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A Specimen-level Analysis of Polymicrobial Bacterial and Viral Detections in Hospital-acquired Pneumonia Using Multiplex PCR

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Abstract

Background: Hospital-acquired pneumonia (HAP), including ventilator-associated pneumonia (VAP), remains a major cause of morbidity and mortality in critically ill adults. Conventional culture-based microbiology is time-consuming and may underestimate the true microbial burden, especially after prior antibiotic exposure. Multiplex molecular platforms such as the BioFire® FilmArray Pneumonia Panel (BFPP) provide rapid pathogen detection, but the specimen-level microbial complexity revealed by these assays in HAP has been less well described. This study was done with the objective to characterize specimen-level microbial complexity in ICU patients with suspected HAP using BFPP, with special emphasis on polymicrobial bacterial detections, viral co-detection, and resistance-gene profiles. **Methods**:This secondary, exploratory, specimen-level analysis was performed on the intervention arm of a previously conducted single-center randomized controlled trial in an adult anesthesia ICU of a tertiary government teaching hospital in Kanpur, India, from January 2020 to October 2021. Thirty respiratory specimens from patients with suspected HAP were analyzed using BFPP. Bacterial targets were considered clinically significant at a semi-quantitative threshold of ≥10^6 copies/mL. The specimen was used as the unit of analysis. Main outcomes included bacterial detection, number of bacterial targets per specimen, polymicrobial detection, viral detection, bacteria-virus co-detection, virus-only detection, and resistance-gene detection. **Results**: Among 30 specimens, 21 (70.0%) showed at least one clinically significant bacterial target, while 9 (30.0%) showed none. Polymicrobial bacterial detection was present in 13/30 specimens (43.3%) and in 13/21 bacteria-positive specimens (61.9%). A total of 43 bacterial detections were recorded, most commonly *Acinetobacter calcoaceticus - baumannii* complex, *Pseudomonas aeruginosa*, *Klebsiella pneumoniae* group, *Escherichia coli*, and *Staphylococcus aureus*. Viral targets were detected in 7/30 specimens (23.3%), including 4/30 (13.3%) with bacteria-virus co-detection and 3/30 (10.0%) with virus-only detection. Six resistance-gene detections were identified. BFPP showed 100% positive percent agreement and 92.9% negative percent agreement compared with conventional culture. **Conclusion**: BFPP frequently identified complex microbial patterns in ICU patients with suspected HAP, including substantial polymicrobial bacterial detections and clinically relevant bacteria-virus co-detection. Rapid multiplex testing may help clinicians interpret lower respiratory tract infections more precisely and support earlier, stewardship-guided antimicrobial decisions.

Keywords: Antimicrobial stewardship; BioFire FilmArray; Critical care; Hospital-acquired pneumonia; Multiplex polymerase chain reaction; Randomized controlled trial; Ventilator-associated pneumonia

Introduction

Hospital-acquired pneumonia (HAP), including ventilator-associated pneumonia (VAP), is one of the leading healthcare-associated infections in adults managed in the ICU. It increases mechanical ventilation, the length of stay in the ICU, the duration of antibiotic use and the outcomes of critically ill adults. Contemporary American Thoracic Society/Infectious Diseases Society of America (ATS/IDSA) and European Society of Clinical Microbiology and Infectious Diseases (ESCMID) guidelines recommend distinguishing HAP/VAP from other forms of pneumonia but recognize that HAP/VAP is not a single entity and has different prognosis due to host factors, the day of presentation, the local bacterial ecology and the microbiological species of bacteria involved in the infection [1-4]. In a large group of ICU patients, patients with ventilator-acquired pneumonia as well as non-ventilator-related ICU-acquired pneumonia were observed to have an increased risk for death at 30 days, indicating the clinical importance to promptly identify the causes of VAP and diagnose pneumonia in such patients early on [4].

In HAP/VAP, physicians are forced to make treatment decisions without definitive results in microbiology. Cultures are standard in most of the hospitals. However, bacterial identification requires incubation until colony formation and determination of its susceptibility, except some microorganisms, such as fungi. Studies about diagnostics for VAP confirm that cultures with bacterial identification and antibiotic resistance patterns take on average 48-72 hours and require an empiric use of

broad-spectrum antibiotics during the time required [3]. This time delay is of great importance in the ICU environment because adequate empirical therapy can help critically ill patients, whereas excessive antibiotic treatment drives antibiotic pressure on pathogens and causes damage to healthy microbiota. Moreover, the bacterial recovery rate in the respiratory samples can be modified by the antibiotic coverage before obtaining such cultures. In one well-protocolized study of the quality of sputum culture obtained from patients with suspected VAP, antibiotics can have a strong positive effect on the detection of pathogens with reduced recovery of most pathogens, starting after hours of therapy (including antibiotics with similar mechanisms of action or resistance profiles as expected for VAP) [5]. This study showed a drastic negative impact of antibiotics on the rate of positive cultures (especially with gram-negative organisms). Routine microscopy and bacterial culture may underrepresent the true bacterial pathogens present in patients who have already received antibiotics [3,5].

Another relevant characteristic of HAP/VAP in the ICU is their complexity. Infections of the ICU with multidrug-resistant gram-negative bacilli and *Staphylococcus aureus* are reported with a significant frequency. Nonetheless, more than half of the cases with nosocomial pneumonia do not solely involve single microorganisms. Mixed polymicrobial bacterial infections have historically been described in mechanically ventilated patients. In one classical cohort using bronchoscopy, Combes and colleagues documented 48% of their patients with polymicrobial VAP. Many bronchoalveolar lavages included three or even four bacterial species [6]. In 2017, Natarajan and coworkers found a polymicrobial VAP rate of 62.9% in a medical ICU and demonstrated that polymicrobial episodes were associated with more duration of mechanical ventilation and hospital length of stay [7]. These data have critical clinical importance, since mixed infections can impact decisions about empiric antibiotic therapy, hide relationships between clinical symptoms and microorganisms and complicate the interpretation of diagnostic tests. There is also a major practical question for rapid molecular tests, including those performed on direct respiratory specimens: When should multiple species of bacteria be detected in the same sample? Which findings are from the infection and which from the colonization of the lung and the airway [6,7]? How to consider the entire bacterial load at the patient's side?

