Improved Detection of Culprit Pathogens by Bacterial DNA Sequencing Affects Antibiotic Management Decisions in Severe Pneumonia

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Data Collection B
Statistical Analysis C
Data Interpretation D
Manuscript Preparation E
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Patient:	**Male, 21**
Final Diagnosis:	**Bacterial pneumonia**
Symptoms:	**Cough • fever**
Medication:	**—**
Clinical Procedure:	**—**
Specialty:	**Critical Care Medicine**

Objective: **Unusual clinical course**

Background: Severe pneumonia requiring admission to an intensive care unit carries high morbidity and mortality. Evidence-based management includes early administration of empiric antibiotics against plausible bacterial pathogens while awaiting results of microbiologic cultures. However, in over 60% of pneumonia cases, no causative pathogen is identified with conventional diagnostic techniques. In this case report, we demonstrate how direct-from-sample sequencing of bacterial DNA could have identified the multiple culprit pathogens early in the disease course to guide appropriate antibiotic management.

Case Report: A previously healthy, 21-year-old man presented with neck pain and fever and rapidly developed acute respiratory distress syndrome (ARDS) requiring mechanical ventilation. He was started on broad-spectrum antibiotics and was found to have septic thrombophlebitis of the left internal jugular vein (Lemierre syndrome), with blood cultures growing *Fusobacterium necrophorum*. While his antibiotics were narrowed to piperacillin-tazobactam monotherapy, his clinical condition worsened, but repeated efforts to define an additional/alternative respiratory pathogen resulted in negative cultures. He eventually developed bilateral empyemas growing *Mycoplasma hominis*. Once azithromycin was added to the patient's regimen, he improved dramatically. Retrospective sequencing of consecutive endotracheal aspirates showed *Fusobacterium* as the dominant pathogen early in the course, but with significant and increasing *Mycoplasma* abundance several days prior to clinical detection.

Conclusions: Had sequencing information been available to the treating clinicians, the causative pathogens could have been detected earlier, guiding appropriate antibiotic therapy and perhaps preventing his clinical complications. Real-time bacterial DNA sequencing has the potential to shift the diagnostic paradigm in severe pneumonia.

MeSH Keywords: **Acute Respiratory Distress Syndrome • Lemierre Syndrome • Pneumonia • Sequence Analysis, DNA**

Full-text PDF: https://www.amjcaserep.com/abstract/index/idArt/912055

Background

Infectious pneumonia remains a leading cause of morbidity and mortality in the United States, accounting for over 500 000 presentations to the Emergency Department and over 50 000 deaths annually [1]. Prompt initiation of empiric antibiotics aimed against bacterial pathogens is the mainstay of therapy, although viral etiology is more common than bacterial [2], in which case antibiotics are unnecessary and potentially harmful. Blood and sputum are routinely collected for microbiologic culture prior to antibiotic administration and remain the standard of care for identification of culprit bacterial pathogens. Unfortunately, standard bacterial cultures require incubation periods of variable lengths of time and offer limited sensitivity; in fact, conventional culture techniques fail to identify a causal pathogen in >60% of pneumonia cases [3]. To overcome the limitations of culture-based diagnostics, culture-independent microbial DNA sequencing techniques have been proposed as novel tools for diagnosing respiratory infections [4,5]. Such next-generation sequencing (NGS) techniques involve either amplification of a highly conserved bacterial gene (16S rRNA gene) or shotgun sequencing of all DNA molecules present in a sample (metagenomics) to obtain a detailed profile of the members of a microbial community without the need for *ex vivo* organismal growth and isolation.

Although utilization of NGS as a diagnostic tool for pneumonia offers substantial theoretical advantages for improvement in diagnostic accuracy and timeliness, the clinical validity of this technology has not been widely tested despite proof-of-concept demonstrations of feasibility [6]. Here, we report the case of a patient with severe bacterial pneumonia in which culture-based techniques failed to elucidate the multiple pathogens responsible in a clinically actionable timeline. In contrast, NGS approaches uncovered community profiles that could have established an etiologic diagnosis to guide precise antibiotic management earlier in the course; had this information been available in real-time, the patient's clinical course of illness could have been substantially altered.

