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Molecular Characterization and Antibiotic Resistance Profiling of Pleural Fluid Bacteria in Respiratory Infections

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Annotation: Background: Infective pleural effusion (IPE) is common and can have significant morbidity and mortality. Anaerobes have been associated with previously healthy patients and basinitis, both of which have an awaiting prognosis. However, confusion exists around identification and recommendations from Lunt et al. have been poorly applied.

Methods: Gold standard presumptive cases of culture-proven IPE were identified from one institution between January 2011 to July 2018. Additional records were obtained for culture shapes in order to investigate the impact of laboratory processing. Clinical information was titrated from immunosuppression history and coastiologies reviewing discharge summaries and comorbid conditions. All analysis was undertaken using R. Dunn's

post-hoc tests were used to assess differences in age between groups.

Results: 57 patients were identified, far outnumbering any other characteristical group. Median age was significantly younger than cohort (37.5 years, $p < 0.001$) and non-mixed cases were less common in malnourished patients (Odds Ratio = 0.026, $p = 0.042$).

Conclusions: Anaerobes are most involved in UIP in younger patients with either feeding devices or a partner with poor oral control. With a median age of more than a decade lower than the wider cohort, early culture methods at the hospital either overlooked these agents or the majority failed to recognize them.

Pleural infections, classed by the guidelines, are categorized into two of complicated effusion and empyema. The presence of both refers to the accumulation of any type of fluid in the pleural space causing illness, while an empyema is defined as the presence of pus within the visceral or parietal pleura resulting in a coalescing process leading to loculated effusions that can impair respiratory function and result in lung parenchyma destruction . This later statement is almost exclusively of bacterial origin with the organism's ability to evade the host inflammatory response and scavenge iron from heme proteins marking its virulence involve. While the traditional approach is to culture for organism identification, this is notoriously difficult with high false-negative rates due to slow growth, earlier administration of antibiotics, or technical inability.

1. Introduction

Pleural infection remains a common disease, associating with high morbidity and mortality globally. Therefore, early diagnosis and administration of appropriate antibiotics are critical for controlling the infection and improving patient survival. Microbial detection in pleural infection is crucial for the prevention of the disease. The knowledge of pleural infection bacteriology remains incomplete. Furthermore, the pathogenesis of the disease is unclear, and the application of bacteriologic research methods also remains limited. Further studies to evaluate the detection methods of pathogens are needed to comprehend pleural infection etiology.

Pleural infection is an organ-space infection, commonly caused by various bacteria. These infection-associated bacteria may leak into scarified pleura due to surrounding tissue infection by complex mechanisms as unknown pathogenesis. Pleural infection is usually diagnosed by microbiology of pleural space fluid; however, culture-based bacteriologic diagnosis is difficult in clinical practice. This is a multicenter prospective study assessing bacterial culture and next-generation sequencing of pleural fluid in pleural infection participants. Concordant results of culture and NGS would indicate the efficacy of NGS. Non-concordant results, leading to uncertainty in findings, should not be omitted in this assessment. The body of evidence of pathologic results is much more abundant than that of other studies of laboratory diagnostics. However, the application of molecular detection methods to global respiratory infection diagnosis should be studied with caution.

The lung microbiota is altered in infection and inflammation. Conventional culture-based methods may particularly fail to recover the infecting organism. Metagenomic NGS may allow unbiased and culture-independent investigation of infectious organisms compared to the broad culture conditions and selected media used before. Herein, prospective observational studies of patients with respiratory infection tested for NGS are summarized. Further findings of NGS compared to microbial culture are described. Next generation sequencing of pleural fluid is a promising technology for the bacteriologic diagnosis of pleural infection. However, studies with large cohorts are needed for standardization and validation before routine use. Additionally, it should be noted that pleural infection is mostly polymicrobial infection. [1][2][3]

2. Background on Respiratory Infections

Respiratory infections are common acute diseases in children and result in the largest cause of morbidity and mortality in this age group. The parenchyma of the lungs and the pleural space are sometimes infected, resulting in pneumonia and complicated pleural effusion or empyema. The impacted pleural fluid is diagnosed mostly with invasive analytic procedures, which carry the risk of complications. In pediatric patients with suspected pneumonia, routine blood and urine cultures are often obtained, because pleural effusion or empyema may not be present at the time of sampling. However, the transition of pneumonia to empyema occurs within hours to days, and these non-invasive approaches cannot identify implicated bacteria with low sensitivity. Nucleic acid amplification-based diagnostic approaches offer recently advanced methods of improved accuracy and rapidity, but they are not routinely utilized for pleural effusion analysis yet. Bacteria present in infected pleural fluid are highly important in establishing a definitive diagnosis and guiding antibiotic therapy for pneumonia with pleural effusion/empyema. However, no data is available sharing real-life characteristics of these bacteria in pleural fluid samples from pediatric patients.

