. Culture independent molecular techniques enable the detection of low abundance and uncultivable respiratory pathogens [6] as amplification and subsequent sequencing of the highly conserved 16S rRNA gene in bacteria and the eukaryotic cytosolic homologue 18S in fungi characterizes the microbial community membership the airway microbiome. In the present study, we performed a comprehensive microbiological characterization of respiratory samples from critically ill COVID-19 patients in order to examine the contribution of amplicon-based microbiome analysis on the diagnosis of respiratory co-infections.

METHODS

This is an observational cohort study conducted on laboratory-confirmed COVID-19 patients who were admitted to ICUs at two hospitals in Region Zealand in Denmark between 11 March and 10 April 2020. Region Zealand is one of the five administrative regions in Denmark. Comprising 17 municipalities, the Region encompasses a population of 837.000 citizens. During the study period of March 11 to April 10, a total of 826 patients had a positive SARS-CoV-2 PCR test in Region Zealand and 254 COVID-19 patients were admitted to a hospital ($n = 6$) in the region of which 34 patients needed intensive care at an ICU. All consecutive patients with laboratory-confirmed SARS-CoV-2 infection referred to hospitals in Region Zealand and subsequently admitted to an ICU were enrolled. A confirmed case of COVID-19 was defined as a positive result on real-time reverse transcriptase–polymerase chain reaction (RT-PCR) assay of nasal and pharyngeal swab specimens or endotracheal aspirate. Only laboratory-confirmed cases were included in the study.

Data collection

Epidemiological, clinical and laboratory data were obtained from patients electronic medical records. The data were reviewed by a trained team of physicians. Information recorded included demographic data, medical history, underlying comorbidities, laboratory findings and treatment measures (i.e. antiviral therapy, antibacterial therapy, respiratory support, kidney replacement therapy). The duration from onset of disease to hospital admission and ICU admission was recorded. Acute respiratory distress syndrome (ARDS) was defined according to the Berlin definition.

Microbiological methods and sampling

Lower respiratory tract (LRT) samples were routinely obtained upon admission to the ICU. Endotracheal

aspirates were subjected to routine culture and antimicrobial susceptibility testing according to European Committee on Antimicrobial Susceptibility Testing (EUCAST) recommendations. Real-time PCR-based detection of *Legionella pneumophila*, *Mycoplasma pneumoniae*, *Chlamydomphila pneumoniae* and *Chlamydomphila psittaci* was performed by a commercial kit, b-CAP assay (Biolegio, Nijmegen, The Netherlands), accompanying the BD MAX system according to the manufacturer's protocol.

In addition, QiaStat-Dx® respiratory panel V2 (Qiagen, Hilden, Germany) detecting 21 respiratory pathogens (including subtypes) was also applied. The pathogens include the following: Influenza A, Influenza A subtype H1N1/2009, subtype H1 and H3, Influenza B, Coronavirus 229E, Coronavirus HKU1, Coronavirus NL63, Coronavirus OC43, Parainfluenza virus 1, 2, 3, and 4, Respiratory Syncytial virus A/B, human Metapneumovirus A/B, Adenovirus, Bocavirus, Rhinovirus/Enterovirus, *Mycoplasma pneumoniae*, *Legionella pneumophila* and *Bordetella pertussis*.

Microbiome analysis (16S/18S rRNA Gene Amplicon Sequencing)

Residual material from the endotracheal aspirates was analysed using combined 16S/18S DNA gene amplicon-based microbiome analysis to evaluate the presence of species-specific sequences [7]. Briefly, DNA was extracted by QIAamp® DNA mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's protocol. For each batch of DNA extraction, a negative control without sample material was included for downstream analysis. DNA was amplified by a two-step PCR using a modified version of the published universal prokaryotic primers 341F (ACTCCTAYGGGRBGCASCAG) and 806R (ACTCC-TAYGGGRBGCASCAG) targeting the V3-V4 regions of the 16S rRNA gene. Amplicons were sequenced on the Illumina MiSeq desktop sequencer (Illumina Inc., San Diego, CA, USA), using the V2 Reagent Kit.