With the wider use of multiplex molecular diagnostic assays for pathogens that causes pneumonia, increasing attention has been placed on the involvement of respiratory viruses in VAP. In the ICU, viral testing was historically done inconsistently and in only selected cases, which certainly underestimated the number of patients affected by viral pneumonia and of co-infection of bacterial and viral pathogens. The review of Cawcutt and Kalil emphasizes that bacterial and viral co-infections have become increasingly evident in the community setting as well as in healthcare settings and contribute to more severe pneumonia, to increased hospitalizations in ICUs and higher rates of mortality [8]. Respiratory viruses were found in 32% of cases with HAP at ICUs by Loubet et al [9]. Using multiplex PCR arrays, these were divided into two relevant scenarios: newly acquired respiratory viral infections and prolongued viral carriers, in immunocompromised patients and the latter cases with high rates of concomitant bacterial infections. In this group, mixed infections have been shown to be linked to longer stays and a trend towards more frequent mortality [9]. In the study by Shen et al. , 42.1%, 24.3% and 22.4% of patients were shown to have bacterial pneumonia only, viral pneumonia only or concurrent bacterial-viral pneumonia, respectively [10]. These results indicate that viral detection in VAP is not merely noise but may accurately determine relevant subgroups when tested simultaneously with contemporary findings for bacterial infections [8-10].

Rapid syndromic molecular testing has emerged in response to these diagnostic gaps. The BioFire® FilmArray Pneumonia Panel/Panel Plus is a multiplex real-time PCR platform that can identify common bacterial pathogens, atypical bacteria, respiratory viruses, and selected antimicrobial-resistance markers directly from lower respiratory tract specimens within approximately one hour [11,12]. Several evaluation studies have shown that these panels detect pathogens in more samples than routine microbiology and frequently identify more than one target in the same specimen. In ICU patients with suspected HAP, Crémet et al. evaluated the FilmArray Pneumonia Plus Panel in mechanically ventilated patients and demonstrated rapid broad-pathogen detection in bronchoalveolar lavage and endotracheal aspirate samples [11]. Webber et al. found that the BioFire® panel identified a viral or bacterial target in 58.5% of lower respiratory samples and detected 92 additional bacteria beyond standard methods, while also identifying bacterial-viral co-detection in a subset of specimens [13]. Lee et al. reported coinfections in 42.3% of specimens from adult ICU patients and showed that the panel markedly outperformed conventional viral isolation for viral detection [14]. In the multicenter INHALE study, FilmArray identified pathogens in 74.2% of nosocomial pneumonia samples compared with 44.2% by routine microbiology and detected more pathogens per sample than conventional testing [15].

These advantages have obvious stewardship implications. If pathogens and selected resistance genes are available within hours rather than days, clinicians may be able to

optimize empirical therapy earlier, avoid unnecessary broad-spectrum escalation, or de-escalate treatment when a more precise microbiological explanation is available. In critically ill patients with HAP, Erich et al. estimated that availability of the FilmArray pneumonia panel results could have prompted a change in therapy in nearly two-thirds of patients, with an anticipated reduction of about 51 hours to optimized treatment [16]. Recent reviews confirm that rapid multiplex molecular panels can shorten turnaround times and increase diagnostic accuracy among critically ill patients [17], but increased sensitivity raises diagnostic issues. Molecular panels detect nucleic acids, not organisms and detection of resistance genes is not a direct replacement for phenotypic antimicrobial susceptibility testing. Lee et al. Emphasize the need to clinically correlate detection of multiple organisms and antibiotic-resistance genes, while Jitmuang et al. point out that multiple bacteria may exist in the same sample and antibiotic-resistance genes may be poorly attributed to a particular organism [12, 14]. Few published studies of the BioFire panel versus culture have examined the specimen-level ecology identified by the test itself, which is an important omission. The positive status of a test, the presence of no bacterial target, a single presumed pathogen, multiple bacterial targets, the presence of virus alone or with bacteria or the presence of resistance genes within these specimens, all may be clinically relevant for bedside decision making. These findings are especially important in ICU hospital-acquired pneumonia, where colonization, prior antimicrobial therapy, resistance to multiple drugs and overlap in the syndromic diagnoses coexist [3,8,14,16]. Further evaluation of polymicrobial bacterial results and viral coinfection could thus have value for patient care in this complex setting, beyond the concordance of the results of the test with results of culture or simple test positivity. In view of the issues raised above, this study was intended to examine results from specimens from ICU patients with hospital-acquired pneumonia suspected by means of BioFire® ® FilmArray Pneumonia Panel. This analysis is secondary to the primary objective of assessing overall clinical superiority of test over culture and is aimed at exploring microbial complexity identified by the panel by defining the proportions of patients with single compared with multiple bacterial identifications, the relative frequency of viral-only or viral-bacterial coinfections and the pattern of associated antibiotic-resistance genes.Such an approach may help refine interpretation of multiplex respiratory diagnostics in critically ill adults and provide a more practical microbiological framework for clinicians confronted with complex panel results in HAP/VAP.

Materials and Methods

Study design and setting: This study was a secondary, exploratory, specimen-level analysis of the intervention arm of a previously conducted single-center, parallel-group randomized controlled trial performed in the adult anesthesia intensive care unit of a tertiary-level government teaching hospital in Kanpur, India, between January 2020 and October 2021. The parent trial evaluated rapid molecular diagnosis using the BioFire® ® FilmArray Pneumonia Panel (BFPP) against conventional semi-quantitative streak culture in patients with suspected hospital-acquired pneumonia (HAP). The present analysis was restricted to the respiratory specimens tested by BFPP in the intervention arm and was undertaken to characterize specimen-level microbial complexity, including polymicrobial bacterial detections, viral co-detection, and resistance-gene detection.