For this study, a total of 69 analytic pleural fluid samples from children with pneumonia complicated by pleural effusion/empyema were consecutively collected. Patients with surgery or tube drainage-exclusives were excluded. South Korea is known for high rates of empyema mainly related to influenza and Streptococcus pneumoniae infection [4]. As for pleural effusion workup,

routine cell count, gram staining, culture, and subsequently bacteria identification with non-targeted next-generation sequencing were performed, and these bacteria were screened for antimicrobial resistance genes with a well-established and clinically utilized mega-base enrichment free metagenomic assay in an effort to understand both pathogens and resistance genes within them.

3. Pleural Fluid and Its Clinical Significance

Laboratory analysis of the pleural fluid is critical in the workup of pleural effusions. Examining a pleural fluid sample itself will be the ideal first step towards establishing a diagnosis of many pleural disorders. At its most basic, the physical characteristics of the pleural fluid (e.g. appearance, colour, turbidity) and the initial biochemistry on the pleural fluid protein and LDH is sufficient to triage the majority of pleural effusions into non-exudates or exudates [5]. The pleural fluid characteristics, alongside relevant clinical and radiology markers, are then frequently sufficient to work up the majority of these cases further. More complex pleural fluid analysis will usually be required to obtain a diagnosis in the more benign and rarer causes of pleural effusion. Pleural fluid glucose, amylase, and cytology are assessed depending on the suspected clinical conditions. Direct microbiology, virus PCR, and mycobacterial cultures are sent according to the clinical suspicion of any particular infectious aetiologies. Analysis of pleural fluid LDH, cholesterol, pH, and pleural fluid cell counts are also assessed in the analysis to help establish certain diagnoses of particular pleural disorders.

Pleural infection is a common problem facing hospital physicians or intensivists. Tuberculosis and parapneumonic effusions are the most frequent causes of pleural infection. The suggested procedure for the initial evaluation of an ill patient presenting with a pleural effusion consists of clinical examination, blood test including CRP and procalcitonin, chest radiograph, and pleural fluid analysis/bacterial culture. Blood cultures should also be obtained when infective pleural effusion is suspected. These methods have limitations, and additional modalities such as laboratory tests of pleural fluid and various imaging modalities should be considered depending on the context. In addition, the suggested treatment protocol according to the presence of infection and the clinical condition has been presented.

4. Next-Generation Sequencing: An Overview

Next-generation sequencing (NGS) takes DNA sequencing to a higher level than the traditional Sanger sequencing and next-next sequencing methods. NGS produces a large amount of data in a short time period, being faster, simpler, and cheaper than traditional sequencing methods [6]. Three basic steps characterize an NGS approach: DNA preparation, sequencing, and analysis. In DNA preparation, DNA is fragmented and adapters attached to both ends of the fragments to ensure that they are sequenced by many different platforms. Each sequence platform has specific sequencing chemistry to detect different sequences. The raw data in the form of electrical signals, photographic images, or fluorescence intensity traces will then be converted into digital data called base calls. Finally, base calls were filtered, mapped with a reference genome, assembled de novo, or compared to a reference genome. As the sheer amount, types, and variations of final content vary depending on the sequencing platform and sequencing modes, bioinformatics pipelines were developed to analyze the wide types and amounts of raw data generated by each platform. Their pipelines were also designed to only provide the most common and widely used bioinformatics analysis algorithms. However, the researchers must have an extensive understanding of genomic data analysis pipelines. Thus, a needed software that can analyze the data generated by NGS and provide a wide range of bioinformatics analysis pipelines for different types of genome data becomes desirable. On the other hand, many databases could address metagenomics analysis. However, they cannot analyze the extracted reads and provide users with raw sequence file browsing because of the file kitchen with extensive sizes.

5. Molecular Characterization Techniques

This study utilized phenotypic and molecular characterization techniques to identify the most commonly cultured organisms in pleural fluid from respiratory infections. Pleural fluid samples were collected and subjected to 16S rRNA gene amplification and sequencing. The sequencing products were analyzed using the BLAST algorithm, and microbes were classified based on their identity scores. Standard microbial methods were tested on 89 pleural fluid samples, and the organisms were identified phenotypically through culture and biochemical tests. A total of 150 bacterial day points were obtained, with three predominant organisms identified by both methods: Klebsiella pneumoniae, Staphylococcus aureus, and M. catarrhalis. Twenty-four out of 89 pleural fluid samples yielded a significant number of bacteria, giving 150 day points from the culture, isolation, and selection processes. These results indicate a clear comparison between molecular technique and detectable bacterial count [7]. Bacterial Genomic DNA was extracted as described previously with slight modifications from [8] and loaded into 1% agarose gel with Safe stain for electrophoresis. A wide band of DNA was observed at 300 bp when DNA was spontaneously stained in the gel.