Case Report

The clinical course (Figure 1A, 1B)

A previously healthy, 21-year-old man with no significant past medical history was evaluated in the Emergency Department with rapidly progressive shortness of breath, fever, and sore throat beginning 5 days prior to presentation. Despite empiric treatment with vancomycin, piperacillin-tazobactam, and azithromycin, he required endotracheal intubation for worsening hypoxemic respiratory failure consistent with acute respiratory distress syndrome (ARDS). CT imaging demonstrated bilateral pulmonary infiltrates and thrombophlebitis of the left

internal jugular vein, and blood cultures drawn on admission grew *Fusobacterium necrophorum* and *Granulicatella*. A clinical diagnosis of septic thrombophlebitis (Lemierre syndrome) was made, with *Fusobacterium* considered the causative pulmonary pathogen, and antibiotics were narrowed to piperacillin-tazobactam on hospital day 3. Despite seemingly appropriate antibiosis, the patient remained febrile with worsening hypoxemia requiring rescue maneuvers including high positive end-expiratory pressure titration and neuromuscular blockade.

Given his clinical worsening, vancomycin was restarted empirically to cover methicillin-resistant *S. aureus* (MRSA) that could have been missed by blood or sputum cultures. In a persistent search for a respiratory pathogen beyond *Fusobacterium*, the treating clinicians performed repeated sampling of the respiratory system for cultures. Routine culture of sputum on hospital day 1 identified normal respiratory flora, and BAL specimens on hospital days 3 and 7 revealed no growth. The patient remained febrile with persistent leukocytosis, and 1 week following admission he developed empyemas with bilateral pneumothoraces requiring surgical chest tube insertions. Bacterial culture performed on pleural fluid drained from these effusions demonstrated growth of *Mycoplasma hominis*. In response to this unanticipated finding, the treating clinicians restarted azithromycin therapy and the patient's fevers subsided within 48 h. The patient clinically improved thereafter, was eventually liberated from mechanical ventilation on hospital day 17, and was discharged from the hospital 1 week later.

The insights obtained by research molecular analyses (Figure 1C, 1D)

To gather retrospective insights into the infectious etiology of our patient's disease and clinical course, we performed bacterial DNA sequencing experiments with respiratory samples collected for research purposes and measured plasma biomarkers on concurrently collected blood samples. Respiratory samples were endotracheal aspirates drawn independent of the 3 aforementioned clinical samples used by the treating clinicians for microbiologic cultures. From these endotracheal samples, we extracted bacterial DNA with standard techniques [7] and conducted sequencing of the 16S rRNA gene (V4 subunit) on the Illumina MiSeq platform. We analyzed the obtained bacterial sequences using custom mothur-based pipelines (calling operational taxonomic units-taxa at 97% identity threshold) [8].