The subsequent inter-sample variable sequence read output compromises the use of a standardized absolute number of sequences to determine the detection of potential clinical important organisms. Instead, we rationalized that it would be valuable to define a cut-off threshold based on a standardized proportion in order to discriminate potential important organisms in a sample. Thus in samples where >50% of the total sequence output exhibiting non-vertebrate DNA (host-DNA) could be consigned to a bacterial or fungal genus/species, this genus/species was considered potential clinical important. This rather cautious approach was justified by the hypothesis that if the majority of the sequences produced for any given sample could be attributed to a single prevailing microorganism, the possibility of this organism being involved in respiratory tract infection might be considered significant.

RESULTS

Table 1 summarizes the demographical and clinical characteristics of COVID-19 patients at the time of admission to the ICU. During the study period, 13.4% ($n = 34$) of all hospitalized COVID-19

patients in the region required ICU level care during their stay. Mean age was 68 years (IQR 59–74y), and 6 patients (18%) were female. Most patients (27 [79%]) had at least 1 comorbidity; however, 7 patients (21%) had no known comorbidities. The Simplified Acute Physiology Score III (SAPS 3) is an ICU scoring system and is used to predict the mortality risk for patients presenting at the ICU. Upon admission to the ICU, the median SAPS III score was 72 (66.5–81.5) corresponding to a predicted mortality >40%. The laboratory measures demonstrated an elevated C-reactive protein (CRP) at 145 (118–233) mg/L, whereas procalcitonin was generally at low levels 0.6 (0.3–2.8) ug/L. The PaO₂ (arterial partial pressure of oxygen)/FiO₂ (fraction of inspired oxygen) ratio was 0.76 (IQR 0.61–0.90) with 50% of the patients having PaO₂/FiO₂ ratio ≤100 corresponding to severe ARDS. Patients were hospitalized a few days (mean 2.7 days [0–16]) prior to ICU admission and stayed a mean of 15 days (range 2–49) at the ICU. The majority of patients (20 [59%]) received antimicrobial therapy prior to ICU admission.

Table 2 summarizes the microbiological results from the analyses performed on LRT samples and

compares patients receiving antibiotics prior to ICU admission with antibiotic-naïve patients.

Pathogens were detected in seven patients (21%) by endotracheal aspirate culture. Among the 20 patients receiving antibiotics prior to ICU admission, fungi (3 *Candida albicans*, 1 *C. tropicalis*, 1 *C. dubliniensis*) were cultured in 5 (15%) endotracheal aspirates; however, bacterial pathogens were not cultured in this patient group. Among the 14 patients who were antibiotic-naïve at ICU admission, two patients (6%) had bacterial respiratory pathogens (*Staphylococcus aureus*, *Streptococcus pseudopneumoniae*) cultured in their endotracheal aspirates.

Co-infection of viral aetiology was evaluated by the QiaStat-Dx® respiratory panel V2 which detected adenovirus in one patient (3%). The commercial PCR targeting *Legionella pneumophila*, *Mycoplasma pneumoniae*, *Chlamydomphila pneumoniae* and *Chlamydomphila psittaci* were unable to detect any of these bacteria of atypical aetiology to LRT infections in the samples.