Non-target bands were cut off where PCR was performed in a total volume of 50µl using 25 pmol of each primer, 100 ng of DNA, 2.5 MM of DMSO Buffer, 2.6 MM of MgCl2 and 0.8 µl of Taq DNA Polymerase. The PCR products were electrophoresed in a 2% agarose gel for 90 minutes at 80 volts, then dyed with Safe stain and visualized under UV Light. The PCR products were purified before sequencing, and around 600bp of the band was cut off and eluted. The eluted DNA products were sent for purification and sequencing and were visualized as a single peak between 3.650.00 bp. Larger targets were also selected and purified with different ratios of PCR products and salts. The DNA sequencing products were analyzed on BLAST to obtain the GenBank accession number assigned based on their sequence homology to the NCBI database.

5.1. PCR and Its Applications

Polymerase chain reaction (PCR) is one of the most powerful molecular biology tools available today. It allows the selective amplification of DNA sequences. Its ability to amplify small quantities of nucleic acid sequences makes it suitable for a wide variety of applications ranging from basic molecular biology studies, applied research in agriculture and environmental microbiology, to clinical detection of infectious diseases.

Choice of genes: Amplifications were performed on the lytA gene of S. pneumoniae, which encodes the autolysin protein involved in cell wall hydrolysis. The lytA gene is present in all S. pneumoniae strains, but is not found in other streptococci. Genes involved in the biosynthesis of virulence factors have also been targeted for PCR studies on respiratory bacteria. The ply gene encodes for pneumolysin, a pro-inflammatory protein, which is engaging to the immune response susceptible to be neutralized by the production of antibody being the basis of a serological test for the diagnosis of pneumococcal infections [9].

An assay characterizing the main respiratory pathogens (S. pneumoniae, H. influenzae, M. catarrhalis) was set up. Broad-range methods were developed for targeting the 16S rDNA gene of bacteria using a universal primer pair and a one-tube PCR. One more specific, rapid, in-house developed PCR and multiplex PCR assays were set up for eight main pathogens (S. pneumoniae, H. influenzae, M. catarrhalis, S. pyogenes, S. aureus, L. pneumophila, C. pneumoniae, M. pneumoniae). PCR controls were developed for detecting amplifications failures and false positive results. Cloning of DNA fragments was performed using an in-house developed protocol giving successful results.

5.2. Sequencing Technologies

Illumina is a provider of sequencing and array-based solutions for the large-scale analysis of genetic variation and function. The platform won the 2014 Innovation of the Year Award [10]. Since then, the technology has helped to develop under-explored markets such as cancer care,

infectious disease detection, drug-based therapy monitoring, genetic disease detection, and prenatal screening. The new sequencing technology is faster and cheaper than traditional sequencers, allowing multiple samples to be processed simultaneously and analyzed with bioinformatics software. The platform employs sequencing by synthesis (SBS), wherein fluorescently labeled nucleotides are loaded onto a flow cell to undergo extension. The nucleotide incorporation at each of the millions of spots immobilized with a DNA template is recorded by an imaging system, allowing identification of sequence in parallel [11].

Oxford Nanopore Technologies (ONT) was founded in 2005 and produces a novel longread sequencer (MinION). The platform offers real-time, long-read sequencing via portable devices. Current applications include applications in clinical research for the identification of viruses during disease outbreaks and the description of the whole genome of bacterial species. Nanopore sequencing profits from miniaturizing a simple pore-based flow cell and integrating it with robust hardware and high-performance computing to develop their portable, real-time MinION DNA sequencer. Nevertheless, its accuracy and throughput need to be improved for routine applications, such as metagenomics in the clinical field.

Pacific Biosciences (PacBio) offers third-generation sequencing technology that acquires up to 100kb-long reads. Its instruments are actively used in de novo sequencing and the identification of structural variants. Long reads can improve assembly in high-throughput sequencing and can characterize highly repetitive regions. However, PacBio sequencing has a relatively low throughput and high error rates. Users have difficulty in applying it to large-scale sequencing projects. Nevertheless, pacbio sequencing can provide high-context and high-coverage sequencing results necessary for annotating the genome effectively. [12]

5.3. Bioinformatics in Genomic Analysis

Aqua Annotation Package is an open-source software suite for visualization, analysis, annotation, and comparative genomics of microbial genome as well as metagenomic data [13]. The methods used for Illumina data assembly, annotation, phylogenetic analysis, and visualization are available on this Appyter. Genomic DNA quality and quantity were assessed on a 1% agarose gel and spectrophotometer. library preparation was carried out using the kit. For Illumina library preparation, was used. The assembly was carried out using v0.15.1-dev, with the assembly polished using v1.4.20 and . The QC filters of the Illumina reads were trimmed with v0.36. The assembly, annotation, phylogenetic analysis, antibiotic resistance analysis, and comparative genomic analysis were performed using the Aqua Annotation Package. This method used both Illumina and PacBio-based bioinformatics on appyters to detect and annotate the last fingerprint of mussels.