Strikingly, we identified that *Fusobacterium* and *Mycoplasma* taxa were the most abundant bacterial genera at all 3 time points. Initially, *Fusobacterium* dominated respiratory aspirates, with 63% relative abundance (*vs.* 23% abundance of *Mycoplasma*), strongly suggesting that *Fusobacterium* was the causative pathogen of the patient's bilateral pneumonia consistent with ARDS, without any evidence of MRSA superinfection.
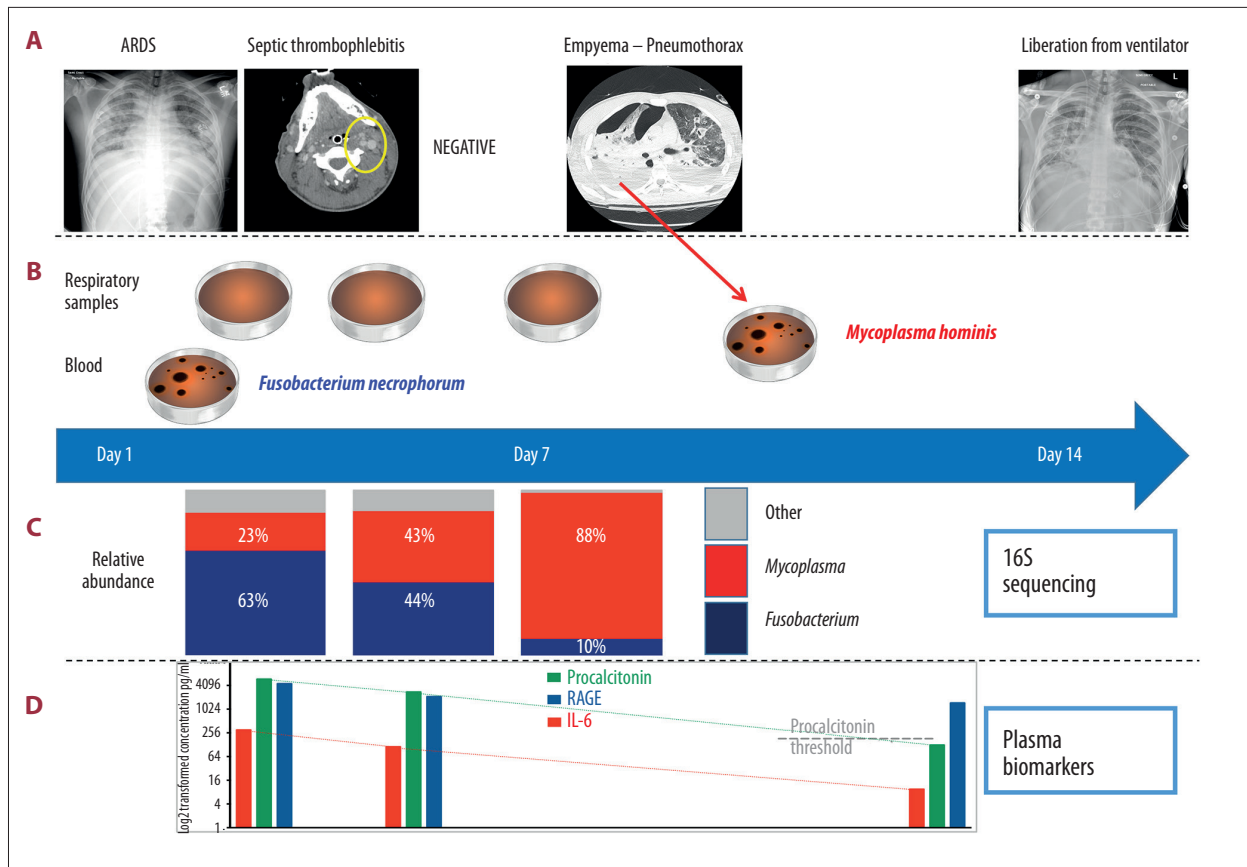
Figure 1. Temporal evolution of the patient's clinical course with clinical data shown in A and B and retrospectively obtained research data in C and D. (**A**) Imaging at baseline showing bilateral pulmonary infiltrates on chest radiograph consistent with ARDS, with CT neck demonstrating thrombophlebitis (yellow circle). CT chest obtained 5 days later showing bilateral empyemas with pneumothoraces requiring chest tube drainage. Chest radiograph on hospital day 17 after being liberated from mechanical ventilation. (**B**) Blood cultures on admission were positive for *Fusobacterium necrophorum*. Three respiratory specimen cultures (endotracheal aspirate or bronchoalveolar lavage) obtained during the first week failed to identify an infectious etiology. Pleural fluid obtained following empyema drainage grew *Mycoplasma hominis*. (**C**) 16S rRNA gene sequencing data, showing the relative abundance of *Fusobacterium* and *Mycoplasma* taxa in endotracheal aspirates on ventilator days 1, 3, and 7. (**D**) Log2 transformed concentrations of plasma biomarkers (RAGE, IL-6, procalcitonin) on ventilator days 1, 3, and 14, showing significant decline overtime with appropriate antibiotics.

The presence of *Mycoplasma* was surprising and likely represented a co-pathogen that grew progressively in abundance as the dominant *Fusobacterium* was being eradicated under the antibiotic pressure of piperacillin-tazobactam monotherapy. Repeat 16S sequencing performed on ventilator day 7 (several days following the discontinuation of azithromycin), demonstrated a marked increase in the abundance of *Mycoplasma* up to 88% of the microbial community, whereas *Fusobacterium* now accounted for only 10% (Figure 1C). As 16S sequencing can only provide bacterial identification information to the genus taxonomic level, we also pursued whole meta-genome sequencing (WMGS) with the Illumina NextSeq platform to identify whether the *Fusobacterium* and *Mycoplasma* taxa corresponded to the clinically isolated species. Notably, we were able to detect sequences that matched the genomes of *Mycoplasma hominis* and *Fusobacterium necrophorum* at the

species level, corroborating our 16S results, despite the overwhelming amounts of human DNA present in these samples.