By applying the standardized threshold for detecting potential clinical important microorganisms, microbiome analysis recognized four potential

Table 1. Demographic and clinical characteristics on ICU admission of COVID-19 patients in Region Zealand, Denmark

	Total (N = 34)
Age, median (IQR), y	68 (59–74)
Female	6 (18%)
Comorbidities	
None	7 (21%)
Hypertension	19 (56%)
Hypercholesterolemia	8 (24%)
Cardiovascular disease ¹	4 (12%)
Lung disease ²	9 (26%)
Diabetes	9 (26%)
Malignancy ³	4 (12%)
Other ⁴	5 (15%)
SAPS III, median (IQR)	72 (66.5–81.5)
White blood cell count, ×10 ⁹ /L, median (IQR)	9.0 (5.0–13.8)
Absolute neutrophil count, ×10 ⁹ /L, median (IQR)	7.5 (4.1–12.3)
Absolute lymphocyte count, ×10 ⁹ /L, median (IQR)	0.8 (0.6–1.1)
C-reactive protein, mg/L, median (IQR)	145 (118–233)
Procalcitonin, µg/L, median (IQR)	0.6 (0.3–2.8)
PaO ₂ , kPa, median (IQR)	9.1 (7.0–11.0)
FiO ₂ , median (IQR)	0.76 (0.61–0.90)
Total days in hospital prior to admission to ICU, mean (range)	2.7 (0–16)
Total days in ICU, mean (range)	15 (2–49)
Total days on invasive mechanical ventilation, mean (range)	13 (0–49)
Patients receiving antibiotics prior to ICU admission	20 (59%)

BMI, body mass index (calculated as weight in kilograms divided by height in meters squared); FiO₂, fraction of inspired oxygen; ICU, intensive care unit; IQR, interquartile range; PaO₂, arterial partial pressure of oxygen; SAPS III, Simplified Acute Physiology Score 3.

¹Cardiovascular disease includes cardiomyopathy and heart failure.

²Lung disease includes chronic obstructive pulmonary disease (COPD) and asthma.

³Malignancy includes active neoplasia and neoplasia in remission.

⁴Other includes epilepsy, inflammatory bowel disease, osteoporosis, endocrine and neurologic disorders.

Table 2. Microbial pathogens detected in lower respiratory tract samples of COVID-19 patients at the time of admission to the ICU

	Total (N = 34)	Antimicrobial therapy prior to ICU admission (N = 20)	No antimicrobial therapy prior to ICU admission (N = 14)
No. of patients with co-infection	12 (35%)	6 (30%)	6 (43%)
Standard culturing	7 (21%)		
<i>Candida albicans</i>		3 (15%)	–
<i>Candida tropicalis</i>		1 (5%)	–
<i>Candida dubliniensis</i>		1 (5%)	–
<i>Staphylococcus aureus</i>		–	1 (7%)
<i>Streptococcus pseudopneumoniae</i>		–	1 (7%)
QiaStat-Dx® respiratory panel V2	1 (3%)		
Adenovirus		1 (5%)	–
Atypical pneumonia PCR ¹	–	–	–
Microbiome 16S/18S sequencing	17 (50%)		
<i>Haemophilus influenzae</i>		–	3 (21%)
<i>Fusobacterium necrophorum</i>		–	1 (7%)
<i>Acinetobacter Iwoffii</i>		2 (10%)	–
<i>Corynebacterium durum</i>		–	1 (7%)
<i>Dialister microaerophilus</i>		1 (5%)	–
<i>Pseudomonas boreopolis</i>		1 (5%)	–
<i>Corynebacterium kroppenstedtii</i>		–	1 (7%)
<i>Faecalibacterium prausnitzii</i>		1 (5%)	–
<i>Candida albicans</i>		3 (15%)	–
<i>Candida tropicalis</i>		1 (5%)	–
<i>Candida dubliniensis</i>		1 (5%)	–
<i>Staphylococcus aureus</i>		0	1 (7%)

¹*Legionella pneumophila*, *Mycoplasma pneumoniae*, *Chlamydomphila pneumoniae* and *Chlamydomphila psittaci*.

