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Santhanakrishnan S
SRM Medical College Hospital and Research Centre
Nalini Jayanthi N
nalinijn@srmist.edu.in

SRM Medical College Hospital and Research Centre
Thyagarajan Ravinder
Government Kilpauk Medical College
Leela Kagithakara Vajravelu
SRM Medical College Hospital and Research Centre
Ravichandran B
Government Kilpauk Medical College
Selvanathan E
Government Kilpauk Medical College

Research Article

Keywords: Ventilator-associated pneumonia, antimicrobial resistance determinants, virulence-associated genes, multidrug-resistant Gram-negative bacteria, TaqMan real-time PCR, molecular epidemiology.

Posted Date: November 25th, 2026

DOI: https://doi.org/10.21203/rs.3.rs-11578181/v1

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Additional Declarations: No competing interests reported.

RESISTANCE–VIRULENCE ARCHITECTURE OF VENTILATOR-ASSOCIATED PNEUMONIA PATHOGENS: A MULTICENTRIC MOLECULAR STUDY

Santhanakrishnan S[1], Nalini Jayanthi N[2*], Thyagarajan Ravinder[3], Leela Kagithakara Vajravelu[4], Ravichandran B[5], Selvanathan E[6]

[1]Santhana Krishnan S , Ph.D. Research Scholar, Department of Microbiology, SRM Medical College Hospital and Research Centre, Faculty of Medicine and Health Sciences, SRM Institute of Science and Technology, Kattankulathur, Chengalpattu, Tamilnadu, India 603203 .Email id: ss4021@srmist.edu.in

[2*]Nalini Jayanthi N, Professor and HOD , Department of Respiratory Medicine, SRM Medical College Hospital and Research Centre, Faculty of Medicine and Health Sciences, SRM Institute of Science and Technology, Kattankulathur, Chengalpattu, Tamilnadu, India 603203.Email id: nalinijn@srmist.edu.in

[3]Thyagarajan Ravinder , Professor and HOD, Department of Microbiology, Government Kilpauk Medical College, Kilpauk, Chennai, Tamil Nadu, India 600010, Email id:- drtravinder67@gmail.com

[4]Leela Kagithakara Vajravelu, Professor and HOD, Department of Microbiology, SRM Medical College Hospital and Research Centre, Faculty of Medicine and Health Sciences, SRM Institute of Science and Technology, Kattankulathur, Chengalpattu, Tamilnadu, India 603203.Email id: leelav@srmist.edu.in

[5]Ravichandran B, Associate Professor, Department of Microbiology, Government Kilpauk Medical College, Kilpauk, Chennai, Tamil Nadu, India 600010, Email id:- drrashyy1974@gmail.com

[6]Selvanathan E, Final Year Post Graduate, Department of Microbiology, Government Kilpauk Medical College, Kilpauk, Chennai, Tamil Nadu, India 600010, Email id:- drselvazeno1986@gmail.com

Corresponding Author:

N Nalini Jayanthi , Professor and HOD , Department of Respiratory Medicine, SRM Medical College Hospital and Research Centre, Faculty of Medicine and Health Sciences, SRM Institute of Science and Technology, Kattankulathur, Chengalpattu, Tamilnadu, India 603203.Email id: nalinijn@srmist.edu.in

Leela Kagithakara Vajravelu, Professor and HOD, Department of Microbiology, SRM Medical College Hospital and Research Centre, Faculty of Medicine and Health Sciences, SRM Institute of Science and Technology, Kattankulathur, Chengalpattu, Tamilnadu, India 603203.Email id: leelav@srmist.edu.in

ABSTRACT

Ventilator-associated pneumonia (VAP) remains a significant complication of mechanical ventilation, driven by a complex interplay between multidrug-resistant bacterial pathogens and their associated virulence determinants. Molecular characterization of these organisms can provide valuable insight into the resistance and pathogenicity landscape underlying VAP.

In this multicentric study, 120 endotracheal aspirate specimens obtained from mechanically ventilated patients were investigated for VAP-associated bacterial pathogens. Bacterial identification and antimicrobial susceptibility testing were performed, followed by molecular characterization of 55 confirmed bacterial isolates comprising *Acinetobacter baumannii* (n=23), *Klebsiella pneumoniae* (n=15), *Escherichia coli* (n=10), and *Pseudomonas aeruginosa* (n=7). TaqMan probe-based real-time PCR was employed to investigate selected antimicrobial resistance determinants together with organism-specific virulence and hypervirulence-associated genes.

Phenotypic resistance was highest against ampicillin (100%), followed by tetracycline (58.2%), levofloxacin (25.5%), ceftriaxone (21.8%), and cefotaxime (20.0%). Molecular analysis showed the predominant detection of blaTEM (100%) followed by tetA and tetB (58.2% each), blaCTX-M (43.6%), qnrA (38.2%), qnrB (34.5%), blaNDM (32.7%) and blaOXA-48-like (20.0%). Virulence-associated determinants showed organism-specific distribution, including ompA/bap**,** khe/rmpA**,** invE/stx2, and lasB/lasR among *A. baumannii*, *K. pneumoniae*, *E. coli*, and *P. aeruginosa*, respectively.

The results revealed a high degree of phenotypic resistance and the presence of a spectrum of molecular resistance and virulence-associated determinants among the VAP-associated pathogens emphasizing the necessity of integrated microbiological and molecular surveillance to gain insights into the changing pathogenic landscape of VAP.

Keywords

Ventilator-associated pneumonia; antimicrobial resistance determinants; virulence-associated genes; multidrug-resistant Gram-negative bacteria; TaqMan real-time PCR; molecular epidemiology.

1. INTRODUCTION

Few problems in contemporary medicine carry the same weight as antimicrobial resistance (AMR). What makes it so unsettling is not simply that certain drugs are losing their punch, but that the entire logic on which modern healthcare rests — the assumption that a bacterial infection can, in most cases, be reliably treated — is being quietly undermined. The World Health Organization has warned that if this erosion continues unchecked, healthcare systems risk sliding back toward something resembling the pre-antibiotic era, a time when a routine infection could become a death sentence [1]. That warning is not abstract for hospitals; it is lived daily in Intensive Care Units (ICUs), where the density of sick, immunocompromised patients, combined with heavy antibiotic use and invasive procedures, creates near-ideal conditions for multidrug-resistant (MDR) organisms to take hold. Once established, these organisms do not stay contained. They contribute to a steady climb in healthcare-associated infections (HAIs), stretch hospital stays well beyond what would otherwise be necessary, and, in the worst cases, tip the balance toward mortality that better antibiotic stewardship might have prevented [2].

Within this already troubling landscape, one organism deserves particular attention: Acinetobacter baumannii. It is, in many respects, an unremarkable-looking Gram-negative bacterium, yet it has earned a reputation among clinicians and microbiologists as an exceptionally resourceful survivor. It persists on hospital surfaces, medical equipment and even human skin for extended periods, resisting standard disinfection efforts in ways that few other pathogens manage, and it seems to acquire new resistance genes almost opportunistically, whenever the chance arises [3, 4, 5]. This adaptability is precisely why its carbapenem-resistant strains, commonly abbreviated as CRAB, have been placed on the critical-priority pathogen lists maintained by both the World Health Organization and the United States Centers for Disease Control and Prevention. That designation is not a bureaucratic formality; it signals that clinicians are running out of dependable treatment options and that the public-health stakes of allowing this organism to spread further are considerable [6,7,8].

Nowhere is the danger posed by A. baumannii more concentrated than in neurosurgical and other critical-care units. Patients admitted to these wards are, almost by definition, already vulnerable — many arrive after trauma, stroke, or major surgery, and their treatment frequently requires a cluster of invasive interventions that, while medically necessary, each open a door for infection. Mechanical ventilation bypasses the body's natural airway defences; central venous catheters provide a direct conduit into the bloodstream; and prolonged antibiotic courses, ironically, can clear out competing flora and leave room for resistant organisms to flourish. The combined effect of these exposures is a markedly elevated risk of Ventilator-Associated Pneumonia (VAP) and bloodstream infection [9]. VAP, in particular, has been documented repeatedly as one of the most frequently acquired infections in ICU settings worldwide, and its consequences extend well beyond the immediate clinical picture — it drives up morbidity, adds to mortality figures that are already sobering in critically ill populations, and imposes substantial costs on healthcare systems that are often already stretched thin [10]. Neurosurgical patients occupy an especially precarious position within this picture: the combination of acute neurological injury and the extended ventilator dependence that so often follows it compounds their susceptibility to infection in ways that are well documented across multiple patient cohorts [11].