In conjunction with 16S rRNA gene sequencing and WMGS performed on respiratory samples, we obtained blood samples and measured plasma biomarkers indicative of epithelial injury (Receptor of Advanced Glycation Products-RAGE), host innate immune response (Interleukin 6) and presence of bacterial infection (procalcitonin) with a custom Luminex panel [7]. The temporal changes of these biomarker levels closely reflected the clinical events: marked elevation of RAGE, IL-6, and procalcitonin on admission with persistently elevated levels during the first week of illness (a time period during which *Mycoplasma* was not being covered by antibiotics) with a dramatic decrease by ventilator day 14, including negative procalcitonin levels after 10 days of azithromycin therapy.

Discussion

NGS technologies are culture-independent methods of identifying bacteria directly from biological samples, as there is no requirement for incubation and growth of organisms for isolation. Instead, identification relies on detection of nucleic acid sequences (DNA or RNA) specific to an organism. As such, NGS methods offer tremendous advantages in comprehensiveness and sensitivity compared to routine microbiologic cultures that may be able to identify only a subset of cultivable bacteria. With emerging rapid point-of-care technologies [6,9], NGS may also alleviate the time-consumption problem with traditional culture techniques. Sequencing technologies are mainly divided into those that include amplification of target marker genes (typically the 16S rRNA gene for bacteria) or those that perform shotgun sequencing of all microbial nucleic acids present in a sample (WMGS) [10]. The 16S method capitalizes on the fact that the gene encoding the 16S ribosomal subunit is present in virtually all bacteria and is highly conserved, but also contains hypervariable regions specific for different bacteria at the genus level. Thus, by targeted sequencing of the 16S hypervariable regions and comparisons against bioinformatics databases, we are able to obtain comprehensive profiles of the constituent bacteria in a community and their relative abundances at the genus level (e.g., we identify *Staphylococcus* taxa but are unable to distinguish between *S. aureus* or *S. epidermis* species). Furthermore, because we only sequence a marker gene, we do not obtain any information on the additional genomic content of the bacteria present in a community, such as antimicrobial resistance genes that would be important in antibiotic decision-making. WMGS, on the other hand, uses a "shotgun" approach enabling micro-organismal identification at a species or strain level, and also enables the detection of antibiotic resistance coding potential in a sample. Nonetheless, WMGS remains challenging for clinical applications because patient samples have significantly more human DNA than bacterial DNA, on the order of 10^9: 1. This means that for every 1 billion DNA reads, only 1 will be bacterial in origin. Consequently, the overwhelming majority of the sequencing signal corresponds to the human host and not the bacteria of interest, thus impairing the rapid and cost-effective acquisition of actionable diagnostic information.

In this case, NGS performed retrospectively on respiratory samples identified both the primary infectious source as well as a serious secondary pathogen that went unrecognized and led to an extended ICU course, ventilator dependence, and complications requiring invasive management. Had NGS data (16S or WMGS) been available to the treating physicians, azithromycin would not have been discontinued and vancomycin likely would not have been restarted. One could argue that azithromycin should not have been discontinued in the first place, as co-infection with community-acquired microbes is not uncommon. However, these decisions could certainly have been aided by culture-independent microbial data. Another limitation of 16S rRNA gene sequencing is that species-level identification of microbes is not possible, nor is assessment of antibiotic resistance. Nonetheless, convincing evidence that *Mycoplasma* taxa were a causative pathogen in this case would have resulted in different antibiotic management decisions even in the absence of a detailed susceptibility profile.

Conclusions

In addition to limiting unnecessary antibiotics, accurate diagnostic information leading to timely etiologic diagnosis may have prevented the need for continued aggressive mechanical ventilation, which, in turn, could have prevented the patient's bilateral pneumothoraces requiring invasive thoracostomy tube placement. Thus, development of efficient technology to provide critical care providers with timely NGS results has the potential to amplify the clinicians' ability to correctly diagnose and appropriately treat serious bacterial infections, and allow for precise and rational antibiotic use in our healthcare systems. As the technology matures, costs decrease, and experience with collection processing improves, the utility of culture-independent techniques for critically ill patients on a broader scale warrant closer investigation. Our case highlights how an emerging technique currently used in epidemiologic studies (NGS) could overcome limitations of the current standard of care (microbiologic culture) to accomplish personalized antibiotic management in the ICU.

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