respiratory pathogens (3 *Haemophilus influenzae*, 1 *Fusobacterium necrophorum*) isolated in samples from four other patients (12%). All four patients did not receive antibiotics prior to ICU admission. Additionally, the microbiome analysis identified 6 bacterial species in respiratory samples from seven patients that were not deemed clinically important as they were recognized as oral (*Acinetobacter Iwoffii*, *Dialister microaerophilus*, *Faecalibacterium prausnitzii*) or environmental commensals (*Corynebacterium durum*, *Corynebacterium kroppenstedtii*, *Pseudomonas boreopolis*), and their role in LRT infections was interpreted as negligible. In accordance with the tracheal aspirate cultures, the microbiome analysis confirmed the sample positive for *Staphylococcus aureus* and all the yeasts (3 *Candida albicans*, 1 *C. tropicalis*, 1 *C. dubliniensis*), however, was not able to identify the cultured *Streptococcus pseudopneumoniae*.

DISCUSSION

This study describes basic demographic characteristics, clinical presentation and microbiological findings of all consecutive patients with laboratory-confirmed SARS-CoV-2 infection referred to hospitals in Region Zealand and subsequently admitted to ICU (n = 34) within the first month of the

Danish pandemic involvement. This study provides additional insight into the possible benefits of using available molecular microbiological panels and tests, including microbiome examination of respiratory tract specimens.

Our findings support the observations of earlier COVID-19 studies, which found a high percentage of hospitalized patients of advanced age with pre-existing conditions, arterial hypertension being the most common. The spectrum of clinical presentation of COVID-19 disease in hospitalized patients is wide going from patients being allocated to internal departments of medicine to patients having acute need for ICU treatment and eventually intubation and artificial ventilation. Not seldom patients with COVID-19 admitted to the ICU have a fulminant clinical presentation and severe changes in their lungs [8]. Accordingly, 50% of the patients in our study had severe ARDS, determined by a P_{aO_2}/F_{iO_2} ratio ≤ 100 . The disease severity upon ICU admission corresponds to a high SAPS 3 score in our study group. The contribution of respiratory co-infection to the pulmonary deterioration is unprecedented, and the recognition of co-infection in critically ill COVID-19 patients is challenged by the thorough pulmonary inflammation in these patients, as conventional inflammatory biomarkers are typically elevated which lowers their sensitivity in predicting bacterial infection. In our study, the

patients had a median CRP of 145 mg/L (IQR 118–233) upon admission to the ICU. Although procalcitonin is proposed as a prognostic marker of severe disease in COVID-19 patients [9], its value in assessing bacterial pneumonia in intubated ICU patients is debatable [10].

Studies performed on patients with influenza infections imply that co-infections with oropharyngeal bacteria such as *Streptococcus pneumoniae*, *Staphylococcus aureus* or pyogenic streptococci occur in a high percentage of cases, including patients who received intensive care therapy [11]. In a study performed on the critically ill patients due to another coronavirus, MERS-CoV, bacterial pathogens from respiratory samples were found in 20–25% of patients [12].

Only limited number of observational studies about co-infections among COVID-19 patients have been published. Co-infection was ascertained in 57 of 221 (26%) COVID-19 patients in Wuhan, China. Among patients with severe disease, 65% had co-infection and of those, 39% had bacterial respiratory pathogens detected [13]. Among 116 SARS-CoV-2-positive specimens from COVID-19 patients in Northern California, 21% were positive for one or more additional pathogens like rhinovirus/enterovirus and non-SARS-CoV-2 Coronaviridae [14]. Opportunistic bacterial pathogens were detected in 31% of respiratory samples from 32 COVID-19 patients in Guangzhou, China, and the proportion of co-infection in ICU patients was significantly higher than patients with milder aetiologies [15]. In our cohort, only one patient had co-infection with another virus, adenovirus. In contrast, 7/34 (21%) patients had a significant growth of potential pathogens by culture. Results from the microbiome analysis identified potential pathogens as predominant bacteria in the respiratory microbiome (3 *H. influenzae*, 1 *F. necrophorum*) in four more patients. Interestingly, none of the four patients received antimicrobial therapy prior to the ICU admission. There was a reasonable compliance between culture and microbiome results as 6/7 (85%) of cultured microorganisms in the aspirates were accordingly identified in the microbiome analysis. The microorganisms identified by microbiome analysis were recognized by employing a conservative pre-defined cut-off threshold based upon a standardized proportion of sequence outputs in LRT samples. This approach might increase the risk of ignoring potential clinical pathogens with low sequence read output. However, lowering the pre-defined threshold as determined in the methods section did not reveal any expected respiratory pathogens. Thus, when combining the findings in microbiome analysis and conventional culturing of

LRT samples, the prevalence of bacterial/fungal co-infections increased from 21% to 33%.