These broader patterns were reflected clearly in the population examined for this study. Multidrug-resistant A. baumannii emerged as the single most common nosocomial pathogen identified, accounting for a disproportionately large share of the clinical isolates recovered and outnumbering every other Gram-negative organism encountered [12]. A closer look at the affected patients revealed a fairly consistent demographic pattern: most were middle-aged or older, and men were represented more often than women. Geographically speaking — or perhaps more accurately, unit-wise — the majority of isolates traced back to high-dependency and intensive-care areas, with the Neurosurgical ICU standing out as a particular hotspot [13]. None of this is especially surprising when set against what is already known about the drivers of MDR A. baumannii transmission: units that combine invasive procedures, extended ventilation, and round-the-clock intensive monitoring tend, almost inevitably, to become the settings where such organisms circulate most freely [14].

Zooming out slightly, the resistance trends observed here are not isolated to a single institution or organism. Rates of multidrug resistance (MDR), extensively drug-resistant (XDR) phenotypes, and carbapenem resistance more broadly have all been climbing across ICUs internationally, to the point where the trend itself is now considered a distinct and pressing public-health concern in its own right. Much of this can be traced back to prescribing habits that, while understandable given clinical urgency, have arguably outpaced the evidence supporting them: broad-spectrum antibiotics are frequently started empirically — that is, before culture results confirm which organism is actually responsible — and this pattern of use, repeated across countless ICU admissions, has steadily nudged resistant strains toward dominance while narrowing the pool of drugs still capable of treating them [15].

The isolates recovered in this study reinforce that broader regional picture rather well. Gram-negative bacilli accounted for the overwhelming majority — 96.5 percent — of all organisms identified, a figure closely aligned with what has been reported elsewhere for ventilator-associated respiratory infections in similar settings. Among these, A. baumannii, Pseudomonas aeruginosa, and Klebsiella pneumoniae were, by some distance, the species recovered most often [6]. What stood out just as much as the identity of these organisms was their resistance profile: antibiotics that clinicians have long relied on for empirical ICU therapy — third-generation cephalosporins, fluoroquinolones, and aminoglycosides among them — showed strikingly high rates of resistance. Colistin was the notable exception, retaining complete activity against every single Gram-negative isolate tested, a finding that, while reassuring in the short term, also raises uncomfortable questions about how much of the therapeutic burden is now falling on a drug once considered a last resort [16].

K. pneumoniae deserves its own moment of attention here, since it did not simply appear frequently among the isolates — it also displayed an unusually broad resistance profile, spanning β-lactams, carbapenems, and even colistin itself [17]. Digging a little deeper, replicon typing of resistant transconjugants uncovered something particularly telling: the K. pneumoniae isolates carried both F-type and K-type plasmids, and each of these plasmids was found to encode multiple resistance genes simultaneously [18]. This is not a trivial detail. Plasmids of this kind function essentially as mobile genetic packages, capable of moving resistance traits not just within a single bacterial lineage but across entirely different species, which helps explain how resistance can spread so quickly through a hospital ward once it takes hold. This capacity fits within a much larger biological story: genetic mutation and

horizontal gene transfer (HGT) are widely regarded as the two principal engines driving bacterial genome evolution and adaptation, and A. baumannii in particular has proven remarkably skilled at exploiting HGT and various mobile genetic elements to acquire and disseminate resistance genes across its population [19].

All of this resistance would be somewhat easier to manage if clinicians could identify the responsible organism quickly — but that, unfortunately, is where another layer of the problem emerges. Traditional culture-based identification and antibiotic susceptibility testing, while still considered the diagnostic gold standard in many respects, are inherently slow, typically requiring several days before results are available. In the meantime, clinicians are left with little choice but to begin treatment empirically, guessing at the most probable pathogen and its likely resistance pattern based on local epidemiology and clinical judgement rather than confirmed laboratory data. That delay carries real consequences: it has been linked to increased mortality and, perhaps more insidiously, to further selection pressure that favours resistant organisms over susceptible ones. The problem is magnified considerably in low- and middle-income country (LMIC) settings, where MDR pathogens are already disproportionately prevalent and where the infrastructure needed to shorten diagnostic turnaround time is often limited. It is precisely this gap — between the urgency of clinical decision-making and the slowness of conventional diagnostics — that has fuelled growing interest in rapid, affordable, PCR-based molecular diagnostic tools capable of guiding antibiotic therapy far closer to real time [17].

Several technologies have emerged in response to this need, and multiplex PCR platforms are among the most promising. The BioFire FilmArray system is a useful illustration of what this technology can offer: it is a fully automated, single-cartridge platform capable of testing a clinical sample for multiple species-specific targets and resistance genes in one integrated run, sparing laboratories the time and effort of running separate assays for each suspected organism [20]. Its Pneumonia Plus Panel, specifically, screens for fifteen of the most frequently encountered Gram-negative and Gram-positive bacteria, reporting them semi-quantitatively through bacterial identification numbers (BIN), alongside a set of atypical bacteria that are reported in the same way. It further extends coverage to nine viruses commonly implicated in pneumonia and other lower respiratory tract infections, although these are reported only qualitatively. On the resistance-gene side, the panel targets an array of clinically significant markers — KPC, NDM, OXA-48-like, VIM, IMP, and CTX-M, which

together capture much of the carbapenemase and extended-spectrum β-lactamase (ESBL) landscape, along with mecA/mecC and MREJ, which flag methicillin resistance [20].

Quantitative PCR (qPCR) offers a complementary, if somewhat more targeted, approach to the same underlying problem. Rather than screening broadly, it relies on primers designed against sequences that are already known, allowing for both the detection and precise quantification of individual antibiotic-resistance genes (ARGs) within a sample. Scaled up, high-throughput qPCR (HT-qPCR) extends this same logic across hundreds of resistance genes at once, making it possible to build a far more comprehensive resistance profile than would be feasible using conventional methods [21]. Taken together with syndromic multiplex panels — tools that test simultaneously for both pathogens and their associated resistance genes — these molecular diagnostics represent a genuine shift in how quickly and how precisely antibiotic therapy could, in principle, be tailored. Whether that promise translates into measurably better patient outcomes and more disciplined antibiotic stewardship in ICU settings is, in many ways, the question this study sets out to explore [22].

The current retrospective study Substantiate the distribution and occurrence of Multiple Antibiotic resistance while addressing the development and prevalence of virulence and hyper virulence factors among the tertiary health care hospitals of South India.

2. MATERIALS AND METHODS:

2.1 DESIGN OF STUDY PLAN:

The present study demonstrate the molecular epidermology of the Ventilator Associated Pneumonia (VAP) coupled Endo Tracheal (ET) isolates from the Teriary Health Care hospitals of South India during the period August 2024 to June 2026, appoved by the institutional Ethical committee of SRM Medical College, Hospital and Research Centre, Chengalpattu, Tamil Nadu, India (IEC No.: 8883, dated 28th August 2024) and Government Kilpauk Medical College, Chennai, Tamil Nadu, India (IEC No.: 1358/2024, dated 27th of December 2024)

Patients who had been in the Mechanical Ventilator for more than 48 hrs were eligible to participate in the Study. All the clinical data of the patients were observed and analysed to satisfy the sampling criteria of 1. Patient of age greater than Eighteen years, 2. Patient admitted in Intensive Care Unit for greater than two consecutive calendar days on the day of event, being the day of Placement of Mechanical Ventilator being Day one and 3. Patient's Radiological studies to observe the Pulmological findings [23].

A total of 120 Endo Tracheal Aspirate (ETA) samples were collected from 4 different hospital centres, transferred to the Laboratory of Department of Medical Microbiology, SRM Medical College, Hospital and Research Centre, Chengalpattu, Tamil Nadu, India and stored at 4˚C.