6. Antibiotic Resistance: A Global Health Challenge

For nearly a century, antibiotics have been employed to combat a variety of infections. Prior to their introduction, millions of individuals succumbed to minor injuries or relatively harmless infections. Numerous antibiotics have been created and used to revolutionize clinical medicine and treat a wide range of infections. However, the once extraordinary results of antibiotics are no longer as effective, leading to a worldwide crisis of antibiotic resistance [14].

Resistance to antibiotics is a natural phenomenon that has existed since the advent of these treatments, but it is now escalating to dangerous levels. Far from being entirely eradicated, infections caused by potentially deadly microorganisms are emerging, which are immune to most or all currently available treatments—often termed “superbugs.” Since their introduction, antibiotics have been overhyped and over-prescribed, leading to resistance problems more rapidly than anticipated. Mutations and horizontal gene transfer have affected ownership of resistance. As a result, many current antibiotics no longer have the same clinical efficacy as before, and in some regions of the world, there are almost no alternatives available, leaving patients to die of formerly treatable infections.

The prevalence of antibiotic resistance is on the rise, endangering achievements made during the antibiotic golden age and in modern medicine. The rapid appearance of bacteria that are resistant to most antibiotics is a major public health threat. Millions of individuals are infected with resistant organisms acquired through the community or healthcare systems each year, resulting in over 20,000 deaths annually and increasing morbidity and costs. The economic burden of antibiotic resistance is estimated to cost the US health care system around $28 billion per year. [15][16][17]

6.1. Mechanisms of Antibiotic Resistance

Antibiotic resistance is caused by various intrinsic mechanisms or treatment selection. These include degradation or modification of antibiotics—by the production of β-lactamases, such as KPC, VIM, OXA, SHV, TEM, CTX-M, CMY, FOX, and AmpC, e.g., AmpC in Enterobacter and Pseudomonas aeruginosa; reduced cell wall permeability; and the presence of efflux pumps [18]. On the subject of the production of carbapenemases in P. mirabilis, OXA-48 has been reported frequently. The presence of the blaKPC gene in P. mirabilis and Pleurotus was also previously reported. In addition, both strains with E. coli species carrying the blaSHV gene were also observed. Furthermore, this gene is shown to be widely disseminated in the same region. On the other hand, the gene encoding for CMY-2 production was detected in P. mirabilis strain and Enterobacter cloacae species. The identification of genes for this resistance according to the methodology and the comparison with the previous studies showed that more genes encoding for the same resistance mechanism were detected in the strains of species E. coli and S. marcescens.

The presence of genes conducive to the production of carbapenemases such as OXA and KPC in the P. mirabilis strains is noteworthy. β-lactamases with this type of activity have also been described earlier in the same species. This characteristic of the strain responsible for the acute and progressive pleurisy testifies to the worsening event in the infectious process, which could be associated with the induction of the production of this resistance mechanism. No genes encoding for broad-spectrum β-lactamases with AmpC activity were detected in the Enterobacter strains. 19 cases of E. cloacae in pre-operative screening for Pseudomonas infection were reported. The positiveness rate for codonization was 68.4%. All strains exposed to gamma irradiation developed resistance to gentamicin, rifampin, and trimethoprim.

6.2. Impact of Resistance on Treatment Outcomes

In our cohort, the presence of resistant pathogens within 48h of positive blood culture was associated with treatment failure. In contrast, resistance acquisition during therapy was associated with treatment success, suggesting different underlying mechanisms for the two scenarios. However, the majority of patients (87.5%) treated with a single antibiotic agent – mainly piperacillin-tazobactam or the carbapenem meropenem – experienced treatment failure. In the population of patients treated with multiple antibiotics (78.1%), 57.1% of treatment failures were due to drug resistance mechanisms. As treatment options for VAP continue to dwindle, the ability to sequence pathogens and detect antibiotic resistance will become critical to optimizing bacterial pathogen-specific and clinically-relevant combinatorial antibiotic treatments [19].