Microbiome analyses has been increasingly used in the clinical microbiology laboratories for identifying rare, difficult-to-detect and co-infecting pathogens directly from clinical samples [16, 17, 18]. Characterization of the airway microbiome have been accomplished in various respiratory diseases. In ventilator-associated pneumonia (VAP), sequencing of 16S rRNA amplicons has enriched the microbiological diagnosis by supplementing standard culturing [17, 19, 20] and demonstrated a potential application as prognostic marker as the microbiome composition at the time of intubation may predict the subsequent development of VAP [19]. In cystic fibrosis (CF), the airway microbiome is more thoroughly studied and indicates that a diminished microbiome diversity is associated with increased inflammation and inferior lung function [21, 22]. Correspondingly in non-CF bronchiectasis, an association between microbiome diversity and disease severity is recognized [23, 24]. Our result support that microbiome analysis could be a valuable supplement to culture. However, to our knowledge, no studies have highlighted the clinical impact of airway microbiome analyses on antibiotic treatment decision-making and subsequent patient outcome. Consequently, the application of microbiome analyses on respiratory samples has yet to be determined in the clinical setting, however, lowered turn-around-time and cost are a prerequisite for routine application to be feasible.

Based on earlier experience from other viral pneumonias and their clinical characteristics, empirical antimicrobial therapy is recommended for patients with COVID-19 [24]. Consequently, more than 80-90% of COVID-19 patients at ICUs receives antimicrobial therapy [25]. In our study population, 59% of patients received broad-spectrum β -lactam antibiotics (piperacillin/tazobactam or meropenem) prior to ICU admission. Hospitalized COVID-19 patients receives broad-spectrum antibiotics with unknown efficacy. The subsequent possible overuse of antibiotics calls for antimicrobial stewardship as a potential increase in antimicrobial resistance rates during this pandemic is a worried consequence [26]. Appropriate microbial characterization of respiratory samples in COVID-19 patients is a prerequisite for antimicrobial stewardship, and microbiome analysis may supplement conventional diagnostics in guiding rational antibiotic management.

To our knowledge, this study is the first study that use microbiome analyses as a supplement to the routine methods to investigate the presence of co-infections in COVID-19 patients admitted to

ICU. Our result showed that microbiome analysis contributed to diagnosing bacterial co-infections in 4/34 cases. One of the limitations of this study is the small number of patients included. Well-designed studies with large sample size are warranted to explore the risk of co-infection based on microbiological characterizations and impact of microbiological findings on the clinical outcomes of critically ill COVID-19 patients.

CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

ETHICAL APPROVAL

The study has achieved approval from the Regional Ethics Committee (SJ-832) and the Danish Data Protection Agency (REG-054-2020).

AUTHOR CONTRIBUTIONS

KT and XCN designed the study, collected data, analysed data and wrote the manuscript. HPP, SI, LW and JJC contributed to the data collection and analysis. KF and HVN performed the microbiome analyses. All authors contributed to the revision of the draft manuscript and approved the final version.

ABBREVIATIONS

SARS-CoV-2: Severe Acute Respiratory Syndrome Coronavirus 2; COVID-19: Coronavirus disease-2019; ICU: Intensive Care Unit; LRT: Lower Respiratory Tract; PCR: Polymerase-Chain-Reaction; ARDS: Acute Respiratory Distress Syndrome.

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