Study Population: Adult patients aged 18-65 years were eligible in the parent trial if they developed clinical suspicion of HAP, defined as a new or worsening pulmonary infiltrate on chest imaging along with at least two of the following occurring more than 48 hours after hospital admission: fever >100.4°F, leukocytosis or leukopenia, and purulent respiratory secretions. Patients with respiratory infection at admission, ICU stay <48 hours, uncontrolled diabetes mellitus, severe immunocompromised state, positivity for HBsAg or HCV, chronic kidney disease, or chronic liver disease were excluded. For the present study, only patients randomized to the BFPP arm were included, yielding a dataset of 30 respiratory specimens from 30 patients.

Specimen collection and BioFire® FilmArray Pneumonia Panel testing: Respiratory samples were obtained at the time of clinical suspicion of HAP and included sputum, endotracheal aspirate, or bronchoalveolar lavage, depending on patient status and airway access. Samples were analyzed using the BioFire® FilmArray Pneumonia Panel on the BioFire® FilmArray 2.0 system according to the manufacturer's protocol. For this analysis, bacterial targets were considered clinically significant only when reported at a semi-quantitative bin value of ≥10^6 copies/mL. Viral targets and resistance-gene markers were recorded qualitatively.

A specimen was the basic unit of analysis in this study, including any bacterial detection, number of bacterial targets per specimen, polymicrobial bacterial detection, viral detection, virus-only detection, bacteria-virus co-detection and resistance-gene detection. To achieve an analysis more focused on active infection than on colonization, two thresholds for bacterial detection were incorporated: bacterial targets at a semi-quantitative bin value of ≥106 copies/mL were considered significant and Polymicrobial was the presence of two or more bacterial targets, each ≥106 copies/mL, detected within a single specimen. Virus-Only detection was the presence of one or more viral targets in the absence of significant bacterial

detection, while bacteria-virus co-detection required the presence of at least one significant bacterial target alongside at least one viral target. Positive Percent Agreement (PPA) and Negative Percent Agreement (NPA) were calculated against semi-quantitative streak culture results as the reference standard.

Data handling and statistical analysis: Data were retrieved from the microbiological records of the intervention group from the parent study. This paper includes secondary exploratory analysis based on the specimen level data obtained from the parent study. In the parent study, there was a randomized controlled patient-based experimental design; however, this secondary exploratory analysis is based on the specimen level data obtained. The dataset comprises 30 distinct respiratory specimens (sputum, endotracheal aspirate, or bronchoalveolar lavage) collected from 30 patients within the molecular intervention arm of the parent study. Only bacterial detections meeting the predefined threshold were entered into the final bacterial dataset. Statistical analysis was primarily descriptive. Categorical variables were summarized as frequency and percentage. The proportions of specimens with 0, 1, 2, 3, 4, and 5 bacterial targets were calculated, along with the proportion of specimens showing polymicrobial bacterial detection. Frequencies of individual bacterial targets, viral targets, and resistance-gene detections were also tabulated. No separate sample size calculation was performed for this secondary analysis because the complete intervention-arm dataset of the parent trial was used. The parent trial had prior approval from the Institutional Ethics Committee , and written informed consent had been obtained from all participants or their legally authorized representatives.

Results

A total of 30 respiratory specimens tested by the BioFire® FilmArray Pneumonia Panel (BFPP) were included in this secondary specimen-level analysis. Using the predefined threshold of ≥10^6 copies/mL for clinically significant bacterial detection, 21/30 specimens (70.0%) showed at least one bacterial target, while 9/30 specimens (30.0%) had no detectable bacterial target.

Analysis of within-specimen bacterial burden showed that 8/30 specimens (26.6%) had one bacterial target, 6/30 (20.0%) had two, 4/30 (13.3%) had three, 2/30 (6.6%) had four, and 1/30 (3.3%) had five bacterial targets **(Table 1).** Thus, polymicrobial bacterial detection, defined as detection of two or more bacterial targets in the same specimen, was present in 13/30 specimens (43.3%). Among bacteria-positive specimens alone, 13/21 (61.9%) were polymicrobial **(Figure-1).** The maximum bacterial burden observed in a single specimen was five bacterial targets.

Table 1: Number of bacteria in each Biofire FilmArray Pneumonia panel specimen

TOTAL NUMBER OF BACTERIA POSITIVE	NUMBER OF PATIENTS	PERCENTAGE
0	9	30%
1	8	26.6%
2	6	20%
3	4	13.33%
4	2	6.6%
5	1	3.3%
Total	30	100%