The samples were streaked on MacConkey Agar (HiMedia Laboratories Pvt Ltd, Cat No.: M082) and post incubation, colonies with distinct morphological colonies were picked and cultured in Nutrient Agar (HiMedia Laboratories Pvt Ltd, Cat No.: M001) to obtain a pure culture.

2.2 IDENTIFICATION OF PURE CULTURE:

The species level identification of the pure isolates were carried out using MALDI-TOF (Bruker MALDI-TOF Ultrafle Xtreme II)

2.3 SELECTION AND DETERMINATION OF MINIMUM INHIBITORY CONCENTRATION (MIC)

The isolates were subjected to Antimicrobial Sensitivity Test by Broth microdilution Method as per CLSI M07 [24] against Panel of Antibiotics targeting specific organism as per CLSI M100 [25].

2.4 GENOMIC DNA EXTRACTION

The Genomic DNA were extracted from the isolates using TrueScreen Bacterial DNA Isolation Kit (TranScience Healthcare Technologies) following Manufacturer's Protocol. Briefly, the overnight fresh inoculum was suspended in 560 µL of lysis buffer followed by addition of 20 µL lysozyme and 20µL Proteinase K and incubated at 65°C for 20 minutes. Subsequently equal volume of Absolute Ethanol was added and transferred to high Sensitive Silica-based Spin Column followed by centrifugation at 10,000 rpm for 3 minutes. The column is washed with 500 µL of Wash I followed by double wash with same volume of Wash II. Dry spin is employed to remove residual salts and eluted with 30 µL of Elution Buffer.

2.5 REAL TIME PCR FOR DETECTION OF GENE:

Target genes were selected according to their established association with antimicrobial resistance, virulence and hypervirulence in the respective bacterial species. Primers were designed to amplify conserved regions of the target genes. Primers probes targeting the selected genes were designed using IDT PrimerQuest™. The designed primer and probe sequences were synthesized by Integrated DNA Technologies (IDT, Coralville, USA). For positive controls, corresponding target sequences were obtained as synthetic gBlocks® from IDT and used as positive-control templates for assay optimization and validation [39].

qPCR was employed with each PCR tube consisting of 10 µL TaqMan^{Tm} Universal PCR Master Mix (Applied Biosystem, CA), followed by 0.8 µL of Primer each (Eurofins Genomics, India), 0.4 µL FAM – Black Hole Quencher 1 Probe (IDT, Coralville, USA), 2 µL of Template DNA and made to the final volume of 20 µL with Nuclease Free Water

(Quiagen, Cat No.: 129115). The process was carried out in Athenese-Dx Realtime PCR System (ALTA, India)

2.6 STATISTICS:

The disparity in gene expression levels and other visual representations were conducted using GraphPad Prism (Version 8.0.1).

3. RESULTS:

3.1 DISTRIBUTION OF VENTILATOR ASSOCIATED PNEUMONIA:

A total of 120 Endo Tracheal Aspirates were collected from 4 different tertiary care hospitals from 9 different ICU wards during 4 different seasons of the study period, with the age group distributed from 19-89 with the average distribution of 51.05 ±20.71**(Table 1).** During the initial screening, 55 distinct colony growth were observed with and confirmed as VAP as per CDC/NHSN surveillance criteria 2026 [26]

Season	Total samples	VAP-positive	No growth	VAP positivity
Monsoon	36	19	17	52.8%
Summer	25	15	10	60.0%
Winter	32	13	19	40.6%
Post-monsoon	27	8	19	29.6%
Total	120	55	65	45.8%

Table 1: Distribution of bacterial isolates among Ventilator-Associated Pneumonia(VAP) positive samples

A total of 120 mechanically ventilated patients were evaluated, of whom 55 (45.8%) developed ventilator-associated pneumonia (VAP) and 65 (54.2%) remained VAP-negative. Patient age ranged from 19 to 89 years, with an overall median age of 49 years (IQR 33.0–69.75) and a mean age of 51.05 ± 20.71 years. The age distribution deviated significantly from normality in both the VAP-positive group (Shapiro–Wilk $W = 0.9375$, $p = 0.0067$) and the VAP-negative group ($W = 0.9333$, $p = 0.0017$). The median age of patients with VAP (46 years, IQR 32–64) was slightly lower than that of patients without VAP (49 years, IQR 33–71). VAP was identified in 25 of 59 males (42.4%) and 30 of 61 females (49.2%), with

Fisher's exact test yielding p = 0.470 and an odds ratio of 0.76 (95% CI 0.38–1.59). Hospital-wise analysis also demonstrated the pattern where Hospital 1 recorded 25 VAP-positive cases among 54 patients (46.3%), Hospital 2 recorded 27/56 (48.2%), Hospital 3 recorded 1/4 (25.0%), and Hospital 4 recorded 2/6 (33.3%) ($\chi^2 = 1.209$, df = 3, p = 0.751). The prevalence of VAP was 28.6% in the ICICU/Post-Surgery ICU and 70.0% in the General ICU, with rates of 63.6% in the Post-Anaesthetic ICU, 52.6% in the Cardiac ICU, 47.1% in the CICU, 50.0% in the Neural ICU, 40.0% in the Orthopedic ICU, and 41.7% in the Respiratory ICU across the nine ICU categories ($\chi^2 = 7.827$, df = 8, p = 0.451). Seasonal variation was also noted, with the highest VAP positivity during summer (15/25; 60.0%), followed by monsoon (19/36; 52.8%), winter (13/32; 40.6%), and post-monsoon (8/27; 29.6%). Thus, in the present cohort, age, sex, hospital, ICU type, and season were significantly associated with VAP occurrence **(Figure 1)**.