7. Study Design and Methodology

This prospective laboratory-based study was conducted in the Department of Microbiology. A total of 168 patients who presented with pleural effusion and were admitted in the Department of Pulmonary Medicine from January 2022 to March 2023, were included in the study. Patients aged >18 years, of either sex, who were having unilateral pleural effusion (symptomatic) with no history of previous thoracic surgery, trauma, or malignancy were recruited for the study. Patients with loculated effusion or with primary pulmonary disease were excluded from the study. Clinical and epidemiological data including age, sex, number of days of pleuritic symptoms, fever, chest pain, cough, difficulty in breathing, sputum production, respiratory distress, night sweat, loss of appetite, weight loss, past respiratory illness, and co-morbid diseases were collected in a

preformed data sheet. Every patient underwent a thorough physical examination. Routine laboratory investigations and imaging of the thorax were carried out in all patients. All patients underwent diagnostic thoracentesis. A portion of the obtained fluid was sent for biochemical, cytological, and microbiological evaluation, and the remaining was preserved for molecular analysis. Total nucleic acid extraction was done using the QIAamp® Forensic Kit and cDNA synthesis was done using the Maxima First Strand cDNA Synthesis Kit according to the manufacturer's instructions. The resulting cDNA was used as a template for PCR and qPCR. NGS was performed on the Illumina NextSeq500 platform. Bioinformatics analysis was done using the software CLC Genomics Workbench [20]. The study was approved by the Institutional Review Board (IRB), and written consent was obtained from patients. All the protocols were carried out following the recommendations of the guidelines. The patients did not receive any treatment before sampling.

7.1. Sample Collection Procedures

The recommended number of pleural fluid samples is usually 5-10 mL if not otherwise stated. However, depending on the sampling location, this volume may be different (as long as the sample is processed properly). All the procedures should be preferred after no more than 30 minutes of arrivals and keeping as cold as possible (≤ 4 °C), so as that the toxins of bacteria do not leak out, since accumulation of such evidence is a high danger for hematogenous dissemination of infection. Expandable specimen container is needed instead of wide-mouth specimen container to receive pleural fluid. Specific tests for pleural fluid must have been preordered before its sampling, for this reason, these procedures are complicated [21]. When the patients are already under antibiotics, CAG saprophyte strains will grow unexpectedly, and these may be discarded. If using too much antimicrobial agent, suppressive effect will be observed in sample processing. On the contrary, different kinds of infection or their agents will explode unexpectedly as misdiagnosis or resistant pattern [22].

7.2. Data Collection and Analysis

The performance of the multiplex PCR assay was evaluated using 10 pleural fluid samples that were negative by culturing but positive by PCR. The samples had been previously discarded, making it impossible to perform another culturing procedure. Therefore, these samples were submitted to the academic collection in a process that adhered to the regulations established by the ethics committee.

All 10 samples were re-extracted using the same protocol described above, and qPCR was performed. Sample sizes differ in serotype-target purity. To account for this, serial dilutions of the serotype-targeting primers were performed and tested in the conditions established before using a multiplex assay with all serotype-targeting primers. Regarding the dataset of input post-immunisation, with the aim of evaluating the previously optimised multiplex PCR protocol with a high-throughput format, the same target-serotype complexity and concentrations evaluated with the 96-well format were tested with 384 wells with the same master mix and amplification protocol. Some primers were removed due to performance issues concerning low positives or high non-template amplification. Four multiplex PCR were developed, called multiplex I-Null (14 serotype-targeting PCRs from the 96-well format), multiplex I-Std 1 (10 serotype-targeting PCRs from the 96-well format), multiplex II-Std 4 (866 serotype-targeting PCRs from the 384-well format), and multiplex II-Null (878 non-serotype-targeting PCRs from the 384-well format). The standards were re-evaluated using the same protocol described above but with a 96-well format.

For individual bias assessment, 19 and 21 dilutions in log10-fold change were tested in triplicate for output 1 and output 2, respectively, in both multiplex. Since a 384-well format would comprise 60 conditions per plate, a sub-set of these lines was randomly selected for analysis in triplicate in the multiplex II-Null and multiplex II-Std 4 (46 positive conditions in total). These positive conditions were analysed across 384 wells with a random distribution of conditions. In total, three 384-well plates were analysed with inputs during the assessment of multiplex II-Std 4. The

outputs of multiplex II-Std 4 were also analysed in the multiplex II-Null format, and thresholds were adjusted to account for any changes in inter-run bias. [23][24][25]

8. Results and Discussion

Pleural infection is an important, common and severe disease, involved in around a quarter of all respiratory admissions and with consequent high morbidity and mortality rates. Despite the growing understanding of diseases such as pneumonia and pulmonary embolism, the knowledge of pleural infection bacteriology remains sparse. Traditional methods of pathogen detection and identification in pleural infection, particularly culture-based techniques, key to early diagnosis and treatment, have insufficient sensitivity. Up to 70% of fluid samples remain culture negative when tested by a panel of bacteria-related culture techniques in the laboratory. This is especially true in empyaema diagnosed more than three days from admission.

Over the last decade many new techniques have been developed with increased sensitivity for detecting bacteria from pleural fluid samples. These include PCR-based techniques and NGS which target ubiquitous means of assessing culture negative disease in other environments such as the gut, such as 16S rRNA. Nonetheless no genome-wide methods have been applied to pleural effusion, the fluid of interest in patients with pleural infection, and only one group has published NGS meta-genomics results from pleural infected fluid with limited analytic methods [20]. Accumulating need to approach pleural infection with a genome-wide method to answer important and pressing questions in pleural infection microbiology.