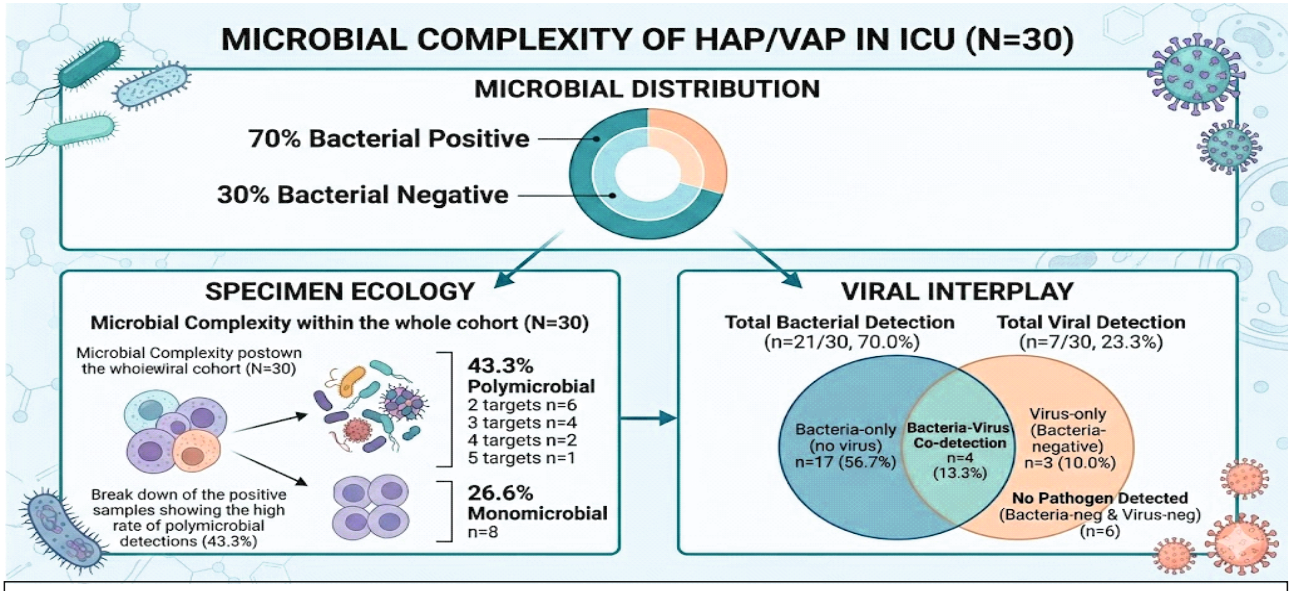

Figure 1. Microbial complexity of HAP/VAP in ICU specimens assessed by multiplex PCR.
Abbreviations: HAP, hospital-acquired pneumonia; VAP, ventilator-associated pneumonia; ICU, intensive care unit; N, total number of specimens; n, number of specimens in each subgroup; PCR, polymerase chain reaction.

A total of 43 bacterial detections were recorded across the 30 BFPP-tested specimens **(Table 2)**. The most frequently detected bacterial target was *Acinetobacter calcoaceticus-baumannii* complex (11 detections), followed by *Pseudomonas aeruginosa* (9 detections), *Klebsiella pneumoniae* group (8 detections), *Escherichia coli* (6 detections), and *Staphylococcus aureus* (6 detections). Less frequent bacterial detections included *Enterobacter cloacae complex (1 detection), Klebsiella oxytoca (1 detection), and Streptococcus agalactiae (1 detection). No detections were noted for Haemophilus influenzae, Klebsiella aerogenes, Moraxella catarrhalis, Proteus* species., *Serratia marcescens*, *Streptococcus pneumoniae,* or *Streptococcus pyogenes.* No atypical bacterial targets, including *Chlamydia pneumoniae, Legionella pneumophila,* or *Mycoplasma pneumoniae,* were identified.

Viral targets were detected in 7/30 specimens (23.3%). Influenza A was the most common virus, detected in 4 specimens, while adenovirus, human metapneumovirus, and parainfluenza virus were each detected in 1 specimen. Bacteria-virus co-detection was observed in 4/30 specimens (13.3%), whereas 3/30 specimens (10.0%) showed virus-only detection. Multiple viral detections were not observed in any specimen. No detections were recorded

Table 2: Summary of Organism in Group B (Biofire filmarray Pneumonia panel) n = 30

PATHOGENS (TOTAL DETECTION IS 57)	TOTAL NUMBER (%)
ANY BACTERIA	43 (75)
Acinetobacter calcoaceticus-baumannii complex	11 (19.2)
Enterobacter cloacae complex	1(1.7)
Escherichia coli	6(10.5)
Haemophilus influenza	0
Klebsiella aerogenes	0
Klebsiella oxytoca	1(1.7)
Klebsiella pneumoniae group	8(14)
Moraxella catarrhalis	0
Proteus species	0
Pseudomonas aerogenosa	9(15.8)
Serratia marcescens	0
Staphylococcus aureus	6(10.5)
Streptococcus agalactiae	1(1.7)
Streptococcus pneumoniae	0
Streptococcus pyogenes	0
Atypical Bacteria *Chlamydia pneumoniae* *Legionella pneumophila* *Mycoplasma pneumoniae*	0
ANY VIRUS	7(12.3)
Adenovirus	1(1.7)
Coronavirus	01
Human Metapneumovirus	(1.7)
Human Rhinovirus/Enterovirus	0
Influenza A	4(7)
Influenza B	0
Parainfluenza Virus	1(1.7)
Respiratory Syncytial Virus	0
Middle East Respiratory Syndrome Coronavirus (MERS-CoV)	0
Antimicrobial Resistance Genes	6(10.5)
CTX-M	4(7)
IMP	1(1.7)
KPC	0
NDM	0
OXA-48-like	0
VIM	1(1.7)
mecA/C and MREJ	0

for coronavirus, human rhinovirus/enterovirus, influenza B, respiratory syncytial virus, or Middle East respiratory syndrome coronavirus (MERS-CoV).

With respect to resistance markers, the BFPP identified 6 resistance-gene detections. These included CTX-M in 4 specimens, IMP in 1 specimen, and VIM in 1 specimen. No detections were recorded for KPC, NDM, OXA-48-like, or mecA/C with MREJ (**Figure-2**).