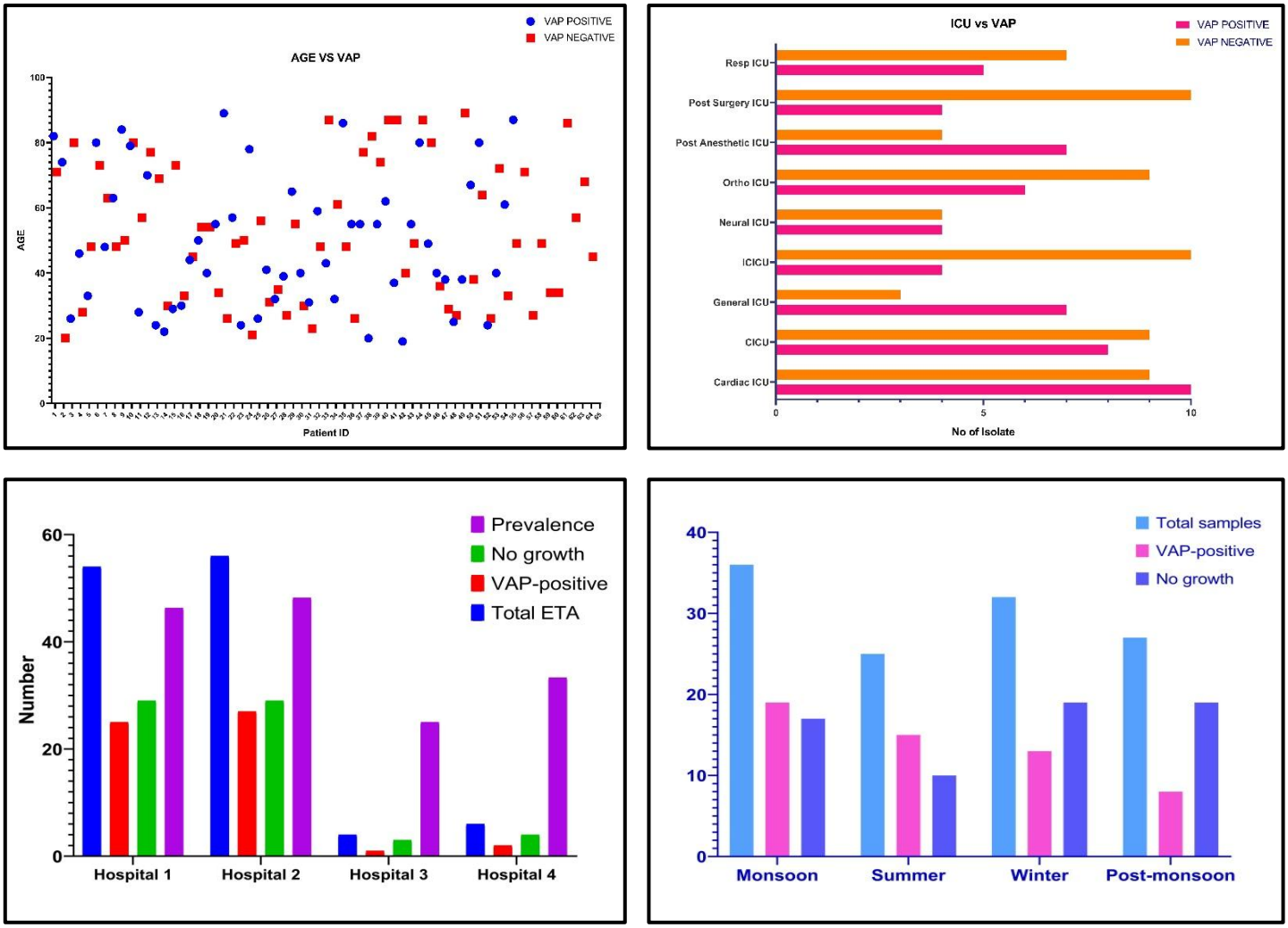

FIGURE 1: Age, Hospital, Ward and Season wise prevalence of ventilator-associated pneumonia among the study population

3.2 DISTRIBUTION OF PATHOGENS:

The MALTI-TOF-MS shows Pathogen distribution among the isolates and reveals that out of 55 positive VAP isolates, 4 major bacterial species were identified demonstrating the prevelance of *Acinetobacter baumannii, Klebsiella pneumonia, Escherichia coli* and *Pseudomonas aeruginosa* **(Figure 2)**.

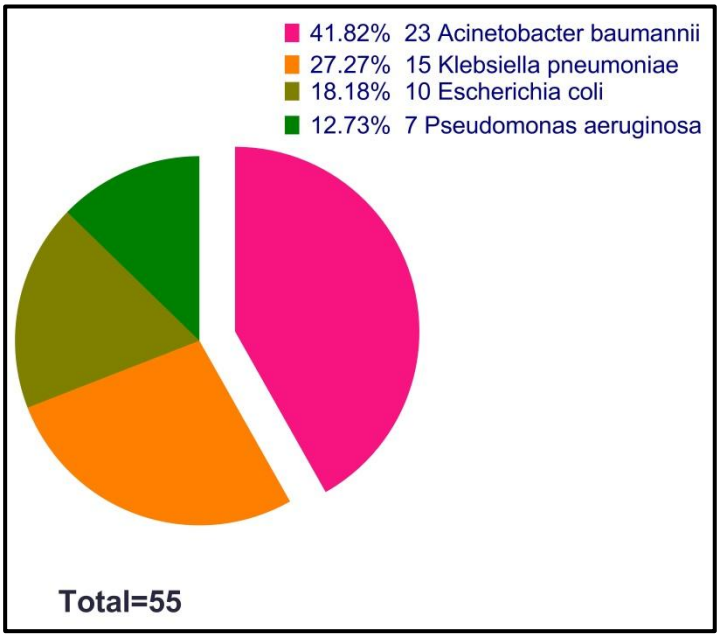

FIGURE 2: Distribution of bacterial isolates among ventilator-associated pneumonia (VAP) positive samples

Among the 55 bacterial isolates recovered from VAP cases, *Acinetobacter baumannii* was the most common organism (23 isolates, 41.8%), followed by *Klebsiella pneumoniae* (15 isolates, 27.3%), *Escherichia coli* (10 isolates, 18.2%), and *Pseudomonas aeruginosa* (7 isolates, 12.7%). This distribution is in line with the common involvement of Gram-negative bacilli, especially *A. baumannii*, in ICU-associated VAP **(Table 2)**..

Organism	Isolates	% of 55 isolates
Acinetobacter baumannii	23	41.8%
Klebsiella pneumoniae	15	27.3%
Escherichia coli	10	18.2%
Pseudomonas aeruginosa	7	12.7%
Total	55	100%

Table 2: Distribution of bacterial isolates among ventilator-associated pneumonia(VAP) positive samples

3.3 ANTIMICROBIAL SUSCEPTIBILITY PROFILE OF VAP-ASSOCIATED BACTERIAL ISOLATES

The antimicrobial panel used in the present study, included several classes of antibiotics including β-lactams, carbapenems, fluoroquinolones, aminoglycosides and tetracyclines **(Table 3).** The highest frequency of resistance was observed for ampicillin (all of the 55 isolates (55/55, 100%) were resistant). Tetracycline had the second highest frequency of resistance with 32 (58.2%) isolates resistant. Resistance to levofloxacin was observed in 14 isolates (25.5%) followed by ceftriaxone (12 isolates, 21.8%) and cefotaxime (11 isolates, 20.0%). Resistance to imipenem was seen in 8 (14.5%) isolates, resistance to doxycycline and amikacin was seen in 7 (12.7%) and 6 (10.9%) isolates respectively. Resistance rates were lower for piperacillin (4/55, 7.3%), gentamicin (3/55, 5.5%) and ciprofloxacin (2/55, 3.6%). No meropenem-resistant isolate was recorded in the present study **(Figure 3).**

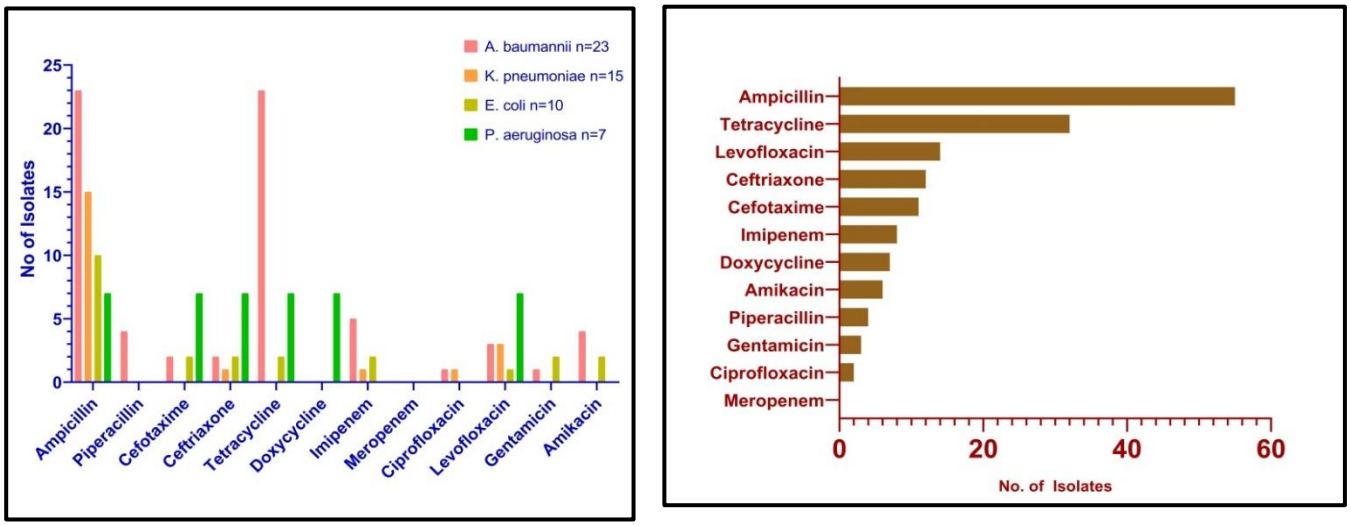

FIGURE 3: Antimicrobial resistance profile of ventilator-associated pneumonia-associated(VAP) bacterial isolates based on minimum inhibitory concentration (MIC) susceptibility testing and organism-specific resistance patterns

Antibiotic	Resistant, n	Resistant, %	Susceptible, n	Susceptible, %
Ampicillin	55	100.0	0	0.0
Tetracycline	32/32	100	0	0.0
Levofloxacin	14	25.5	41	74.5
Ceftriaxone	12	21.8	43	78.2
Cefotaxime	11	20.0	44	80.0
Imipenem	8	14.5	47	85.5
Doxycycline	7	12.7	48	87.3
Amikacin	6	10.9	49	89.1
Piperacillin	4	7.3	51	92.7
Gentamicin	3	5.5	52	94.5
Ciprofloxacin	2	3.6	53	96.4
Meropenem	0	0.0	55	100.0

Table 3: Overall antimicrobial resistance and susceptibility profile of bacterial isolates associated with ventilator-associated pneumonia (VAP)

Of the 23 A. baumannii isolates, 5 isolates (21.7%) were resistant to Imipenem, piperacillin and amikacin were resistant in 4 isolates (17.4%) respectively. Resistance to levofloxacin was seen in 3 isolates (13.0%). Resistance to cefotaxime and ceftriaxone was observed in two isolates each (8.7%). In contrast, no resistance to meropenem or doxycycline was recorded among the *A. baumannii* isolates. Of 15 K. pneumoniae isolates, 3 (20.0%) were resistant to Levofloxacin, and each one isolate was resistant to ceftriaxone, imipenem and ciprofloxacin (6.7%). No resistance was recorded to meropenem, amikacin, gentamicin, tetracycline or doxycycline.