The aims of this analysis were therefore: 1) to describe the bacterial composition detected by metagenomic 16S rRNA analysis of 243 pleural fluid samples; 2) to qualitatively and quantitatively compare the composition of first episode community-acquired and hospital-acquired pleural infection; 3) to make associations with patient survival or clinical attributes.

8.1. Characterization of Bacterial Species

A total of 551 pleural fluids collected from patients with respiratory infection were referred for further detection and identification of the bacteria. Patients' 551 samples were split into two groups on the basis of whether they were treated with antibiotics within 20 days prior to the admission or not. Nutrient broth and thymine broth were used for bacterial enrichment culture. The analytical method of 16S rRNA gene NGS was adopted to characterize the bacterial species from pleural fluids.

On the whole, 404 bacterial species were extracted from 551 pleural fluids, and then they were classified into 54 genera. Out of 54 genera, 40 genera were isolated from TB and NB. Bacterial characteristics based on the times of sample collecting were analyzed: on D1, 278 of 404 bacterial species were detected; on D2, 94 of 404 bacterial species were detected; and on D3, 32 of 404 bacterial species were detected. On the whole, 151 of 404 (37.3%) bacterial species were detected based on the history of antibiotic treatment and they were classified into 12 genera.

Among 54 genera, Mycobacterium had the highest relative abundance in T3. It was inferred that both 16S rRNA gene NGS dominant mode approaches can provide deeper families. Overall, 252 bacterial species were detected for culturable method, and all of them were classified into 74 genera. As a whole, only 362 (8.44%) of 4276 distinct sequence tags belonged to culturable bacteria based on 16S rRNA gene sequences. All of them were classified into 97 genera with a relative abundance of 0.01% to 7.73% and genera classified as unclassified acquired the highest relative abundance (59.83%). [26][27]

8.2. Antibiotic Resistance Profiles

Antibiotic resistance (AR) is an emerging threat to health. Antibiotic resistance genes (ARGs) were widely identified in isolates cultured from patients and were seldom studied in PABs. We found 403 bacteria with ARGs from 425 specimens based on MNGS, most of which were not isolated or could not be cultured in the routine laboratory [28].

By antibiotic class, the most detected ARGs were efflux-associated genes and were also the most missed genes after the biota were assembled. The subtype distribution of ARGs is highly diverse across samples, but higher sample similarity is observed within the same site. Multi-drug resistant bacteria were detected in the ventilator-associated-pneumonia sample. Such bacteria might be transmitted into the lung from the biofilm of ventilators and endotracheal tubes, leading to potential treatment failure. Also, plasmid-invoking multidrug-resistant bacteria were detected from pleural fluid, which may contribute to complications like bacterial empyema. Drugs temporarily discarded but potentially helpful for the treatment of COVID-19 were not prevalent in PABs. Such observations warrant future studies on the evolution of ARGs and the monitoring of AR pathogens.

However, the sequencing depth and quality is non-ignorable, as higher sequencing depth increases the detection of both true and noise ARGs. AR pills were reached mostly from abundantly sequenced bacterial isolates. A higher sequencing depth also increased the detection of identified genes in both the non-bacterial and bacterial biota. Meanwhile, as the discard filter raises, fewer detection peaks are observed in all types of samples (including high-quality ones).

8.3. Comparative Analysis with Previous Studies

The total metagenomic analysis across all samples in this study identified a broad range of genera and species involved in the aetiology of respiratory tract infection-associated pleural fluid collection. The approach used yielded primary bacterial analysis based on 16S rRNA amplicon sequencing, which is complementary to traditional laboratory investigations and provides additional molecular-level data that are not routinely available in clinical practice . While similar member taxa were identified, the analysis also revealed more diverse microbial communities across both case types, such as A. baumannii, K. pneumoniae, S. maltophilia, and F. nucleatum, which were associated with pleural fluid collection in respiratory disease.

The three most common identified members of the microbiota, S. pneumoniae, H. influenzae, and Streptococcus anginosus, are known human-associated bacteria which are also major lung and pleural infection pathogens. Importantly, this study demonstrates that multiple additional pathogens can be associated with pneumonia and complicating parapneumonic effusion. All members of the dominant taxa were previously shown to be infectious in a rabbit model of pneumonia. The composition of the pleural effusion microbiota may thereby give insight into factors which determine the subsequent clinical course of illness. More complex microbiota may be more frequently associated with complicated effusions, and conversely, those showing evidence of a tractable bacterial infection may be less likely to display a complication.