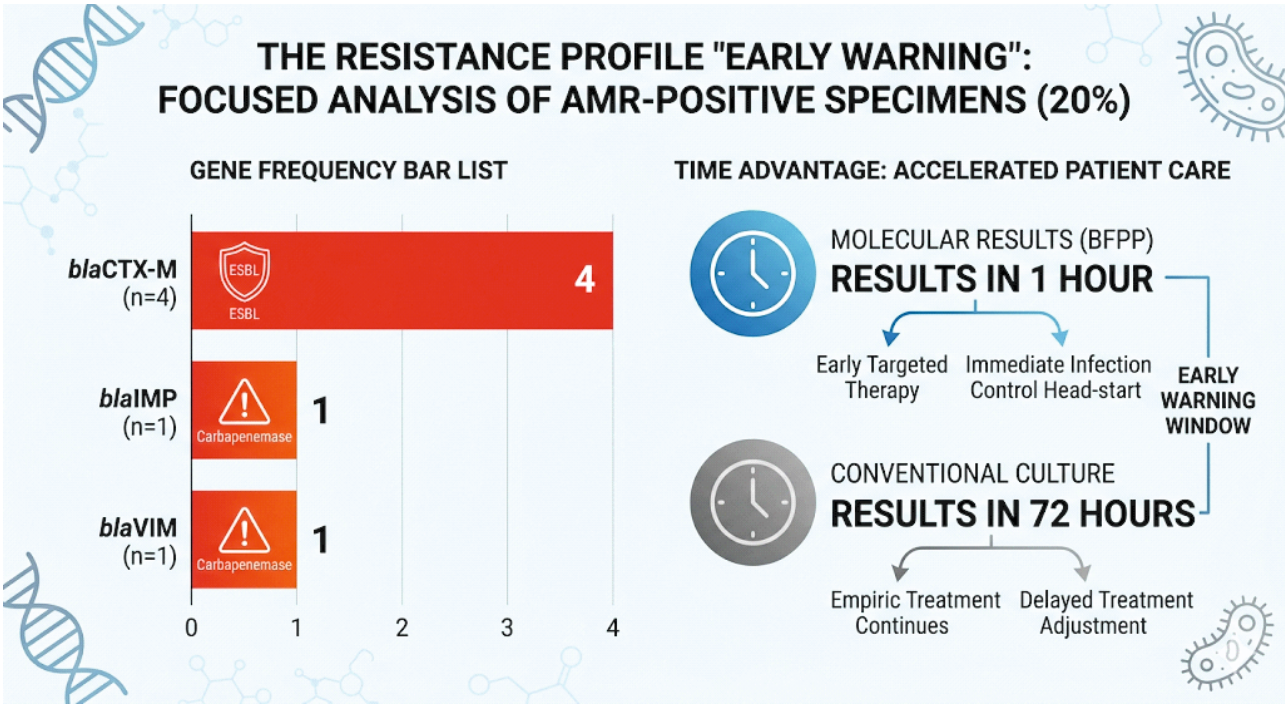

Figure 2. Resistance-gene profile and early-warning value of BFPP in AMR-positive HAP/VAP specimens. Abbreviations: AMR, antimicrobial resistance; BFPP, BioFire® FilmArray Pneumonia Panel; HAP, hospital-acquired pneumonia; VAP, ventilator-associated pneumonia; bla, beta-lactamase gene; CTX-M, cefotaximase-M; IMP, imipenemase; VIM, Verona integron-encoded metallo-β-lactamase; ESBL, extended-spectrum beta-lactamase; n, number of specimens.

Overall, this specimen-level analysis demonstrated that BFPP frequently identified complex microbial patterns in suspected hospital-acquired pneumonia, including a substantial proportion of polymicrobial bacterial detections, occasional bacteria-virus co-detection, and a limited but clinically relevant number of resistance-gene signals.

When compared against conventional streak culture, the BioFire® FilmArray Pneumonia Panel (BFPP) demonstrated excellent overall diagnostic performance, with an overall positive percent agreement (sensitivity) of 100% and negative percent agreement (specificity) of 92.9% (Table 3). Notably, there were no false-negative results for any culture-positive bacterial target, resulting in an overall negative predictive value of 100%, which suggests that a negative BFPP result was highly reliable in ruling out bacterial pathogens detected by culture. Organism-wise, sensitivity was 100% for all culture-positive detections including *Acinetobacter calcoaceticus-baumannii* complex, *Escherichia coli, Klebsiella pneumoniae group, Pseudomonas aeruginosa, and Staphylococcus aureus. However, the positive predictive value was relatively low overall (27.9%), with particularly low values for Pseudomonas aeruginosa (11.1%), Staphylococcus aureus* (16.7%), and *Klebsiella pneumoniae* group (25%), indicating that BFPP detected additional organisms not recovered by culture. Overall, these findings suggest that BFPP is highly sensitive and has excellent rule-out ability, while also identifying a broader range of bacterial targets than conventional culture.

Discussion

In this analysis of a secondary data set that includes samples from ICU patients with suspicion for HAP, the highest rate of intra specimen polymicrobial bacterial detection observed by the BFPP was 43.3% for all BFPP-tested specimens and 61.9% for bacteria-positive specimens, with a maximum of five bacterial targets detected within one specimen. Therefore, based on this analysis, HAP in at least a substantial proportion of these critically ill patients appears to be a microbiologically complex illness with several common bacterial species, not an exclusive monomicrobial event. Similar findings of polymicrobial infection have been reported in earlier VAP studies, including with three and four bacterial species reported by Combes et al. And by Natarajan et al. (62.9%) in a cohort of an Indian ICU [6,7]. These findings collectively suggest that polymicrobial infection is a common finding in severe

Table 3: Diagnostic accuracy of Biofire FilmArray Pneumonia panel

BACTERIA	PP2+ Culture+	PP2+ Culture-	PP2- Culture+	PP2- Culture-	PPA/ Sensitivity %	NPA/ Specificity %	PPV%	NPV%
Acinetobacter calcoaceticus-baumannii complex	5	6	0	19	100	76	45.4	100
Enterobacter cloacae complex	0	1	0	29	-	96.7	-	100
Escherichia coli	3	3	0	24	100	88.9	50	100
Haemophilus influenza	0	0	0	30	-	100	-	100
Klebsiella aerogenes	0	0	0	30	-	100	-	100
Klebsiella oxytoca	0	1	0	29	-	96.7	-	100
Klebsiella pneumoniae group	2	6	0	22	100	78.6	25	100
Moraxella catarrhalis	0	0	0	30	-	100	-	100
Proteus species	0	0	0	30	-	100	-	100
Pseudomonas aerogenosa	1	8	0	21	100	72.4	11.1	100
Serratia marcescens	0	0	0	30	-	100	-	100
Staphylococcus aureus	1	5	0	24	100	82.8	16.7	100
Streptococcus agalactiae	0	1	0	29	-	96.7	-	100
Streptococcus pneumoniae	0	0	0	30	-	100	-	100
Streptococcus pyogenes	0	0	0	30	-	100	-	100
ALL	**12**	**31**	**0**	**407**	**100**	**92.7**	**27.9**	**100**

nosocomial pneumonia and may be more readily visualized with molecular-based testing than with the use of only standard culture.