Resistance to cefotaxime, ceftriaxone, imipenem, gentamicin, amikacin and tetracycline was observed in two isolates of E *coli* (20.0%) each, while one isolate (10.0%) was resistant to levofloxacin. No resistance was recorded to piperacillin, meropenem, ciprofloxacin or doxycycline. The seven *P. aeruginosa* isolates demonstrated a distinct susceptibility profile. All seven isolates were resistant to ampicillin, cefotaxime, ceftriaxone, levofloxacin, tetracycline and doxycycline. In contrast, all isolates were susceptible to piperacillin, imipenem, meropenem, ciprofloxacin, gentamicin and amikacin **(Table 4).**

Antibiotic	***A. baumannii*** **n=23**	***K. pneumoniae*** **n=15**	***E. coli*** **n=10**	***P. aeruginosa*** **n=7**
Ampicillin	23 (100%)	15 (100%)	10 (100%)	7 (100%)
Piperacillin	4 (17.4%)	0*	0	0
Cefotaxime	2 (8.7%)	0	2 (20%)	7 (100%)
Ceftriaxone	2 (8.7%)	1 (6.7%)	2 (20%)	7 (100%)
Imipenem	5 (21.7%)	1 (6.7%)	2 (20%)	0
Meropenem	0	0	0	0
Ciprofloxacin	1 (4.3%)	1 (6.7%)	0	0
Levofloxacin	3 (13.0%)	3 (20%)	1 (10%)	7 (100%)
Gentamicin	1 (4.3%)	0	2 (20%)	0
Amikacin	4 (17.4%)	0	2 (20%)	0
Tetracycline	23 (100%)	-	2 (20%)	7 (100%)
Doxycycline	0	-	0	7 (100%)

Table 4: Organism-specific antimicrobial resistance patterns among bacterial isolates associated with ventilator-associated pneumonia(VAP)

The resistance distribution differed highly significantly across the antimicrobial agents tested ($\chi^2 = 334.9$, df = 11, $p < 0.000001$), demonstrating a strong antibiotic-dependent variation in susceptibility phenotype. The observed contingency pattern was characterized by concentration of resistance toward selected agents, particularly ampicillin and tetracycline, whereas susceptibility predominated for most of the remaining antibiotics, with complete susceptibility observed for meropenem.

The finding of resistance to multiple antimicrobial classes points to an important MDR phenotype in the bacterial isolates associated with VAP. These findings highlight the occurrence of broad antimicrobial resistance among the major VAP-associated pathogens.

3.4 REAL TIME PCR FOR DETECTION OF GENE:

Based on the observed phenotypic antimicrobial resistance patterns, genes corresponding to the predominant resistance phenotypes were selected and designed for subsequent molecular characterization by TaqMan real-time PCR. The details of the designed PCR Primer and probes and its thermal cycling parameters is given in **Table 5.**

Category of gene to detect	Gene	Forward primer (5′→3′)	Reverse primer (5′→3′)	6-FAM – Black HoleQuencher probe (5′→3′)	Annealing temp. (°C)	NCBI Accession No.
β-lactam	blaTEM	CGGATGGCATGACAGTAAGA	TCCGATCGTTGTCAGAAGTAAG	TGCAGTGCTGCCATAACCATGAGT	60	AF309824
β-lactam	blaCTX-M	CTGTCGCCCAATGCTTTAC	GACGTTAAACACCGCCATTC	CCATTGCCCGAGGTGAAGTGGTAT	59	DQ663489.1
Carbapenemase	blaNDM	TGACTTTCGCCGCCAAT	GTCGATCCCAACGGTGATATT	CAACTTTGGCCCGCTCAAGGTATT	60	OR435270.1
Carbapenemase	blaOXA-48-like	GAGGCACGTATGAGCAAGAT	GTCGAGCCAGAAACTGTCTAC	TGGTAATGAGGACATTTCGGGCAA	60	MG436953.1
Fluoroquinolone	qnrA	GATTTCTCACGCCAGGATTTG	ATGAAACTGCAATCCTCGAAAC	TGTGACTTCAGCCACTGTCAGCTG	59	KC493118.1
Fluoroquinolone	qnrB	GCACAAGGCGCAGATTTC	TGGCGTAGCTTAGATTGGTATT	TGAATATGATCACCACGCGCACCT	60	EF634464.1
Aminoglycoside	aac(3)-Ia	AGAGTCCGTCACTCCATACA	GTACTCCTGGATCGGTTTCTTC	AATGGAGAGCCGATTGGGTATGCC	58	DQ370505.1
Aminoglycoside	aac(6′)-Ib	CGATGCTCTATGAGTGGCTAAA	CTTGGCAAGTACTGTTCCTGTA	AAGTGTCGGGCGTGCTTCTTCTC	58	DQ303918.1
Tetracycline	tetA	CATTTCGCTTGCCGCATTT	ATGAGTGCCCGCCTTTC	ATTCACTCGCCCAGGCAATGATCA	60	MW653741.1
Tetracycline	tetB	GCGTTTATATCTGAAGGTTGGTTAG	GGATAGACATCACTCCCTGTAATG	CTGGTGGTGGGATCGCTTTACCTG	60	PZ020785.1
A. baumannii-specific virulence factor	ompA	GTGGTACACGTGGTACTTCTG	CGAGCTTCAGTACGAAGAGATAAA	TAGGTAACGCTGGTGTTGGTGCTT	60	PX680608.1
A.	bap	GTGAATAGGGAGGGTAC	GGCTCAGCTGTTCCACTAAA	TGGCGAGACCATTGTTAT	60	LC57682

baumannii-specific virulence factor		CAATG		TGAAGGCT		8.1
K. pneumoniae-specific virulence factor	khe	GAGAGCGATGAGGAAGAGTTC	GCTTCCAGAGATAGCCGTTTA	ATCTACGTGCTGGAGGGCTATCCA	60	KX842080.1
K. pneumoniae-specific virulence factor	rmpA	GAGTATTGGTTGACAGCAGGA	ATGTCATAATCACACCCTTTAGGA	AAACGTCAAGCCACATCCATTGGC	60	KJ469368.1
E. coli-specific virulence factor	invE	TTTCAGGCAGCAAGCGTT	GTGTAGAACTAAGAGATTCATTAGCCTT	TCTCTATTTCCAATCGCGTCAGAACT	61	MN088823.1
E. coli-specific virulence factor	stx2	CTGCTCCTGTGTATACGATGAC	CGATACTCCGGAAGCACATT	ATGTGGACCTCACTCTGAACTGGG	60	OL872176.1
P. aeruginosa-specific virulence factor	lasB	ACAACCGTGCGTTCTACC	GCGTCGACGAACACCTC	TGGGATACCCGCAAGGCCTT	60	OK146925.1
P. aeruginosa	lasR	CGAGCGACCTTGGATTCTC	GATGAAGGCGTTCTCGTAGTC	TTAGGCAACAGGCCGAACAGGATC	60	PP786282.1

-specific virulence factor						
Housekeeping	16S rRNA	GAGTATGGGAGAGGATGGTAGA	CGTACCTCAGCGTCAGTATTAG	AGGTGTAGCGGTGAAATGCGTAGA	55	OQ061723.1

Table 5: Primer and TaqMan probe sequences designed for the molecular detection of antimicrobial resistance, virulence, and hypervirulence-associated genes

3.4.1 DISTRIBUTION OF AMR GENE:

A high heterogeneity of antimicrobial-resistance gene carriage was observed in the 55 VAP-associated Gram-negative isolates by real-time qPCR **(Figure 4)**. The bla CTX-M was distributed significantly differently between species (χ^2 = 8.122, df = 3, p = 0.0436) as was qnrA (χ^2 = 10.61, p = 0.0140) and qnrB (χ^2 = 15.66, p = 0.00133). The strongest species-specific differences were observed for tetA and tetB, both showing highly significant variation across the applicable organisms (χ^2 = 30.00, df = 2, p < 0.000001). In contrast, blaNDM (χ^2 = 2.846, p = 0.416), blaOXA (χ^2 = 1.228, p = 0.746), aac(3) (χ^2 = 6.345, p = 0.0960), and aac(6′)-Ib (χ^2 = 6.624, p = 0.0849) did not show significant species-level differences. This indicate that quinolone- and tetracycline-resistance determinants were more strongly structured by species, whereas carbapenemase and aminoglycoside-resistance genes were more diffusely distributed across the isolate collection.