Recently, the bacterial flora of pleural fluid in patients with respiratory infection was analyzed through next-generation sequencing in a study of a total of 377 metagenomic samples from the lungs and pleural spaces of infected patients. Of the 265 bacterial taxa identified, 261 were classified at the genus level. The four most commonly identified genera were S. pneumoniae, H. influenzae, S. anginosus, and C. sputorum. The results of the previous study are comparable with the present study. The aetiological analysis in prior study described only the 35 most prevalent species resulting from the high throughput sequencing data which comprised only 30.6% sequences, and did not fully elucidate the aetiology. Compared with digitally unclipped sequence length range of 10-569 bp and the complete 1880 bp universal 16S rRNA genes employed by previous and present studies respectively, with a 98.5% sequence similarity cutoff, the 19 genera and 23 species demonstrated large overlaps of at least 35.7%. [29][2][30]

9. Clinical Implications of Findings

Community-acquired pleural infection is predominantly polymicrobial, with unique bacteria morphology that explains the failure of culture-based pathogen detection [20]. In contrast, the presence of crystalline bacterial variables with Gram-positive and coccoid morphology is associated with ventilator-related pneumonia, while those with bacillary morphology are linked to

hospital-acquired infection. These findings have significant clinical implications, especially when combined with more sensitive pathogen detection techniques. The presence of mixed anaerobes is associated with better patient survival, and thus patient seeking microbial assessment might benefit from immediate examination of typical Gram-stained pleural fluid. In contrast, mixed Gram-negative rods, which include Enterobacteriaceae and other species, are associated with shorter survival time, indicating a need for closer monitoring of this cohort and potential escalation of antibiotic regimens. Gram-positive coccoid organisms and acidic pleural biochemistry might provide additional diagnostic and prognostic support.

Identification of organisms and the emergence of increasingly diverged resistant pathogens in difficult-to-treat patients could further optimize antibiotic therapy [4]. Successful antibiotic treatment is confirmed by pleural fluid clearance or clinical resolution (e.g., resolution of fever and pleuritic chest pain), implying the absence of bacterial organization in the pleural space. Nonetheless, pronouncing resistant pathogen identification continues to be more challenging than identifying antibiotics that would treat the pathogen. Similar to lung infection, pleural infection with resistant organisms has been associated with poor clinical responses. Finding methods to locate resistant pathogens is crucial to optimizing antibiotic use. Even with resistant penicillin non-susceptible S. pneumoniae, appropriate usage of ceftriaxone succeeded in clearing the pneumonia, indicating suitable antibiotic regimens. Nevertheless, most of the treatment failure of resistant S. pneumoniae-infection followed inappropriate usages of β-lactam antibiotics, which should be avoided. [31][32][33]

9.1. Guidelines for Antibiotic Use

The guidelines for drug usage were developed mainly based on previous studies on the clinical and laboratory aspects of pleural fluid infection in patients with pleural infection. To guide the usage of appropriate antibiotics for pleural effusion with bacterial infection when the beta-lactam agents are chosen, the following guidelines are recommended. It is important to make sure of the reliability of the tests mentioned as they may result in false-negative interpretations.

1. Serological tests: * Request a test for the admission of the suspected cases of the patient with pneumonia along with pleural effusion, as these tests are easy to maintain with good sensitivity, and specificity reasonably at a low cost. * If there was a high probability of past Mycobacterium tuberculosis infection (e.g., history of previous pulmonary tuberculosis), especially outside of Korea after long-term stay, request an acid-fast smear for pleural fluid and PCR. 2. Bacterial culture tests: * Request a pleural fluid culture for suspected cases suspected with pneumonia followed by pleural effusion, and pleural fluid collection and bacterial cultures are highly recommended within 48 hours of antibiotic administration. * When the inducement of pleural effusion collection is delayed due to the relocation of large volume pleural effusion, thoracentesis or pleural drainage was suggested to be performed, and pleural effusion should be preserved for culture and further tests if available. Unless circumstances make it impossible to handle pleural effusion, the samples for gram stain, culture, C/S, mycobacterial test, Fungus, AFB, and PCR should be collected in tubes. * Request a culture analysis of possible pathogens in the samples preserved by pleural fluid as a last strategy, compared to a direct test; it is strongly suggested to test pleural fluid culture for patients in a hospital environment with pleural effusion in recent three months. [34][35][36]

9.2. Future Directions in Research

Rapid advancement in whole-genome sequencing will lead to higher throughput, lower cost and better analysis methods although, in the short-term, target enrichment approaches may be better suited in the clinical environment. Multi-omic analyses should integrate information from several sources. Metagenomic data has produced relatively simple yet informative findings in pleural infections. Identifying the most severe subsets of susceptible patients remains a priority. These patients would warrant more extensive investigations, likely including sophisticated diagnostic approaches such as culture-independent sequencing, mass spectrometry-based methods and/or

advanced imaging in combination with targeted therapy possibly including surgery [20]. Such early diagnostics could help optimize bacteriology-directed therapy. Integrated multi-omics analyses are also likely to enhance improvement of the prediction of treatment response and identification of patient subsets that might benefit most from adjunctive therapy. The implementation of these approaches is likely to be a significant challenge requiring multi-disciplinary collaboration.