Our findings are also consistent with the clinical utility of the Multiplex molecular testing as an indicator of bacteria at the specimen level, instead of a simple yes/no result for positivity. Multiplex molecular testing offer detection of a broad spectrum of pathogens rapidly and can categorize bacteria semiquantitatively, in contrast to routine culture methods that often underestimate the extent of diversity, when used in conjunction with antibiotics. Lee et al. state that the FilmArray provides rapid, sensitive detection of pathogens in lower respiratory tract samples but stress that interpretation of the presence of multiple pathogens requires correlation with the clinical circumstances [14]. Gong et al. described higher semiquantitative bacteria as predictive of a closer relationship with conventional cultures and suggest a basis for thresholds in interpreting bacterial results [18]. Cojuc-Konigsberg et al. also reported improved specificity when assessing higher genetic loads among panel-positive samples [19]. These data continue to support the interpretation of a semiquantitative signal value as a tool to assist in differentiating greater clinical significance from minor bacterial findings or background signal.

Because a threshold for clinically relevant bacterial detection was implemented in our current analyses, it further reduces concerns that the polymicrobial bacterial burdens observed represent primarily assay limitations and instead further supports our interpretation as evidence of true clinical infectious complexity, although it cannot wholly exclude that the increased burden detected represents a result of bacterial colonization. Detection of viral organisms in an ICU HAP cohort was an additional noteworthy finding: 10.0% of samples had only a viral detection and another 13.3% had evidence of bacteria and virus together. The occurrence of respiratory viruses is rarely emphasized in nosocomial pneumonia, as testing is frequently reserved for patients with influenza in settings that involve the use of ventilators in adult ICUs. Cawcutt and Kalil mention the significance of bacterial-viral co-infection in altering the disease severity and increasing the ambiguity surrounding the differential diagnosis of pneumonia [20]. Loubet et al. found that the viruses were present in 32.0% of cases of HAP in patients admitted to an ICU and showed that those who had combined viral bacterial pneumonia spent longer durations in the hospital and had higher mortality [9]. Shen et al. also found separate categories of only bacterial pneumonia, only viral pneumonia or bacterial viral mixed infection in patients admitted to an ICU who were mechanically ventilated [10]. This information confirms that the diagnosis and care of

patients can be changed by the finding of viral disease in addition to bacterial disease in severe pneumonia. Our observation that some patients have only a viral illness, as others have evidence of bacterial-viral infections, strongly suggests that the rapid, broad pathogen detection capabilities of Multiplex molecular testing may identify subgroups of patients who differ from one another in ways that standard bacterial testing would otherwise leave unnoticed and unaddressed from a therapeutic viewpoint.

The frequency of viral detections observed in our cohort was comparatively lower than that reported in certain published ICU series, however, this variation is clinically plausible. Viral prevalence in HAP/VAP depends on case-mix, seasonality, local epidemiology, immune status, and the intensity of viral testing. Our study was relatively small and limited to a single ICU population, which may partly explain the lower proportion of viral findings. Even so, the presence of virus-only samples is clinically meaningful. In real-world decision-making, a virus-only BFPP result does not by itself rule out bacterial infection, but in the right clinical context it may increase confidence in withholding, narrowing, or discontinuing unnecessary antibacterial therapy, especially when serial clinical assessment does not support ongoing bacterial sepsis. That implication is particularly relevant for stewardship-focused use of syndromic panels in HAP. [9-10, 20-21].

The present analysis also helps explain why rapid molecular diagnosis may have clinical value beyond simple acceleration of microbiology reporting. In the parent randomized report from the same cohort, BFPP-guided diagnosis was associated with lower mortality, shorter ICU stay, shorter antibiotic exposure, and lower total cost [22]. The current specimen-level analysis offers a plausible microbiological rationale for those findings. When clinicians are confronted early with evidence of multiple bacterial targets, virus-only detection, or bacteria-virus co-detection, they may be better positioned to tailor empirical therapy more precisely than when relying on delayed culture alone. This analysis finds substantial empirical evidence in the existing literature on BFPPs. In one study by Van der Westhuyzen et al., the FilmArray panel detected significantly more pathogens compared to culture, as well as demonstrated bacterial and viral coinfections in 27% of cases [21]. Moreover, Ferrer et al. argued that the use of FilmArray findings would help facilitate changes in empiric antimicrobial treatment and possibly even save money [23]. Likewise, Matsuura et al. suggested that timely identification of antibiotic resistance through the pneumonia panel, along with Gram stain and clinical judgment, might affect antimicrobial step-up decisions [24].

On the other hand, the findings in this study should not be considered as an indication that all multiple detections would always result in actionable pathogens. One of the most difficult aspects of multiplex respiratory testing is being able to tell infection from colonization, particularly among patients under intubation in ICUs. This problem can become even more complex when there is a presence of multiple bacteria in the same sample. As a matter of fact, Lee et al. emphasized that multiple pathogens and resistant genes should not always be taken at face value [14]. Berteau et al. showed that in culture-positive VAP, non-targeted bacteria not included in the FilmArray panel were present in 23% of cases, and that polymicrobial VAP carried a higher risk of such non-targeted organisms [25]. Thus, a molecular panel may reveal more than culture, but it does not reveal everything. The implication is not that BFPP should replace conventional microbiology, but that it should be used as an adjunctive rapid test interpreted alongside clinical findings, conventional culture, and local epidemiology.