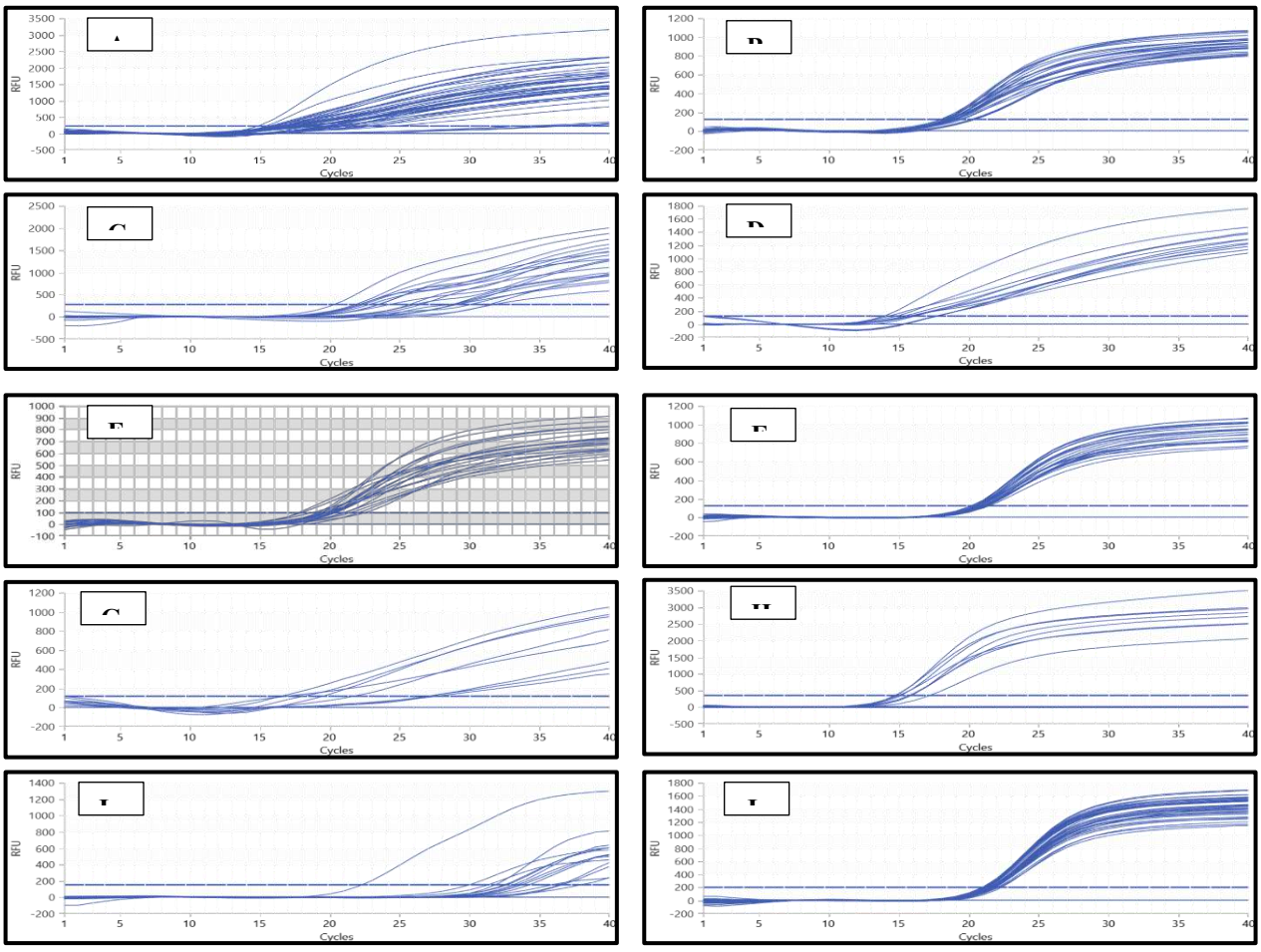

Figure 4: Real-time PCR amplification plots of antimicrobial resistance genes (a) *blaTEM*; (b) *blaCTX-M*; (c) *blaNDM*; (d) *blaOXA-48-like*; (e) *qnrA*; (f) *qnrB*; (g) *aac(3)-Ia*; (h) *aac(6')-Ib*; (i) *tetA*; and (j) *tetB*.

3.4.2 AMR GENE BURDEN:

The cumulative AMR gene burden differed significantly among the four bacterial species (Kruskal–Wallis H = 29.00, p = 0.000002) **(Figure 5)**. Median AMR gene counts were 4 genes/isolate in A. baumannii (IQR 4–6), 2 in K. pneumoniae (IQR 1–4), 3 in E. coli (IQR 2–5), and 7 in P. aeruginosa (IQR 6–7). Dunn's post-hoc analysis showed significant differences between A. baumannii and K. pneumoniae (adjusted p = 0.000401), K. pneumoniae and P. aeruginosa (p = 0.000018), and E. coli and P. aeruginosa (p = 0.003739). The remaining pairwise comparisons were not significant. This pattern indicates that AMR determinants accumulated to different degrees across species, with the greatest molecular resistance burden observed in P. aeruginosa. AMR-gene burden per isolate AMR-gene burden per isolate AMR-gene burden per isolate