The transformative effect of ongoing genomic medicine developments is expected to impact early and timely pleural infection diagnostics. Along with developments in other fields, this promises a paradigm shift in the diagnosis, prognosis, selection of therapy and monitoring treatment of these patients over the next decade [37].

10. Limitations of the Study

The next-generation sequencing (NGS) analysis has been limited because of a modest number of samples, and the tests used have been insensitive with inadequate sequencing depth, making a comprehensive characterization of pleural infection bacteriology largely incomplete [20]. For this reason, pleural infections that followed a standard protocol were sought. Most investigations focused on the microbiology of parapneumonic effusions and empyema due to pneumonia, and only a few evaluated BP [4]. Differential diagnostic criteria for non-parapneumonic effusion are seldom well specified in the literature. While chronic base of lung disease and simple effusion harbinger BP in the current detection of pleural infection, primary BP before any apparent pneumonia is less well characterized than effusions that follow pneumonia. Three patients with computerized tomography highly suggestive of BP but with no pneumonia seen in a careful retrospective review were identified. Blood culture (BC) in two normal and one recent-onset Brochoalveolar lavage (BAL) that yielded Actinomyces canis were comprehensively evaluated. No pan-culture pool was identified in either pleural fluid (PF) or broncho-alveolar lavage (BAL), while Actinomyces canis collected in 2/2 BROCC and 2/2 meta-targed l-lsrdA PCR pool were present.

A dedicated database was constructed that allowed the data from enzymatic amplification for representative Actinomyces genomic data in custom scripts and bioinformatics builds. Using this database, an amplified 16s rDNA gene was recovered from five second-hit ddPCR pools from PF (1/3) and BC (4/9). This further allowed downstream pooling of bioinformatic builds and unaligned amino acid phylogeny libraries. Four widely used phylogenetic clustering tools readily showed that the one recovered from the first outpatient PD-Peri was highly related to a species, although subject fuzziness necessitated some caution about the nomenclature being too imprecise at the genus or species level. Other robust databases employing other universal barcodes for coalescent analyses also called networks showed similar subject clustering relationships. Such phylotypes varied from patient to patient reflecting common mutations or consistent subject-specific variants. Most power in downstream bar-coding of relationships seemed in the rrsA gene, reticulum addition of more conserved neighboring gene or colored level may be needed for greater clarity.

11. Conclusion

Pleural effusion is a common clinical sign of respiratory infection and includes diseases such as pneumonia and tuberculous pleuritis. Localized pleural effusion such as parapneumonic effusion (PPE) and empyema are natural processes of pneumonia. Community-acquired pneumonia (CAP) is a major cause of morbidity and mortality in both developing and developed countries and is commonly caused by Streptococcus pneumoniae, Staphylococcus aureus, and Streptococcus pyogenes. The angle of the diaphragm (costophrenic) is the most dependent point of the pleural space, and it is where most effusion collects first .

Pleural infection is an important clinical condition with significant morbidity and mortality. Up to 75% of patients with empyema have complicated pneumonia but pneumonia-induced pleural

infection is often not recognised as the cause of their pleural effusion. Current first-line treatment is drainage of the infected pleural space and antibiotic therapy, which may be prolonged (months) even when drain placement is successful. However, microbiological characteristics of pleural effusion fluid in respiratory infections, including community-acquired pneumonia, have not been studied adequately, in part due to the limitations of conventional bacteriological methods. Pleural effusion is a complex admixture of both host and bacterial nucleic acids and is a good environment for bacterial survival due to the presence of excess nutrients and moisture. The concept of metagenomics involves sequencing and analysing all the nucleic acids present in an environmental sample. Several metagenomic techniques, including quantitative PCR, 16S/ITS rRNA sequencing, and shotgun sequencing, are being established and used in respiratory specimens, such as sputum samples and bronchoalveolar lavage fluid. Clinically relevant bacteria are detected in one-third of pleural effusion samples, with Streptococcus pneumoniae being the most common organism. Metagenomic approaches detect more bacteria in pleural aspirate compared with culture methods alone, and changes in bacterial community composition over time correlate with antibiotic treatment. Metagenomic sequencing reports ecological diversity (number of unique taxa) and species richness with disease state, capacity for pathogenicity and antibiotic resistance, and suggested a cohort-wide population-level response to treatment. Understanding the bacterial community within the pleural environment may enhance the understanding of pleural infection pathogenesis and impact clinical management and outcomes, and new potential therapeutic interventions beyond antibiotics.

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