A similar principle applies to resistance-gene interpretation. Although the current manuscript is not centered on detailed resistance analysis, the presence of resistance markers in this cohort adds to the clinical significance of rapid syndromic testing. Gong et al. found good positive agreement between molecularly detected antimicrobial resistance determinants and phenotypic resistance, especially for CTX-M and mecA/C-MREJ [18]. According to Matsuura and colleagues, a rapid identification of pathogens that are resistant to common antibiotics could lead to a more targeted antimicrobial treatment at an earlier stage of therapy [24]. Nevertheless, this does not imply that the resistance genes identified in the samples definitively come from a specific organism in the mixed sample and the results do not replace conventional phenotypic susceptibility tests. Therefore, data on resistance genes would be most beneficial as an early indicator and to help guide the early phase of antibiotic selection, pending a full susceptible test on the organisms isolated. The overall impact of pneumonia diagnostic panels on clinical care, though, hinges on the level of adoption into the process by which a physician makes decisions about antibiotics. While our study concluded there was a clinical benefit, not all outside researchers have been so convinced about the potential role of panels as part of the diagnosis in solely improving mortality, Riaño-Sánchez and coworkers observed no improved mortality, shorter ICU stay or fewer days of antibiotics associated with using an multiplex pneumonia panel in intensive care units in Spain, and, conversely, found an increased use of Infectious Disease consults to be associated with superior outcomes [26]. This underscores the fact that rapid molecular tests are not self-implementing tools, their value is likely enhanced when accompanied by stewardship oversight, thoughtful analysis by a clinician at the bedside and defined pathways for

interpreting conflicting and mixed results.

Strengths and Limitations

The case-by-case analysis revealed the complexity of the microbes encountered in ICU-acquired pneumonia specimens, but several limitations must be mentioned to properly interpret the findings. We conducted the study within one adult medical anesthesia ICU in a tertiary teaching medical center, with 30 samples in the molecular analysis portion. Thus, the results presented in this study should be considered exploratory. Although the study showed excellent sensitivity (100% PPA) with a reasonable N of 12, this overestimates the accuracy and findings must be confirmed with larger multi-center studies to ensure external generalizability to different ICUs with different microbial ecosystems. Being a tertiary care referral hospital, we tend to treat more critically ill patients who might have undergone previous health care exposure, thereby having a higher possibility of exposure to Multi drug resistant organisms and polymicrobial pneumonia. However, conventional streak culture is not the best gold standard. Because molecular panels detect DNA from both dead/viable and nonculturable/viable bacteria, the many false positives we found (27.9% PPV) are often real pathogens that had been exposed to antibiotics and were unable to culture. We recommend the next logical steps: use another type of secondary molecular investigation to confirm Discordant cases such as rRNA (16S sequencing) analysis. Differentiating true infection from colonization remains problematic for all molecular tests. Although we used the high threshold of 106 copies/mL with high confidence in increasing the specificity of tests about the presence of DNA from only the viable organism (pathogen), it still remains critical that the test results should only be evaluated with clinical evolution, radiographic features, inflammatory marker values and procalcitonin. The BioFire® panel only tests for the following 15 bacteria and 9 viruses, most of which are commonly encountered pathogens in the ICU. Other pathogens like Stenotrophomonas maltophilia or various fungi cannot be tested on the panel, which leads to an incompletely identified microbial agent in polymicrobial samples. Finally, because some microbiological components of the parent trial have already been reported in the page-proof manuscript, the present discussion deliberately focuses on microbial complexity and co-detection patterns rather than repeating the main between-group diagnostic-yield narrative.

Overall, the present secondary analysis suggests that the major added value of BFPP in suspected ICU-acquired pneumonia may lie not only in faster positivity, but in its ability to reveal how microbiologically complex a given specimen is. The high rate of polymicrobial bacterial detection, the presence of virus-only samples, and the identification of bacteria-virus co-detection together support the concept that HAP/VAP in critically ill adults often represents a mixed and layered infectious syndrome. In this setting, rapid multiplex testing may help clinicians move beyond a binary culture-positive/culture-negative framework toward a more nuanced interpretation of lower respiratory tract infection (**Figure-3**). Future studies should validate whether explicit specimen-level interpretation algorithms based on bacterial burden, co-detection patterns, and stewardship review can further improve antibiotic precision and patient outcomes.