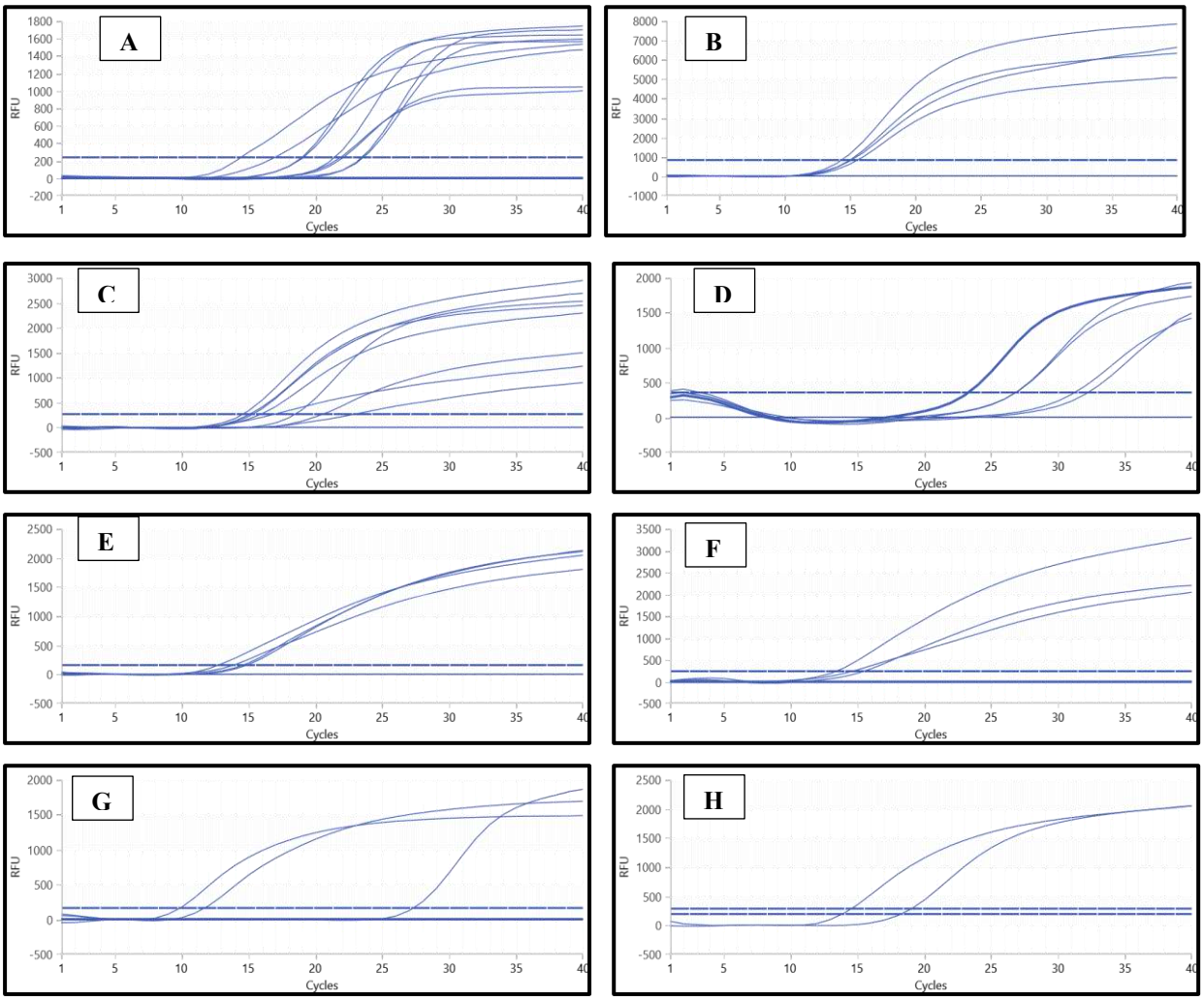

Figure 5: Real-time PCR amplification plots of virulence-associated genes in bacterial isolates. (a) *ompA*; (b) *bap*; (c) *khe*; (d) *rmpA*; (e) *invE*; (f) *stx2*; (g) *lasB*; and (h) *lasR*.

The 16S rRNA gene demonstrated successful amplification in all bacterial isolates, confirming the presence of amplifiable bacterial DNA in every isolate. This uniform amplification supported the reliability of the extracted DNA and its suitability for subsequent real-time PCR-based detection of antimicrobial resistance and virulence-associated determinants **(Figure 6)**

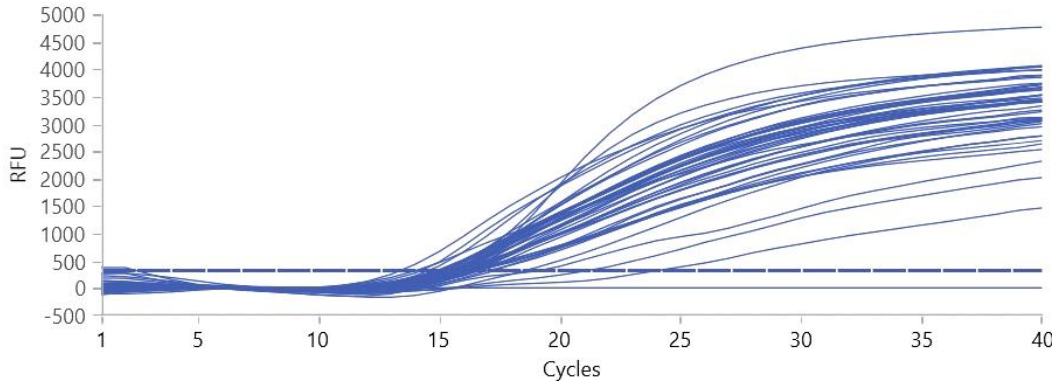

Figure 6: Real-time PCR amplification plot for 16S rRNA gene detection in bacterial isolates

3.4.3 GENOTYPE–PHENOTYPE CONCORDANCE

A strong relationship was observed between several resistance determinants and their corresponding phenotypic resistance profiles. blaCTX-M carriage was significantly associated with cefotaxime resistance (Fisher's exact $p = 0.00632$; OR = 8.70, 95% CI 1.724–42.43) and ceftriaxone resistance ($p = 0.000529$). Carbapenemase genes showed particularly strong concordance with imipenem resistance: blaNDM was significantly associated with imipenem resistance ($p = 0.000036$), while blaOXA showed an even stronger association ($p < 0.000001$). Similarly, qnrA and qnrB were both strongly associated with levofloxacin resistance (both $p < 0.000001$). Among aminoglycoside-resistance determinants, aac(3) was associated with gentamicin resistance ($p = 0.0398$), whereas aac(6′)-Ib was strongly associated with amikacin resistance ($p < 0.000001$). These associations support a high degree of molecular–phenotypic concordance for several major resistance mechanisms in the isolate collection **(Figure 7)**.

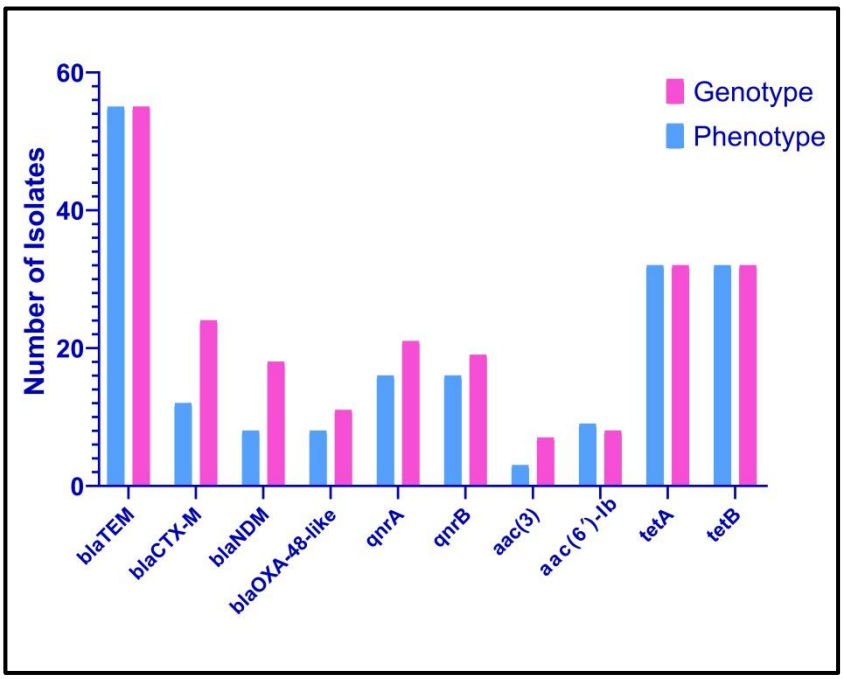

FIGURE 7: Comparison of phenotypic antimicrobial resistance with corresponding AMR gene detection by TaqMan real-time PCR among ventilator-associated pneumonia-associated bacterial isolates

3.4.4 VIRULENCE AND HYPERVIRULENCE GENE BURDEN

Virulence-associated genes showed a more restricted and heterogeneous distribution than AMR determinants. However, the cumulative virulence-gene burden did not differ significantly among species (Kruskal–Wallis $H = 2.490$, $p = 0.477$). Median virulence burden was 0 in A. baumannii, E. coli, and P. aeruginosa, and 1 in K. pneumoniae. Dunn's multiple-comparison analysis showed no significant pairwise differences between any of the four species, with all adjusted p-values > 0.999999. This suggests that, within the limited virulence panel examined, no species carried a statistically greater overall virulence-gene burden than the others.

3.4.5 VIRULENCE-GENE CO-OCCURRENCE

Pairwise analysis of species-specific virulence determinants showed no statistically significant co-occurrence. In A. baumannii, ompA and bap were not associated (Fisher's exact $p > 0.999999$; OR = 0.571, 95% CI 0.039–4.617). Similarly, khe and rmpA were not significantly associated in K. pneumoniae ($p = 0.569$; OR = 0.278, 95% CI 0.0187–2.601). In E. coli, both of the stx2-positive isolates were also invE positive, but this association was not statistically significant ($p = 0.0667$), likely due to the small number of isolates that were positive. In P. aeruginosa, lasR occurred only in a lasB-positive isolate, but the association was likewise non-significant ($p = 0.286$). Overall, these findings indicate that the virulence determinants examined were distributed independently rather than as consistently co-occurring gene combinations.

3.4.6 RELATIONSHIP BETWEEN AMR AND VIRULENCE BURDEN

Spearman analysis showed an almost null correlation ($r_s = -0.0460$, 95% CI −0.3148 to 0.2296, $p = 0.7386$; $n = 55$). Thus, isolates with a high AMR gene burden did not necessarily carry a greater number of virulence-associated determinants. This contrasts with the strong interspecies variation observed for AMR gene burden and suggests that resistance-gene accumulation and carriage of the virulence markers evaluated in this study represent largely independent molecular characteristics.

The combined molecular and phenotypic findings therefore suggest that antimicrobial resistance was a more strongly differentiated feature of the isolate population than the virulence-marker profile examined in this study.

4. DISCUSSION:

Ventilator-associated pneumonia (VAP) is still a major healthcare-associated infection, as it is often caused by Gram-negative pathogens with complicated patterns of antimicrobial resistance. In the current multicentric study the main bacterial pathogens were Acinetobacter baumannii, Klebsiella pneumoniae, Escherichia coli and Pseudomonas aeruginosa. The dominance of these organisms is in line with the recognized importance of resistant Gram-negative pathogens in VAP. And also in an multi-center retrospective cohort study done by Rongrungruang Y, et al, 2025 [31]. The top three causative pathogens of NP were *Acinetobacter baumannii* (44.2%), *Pseudomonas aeruginosa* (34.6%), and *Klebsiella pneumoniae* (28.3%). Similarly in the article done by Çam B, et al. 2025 [18], they have mentioned that The predominant pathogens responsible for VAP are multidrug-resistant (MDR) Gram-negative bacteria, including Acinetobacter baumannii, Klebsiella pneumoniae, Pseudomonas aeruginosa, and Escherichia coli and also the current WHO priority for carbapenem-resistant A. baumannii, P. aeruginosa, and resistant Enterobacterales discussed the same [27]. The Acinetobacter baumanii showed more prevalence than any organism in our study, similarly a study conducted by the Agarwal et al 2026, Among gram-negative isolates, *Acinetobacter baumannii* was the most common pathogen in both groups, identified in 40 (35.3%) early-onset and 30 (36.2%) late-onset VAP cases.[32]

Phenotypic susceptibility profile revealed significant resistance to ampicillin and tetracycline, and other resistance to third generation cephalosporins, fluoroquinolones, aminoglycosides and imipenem. Similarly in the study done by Rao et al. 2024, The *Klebsiella* species showed increased resistance to amikacin (P = <0.001), levofloxacin (P = 0.002), β-lactam β-lactamase inhibitor combination (0.001), and carbapenems (<0.001)[33]. This pattern emphasizes the importance of local antimicrobial susceptibility surveillance in VAP, since empiric treatment recommendations need to account for institution-specific resistance patterns rather than relying exclusively on generalized epidemiological estimates [28].

In our study the biofilm formation on the ET tubes becomes a greatest barrier for antimicrobial therapy . similarly in the study conducted by Bollen et al, 2023 and Shabana Sharif et al, 2025. The challenge is further enhanced by biofilm formation on ETTs and other medical devices. Biofilm-associated bacteria exhibit markedly increased tolerance to antibiotics and host immune responses because the EPS matrix restricts antimicrobial penetration, promotes metabolic dormancy, and facilitates horizontal transfer of resistance genes [34, 35].

A major feature of the present study was the phenotype-guided molecular target selection**.** AMR genes were selected according to the resistance patterns identified initially, followed by specific primer and TaqMan– FAM-Black Hole Quencher probe design. blaTEM was detected in all isolates and corresponded with the universal ampicillin-resistant phenotype. The high prevalence of blaCTX-M detection is also of relevance being CTX-M enzymes the most common ESBL determinants worldwide and highly related to spread by mobile genetic elements [29]. The detection of blaNDM and blaOXA-48-like also indicates the presence of clinically important carbapenemase-associated determinants. NDM and OXA-48-like enzymes have become widely disseminated among Gram-negative pathogens and represent major challenges for antimicrobial therapy [30].

The detection of qnrA and qnrB along with fluoroquinolone resistance suggests the presence of circulating plasmid-mediated quinolone resistance determinants. However, the increased rate of qnr detection compared to phenotypic fluoroquinolone resistance, indicates that gene carriage cannot be correlated with phenotypic resistance. Similarly, detection of aac(3) and aac(6′)-Ib provided molecular evidence for the potential of aminoglycoside resistance while detection of tetA and tetB correlated well to observed tetracycline resistance. These findings provide a rationale for combining phenotypic and molecular approaches rather than employing either approach in isolation.

Furthermore the virulence associated analysis added another level of characterization. Detection of ompA and bap in subsets of isolates indicated the presence of adhesion- and biofilm-associated determinants in A. baumannii. Similarly in a study done by Gaddy et al., 2009. Among the well-characterized virulence determinants, the outer membrane protein A (AbOmpA) of *A. baumannii* stands out as a key virulence factor, contributing directly or indirectly to biofilm formation[36, 37]. In *K. pneumoniae*, detection of **rmpA** identified a subset carrying a recognized hypervirulence-associated determinant.similarly in a study conducted by Russo et al., 2015 Virulence gene clusters, including regulator of hypermucoid phenotype in hvKp (*rmpA/rmpA2*), are carried in by this virulence plasmid[38]. However, rmpA positivity alone should not be interpreted as definitive confirmation of hypervirulent *K. pneumoniae*, since contemporary hypervirulence assessment generally benefits from a broader genetic and phenotypic characterization. Detection of invE/stx2 in E. coli and lasB/lasR in P. aeruginosa further revealed organism-specific differences in virulence-associated profiles.

5. CONCLUSION:

The present study reveals that in the studied ICU population VAP was predominantly caused by Gram-negative pathogens and Acinetobacter baumannii was the most frequently isolated organism. The bacterial isolates were highly heterogeneous in their antimicrobial-resistance profiles. Real-time qPCR revealed substantial carriage of clinically relevant AMR determinants, with significant species-specific differences in several resistance genes and in overall AMR-gene burden, while strong genotype–phenotype concordance was observed for major resistance mechanisms. In contrast, virulence and hypervirulence determinants were more selectively distributed, with no significant difference in overall virulence-gene burden among species. These data together emphasize the need for phenotypic susceptibility testing coupled with molecular surveillance to better characterize VAP pathogens to inform antimicrobial stewardship and improve infection-control practices in critical care settings.

ACKNOWLEDGEMENT:

The authors gratefully acknowledge the financial support of SRM Medical College Hospital and Research Centre, Faculty of Medicine and Health Sciences, SRMIST, Kattankulathur, for bearing the defrayed costs of publishing this article.

ETHICS APPROVAL AND CONSENT TO PARTICIPATE:

A written informed consent was obtained from all participants at the time of sample collection and the study was approved by the Institutional Ethics Committee (IEC No: ECR/8883/INST/TN/2013/RR-19), SRM Medical College Hospital and Research Centre and Institutional Ethics Committee (IEC No: ECR/1385/INST/TN/2020), Government Kilpauk Medical College, Chennai. All procedures involving human participants were conducted in accordance with the ethical standards of the Institutional Ethics Committee and the principles of the Declaration of Helsinki.

AUTHOR CONTRIBUTIONS

All authors contributed to the conception and design of the study. Data collection, analysis, and interpretation were performed by the authors. The first draft of the manuscript was written by the corresponding author, and all authors critically reviewed, revised, and approved the final version of the manuscript. All authors have read and approved the final manuscript and agree to be accountable for all aspects of the work.

DECLARATIONS

Consent for publication

Not applicable

Availability of data and materials

The main document or supplementary data supporting the article's conclusions. Institutional Ethics Committee letter also included in the supplementary data section.

Funding

No funding was received for conducting this study.

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