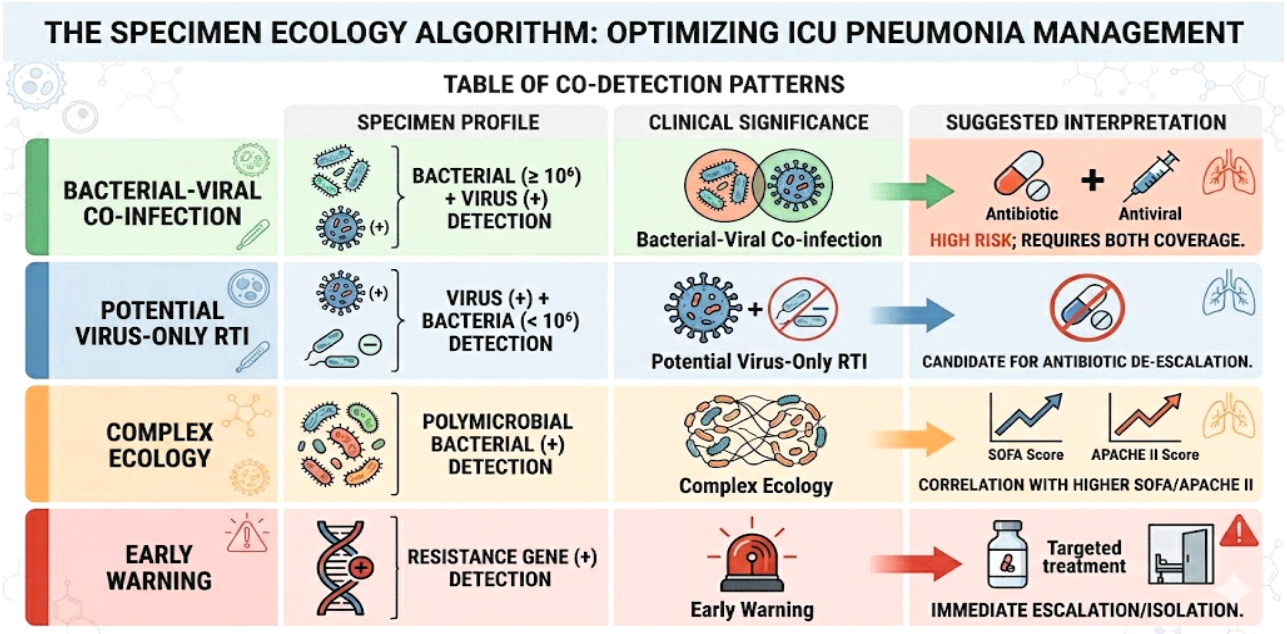

Figure 3: Specimen ecology algorithm for optimizing ICU pneumonia management based on co-detection patterns.
Abbreviations: ICU, intensive care unit; RTI, respiratory tract infection; SOFA, Sequential Organ Failure Assessment; APACHE II, Acute Physiology and Chronic Health Evaluation II; n, number; >10^6, bacterial load greater than 10^6 copies/mL

CLINICAL, ETHICAL AND SOCIAL IMPLICATIONS

1. The study supports earlier and more precise antimicrobial decision making in critically ill ICU patients with suspected HAP/VAP by using multiplex PCR to rapidly detect their bacterial burden, whether they are polymicrobially infected, have viral coinfections or carry resistance genes, all information that may not fully be included with conventional culture.
2. The authors' findings suggest that HAP is often polymicrobially and not monomicrobially suspected in critically ill patients and that physicians should interpret findings on their pneumonia panel by combining quantitative information about bacterial and viral loads with resistance markers, clinical condition, radiology and culture results.
3. Because this test results in faster molecular diagnosis, it encourages more prudent use of antibiotics by allowing clinicians to avoid unnecessary broad-spectrum agents, reinforcing antimicrobial stewardship.
4. The study findings create an ethical responsibility for physicians to carefully interpret molecular test results, given that detection of bacterial and/or viral nucleic acids does not equate with active bacterial or viral infection and that over-treatment with the panel could cause patients harm.
5. Given that molecular testing quickly delivers test results, there arises a duty to be timely, as prompt administration of appropriate antibiotic treatment in critically ill patients often improves outcomes.
6. Better antimicrobial stewardship has the potential to mitigate the societal burden of antimicrobial resistance in ICU patients as well as throughout hospitals and communities.
7. In this manner, use of the multiplex panel may contribute to decreasing length of ICU stay, duration of antibiotic therapy and healthcare costs, thereby relieving patients' families of financial burdens and patients of emotional distress.

Conclusion

This secondary, specimen-level analysis has shown that the BioFire FilmArray Pneumonia Panel often finds complicated microbiological profiles among patients with ICU-acquired pneumonia, involving many instances of polymicrobial bacterial infection as well as significant cases of bacteria-viruses co-detection. These findings suggest that suspected HAP in critically ill adults is often microbiologically more complex than conventional binary pathogen reporting may indicate. By identifying polymicrobial bacterial loads and viral co-detections within the first hour of suspicion, clinicians can move away from "one-size-fits-all" empiricism toward a targeted, stewardship-guided treatment algorithm. This approach not only reduces mortality and antibiotic duration but also addresses the significant economic burden of ICU care. Larger prospective studies are needed to validate how polymicrobial burden and co-detection patterns can be integrated into stewardship-guided treatment algorithms and whether such specimen-level interpretation improves clinical outcomes.

Declarations

Acknowledgment: We acknowledge the nursing and laboratory staff of the LLR, Hospital Intensive Care Unit.

Artificial intelligence uses declaration statement: The graphical illustrations and visual schematics used in this manuscript were created with the assistance of Google Gemini, a generative artificial intelligence tool. The tool was used only for designing and refining visual representations of the study concepts, including microbial complexity, resistance-gene profiling, and specimen-level interpretation algorithms. All scientific content, numerical data, labels, interpretations, and final figure legends were reviewed, verified, and approved by the authors. No patient-identifiable information or raw clinical data were entered into the AI tool. The authors take full responsibility for the accuracy, integrity, and scientific interpretation of all figures included in the manuscript.

Author Contribution: SS and AA designed the study, SS and PS collected the data, and AKM performed the analysis. SS, SP, AA, and PS wrote the first draft of the manuscript. All authors reviewed and approved the final manuscript with PS being the guarantor.

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Ethical Approval: The study was conducted in accordance with the ethical standards of the responsible Ethics Committee and in compliance with the Declaration of Helsinki. Ethical approval was obtained from the Ethics Committee for Biomedical Health & Research, GSVM Medical College, Kanpur for the study titled “Comparison of the BioFire FilmArray Pneumonia Panel versus Traditional Streak Culture Method for Detection of Hospital-acquired Respiratory Infection in Anaesthesia Intensive Care Unit of a Tertiary Care Center: A Randomized Control Trial” (Ref. No.: EC/205/Oct./2021, dated 16-10-2021). The Ethics Committee approval stated

that the study should be conducted as per the submitted protocol and in accordance with applicable regulatory and ethical guidelines. Written informed consent was obtained from all participants or their legally authorized representatives prior to inclusion in the study.

Conflict of interest: None to declare

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