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Original Research Article

Biofilm Formation and Antibiotic Resistance: Prevalence of blaTEM and blaCTX-M Genes in Clinical Isolates at tertiary care Hospital

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Abstract: Biofilm formation is a most important virulence factor that enhances antimicrobial resistance dissemination among community and health care setting. Gram negative and gram-positive organisms lead to the formation of ESBL genes and biofilm formation due the routinely used antibiotics. This shows the significant therapeutic challenges in clinical management. This study focused on the prevalence of biofilm producers among Gram-positive and Gram-negative organisms by phenotypic analysis and to assess the prevalence of extended-spectrum β-lactamase (blaTEM, blaCTX-M) gene among biofilm producing organism. The present study was carried out from September 2024 - September 2025. The Clinical bacterial isolates were identified using standard microbiological methods. Phenotypic biofilm detection was done by tube adherence method. ESBL production in gram-negative isolates was determined phenotypically by Kirby-Bauer disk diffusion method according to CLSI guidelines. Then molecular detection was performed for blaTEM, blaCTX-M genes. Notably the biofilm producing gram negative bacteria expressed more ESBL gene than non -biofilm producing bacteria and the prevalence of blaCTX-M gene is comparatively higher than blaTEM. Biofilm production is observed significantly high in gram negative bacteria than gram positive. This study highlights the routine screening of biofilm and antimicrobial resistance genes for improving therapeutic approaches.

Keywords: ESBL, Biofilm, Antimicrobial resistance, Healthcare.

INTRODUCTION

Biofilm formation is a complex virulence factor exhibited by microbial communities. In this process, cells are encased in an extracellular matrix or slime (a self-produced network of polysaccharides and proteins) that adheres both to each other and to surfaces. Biofilm formation plays a crucial role in the dissemination of multidrug resistance genes. It enables bacteria to alter their phenotypes (observable traits and behavior), which leads to therapeutic failure and challenges in clinical management. Quorum sensing (QS) is the chemical communication between biofilm-producing bacteria. This communication allows bacteria to monitor their environment for other bacteria and alter their behavior in response to changes. The formation of biofilm is influenced by the availability of key nutrients, chemotaxis (chemical stimuli which helps in movement) towards surfaces, motility of bacteria, surface adhesions, and the presence of surfactants[1]. Both Gram-positive and Gram-negative bacteria, such as *Staphylococcus aureus*, *Escherichia coli*, and *Pseudomonas aeruginosa,* play an important role in conferring biofilm formation. A major concern in clinical microbiology is the emergence and spread of extended-spectrum β-lactamase (ESBL)-producing bacteria, particularly among Gram-negative pathogens. ESBL production is often carried out by mobile genetic elements (segments of DNA that can move within or between genomes), leading to rapid dissemination among the bacterial community. Routine detection of biofilm producers and ESBL in clinical laboratories will help in the clinical management of chronic or recurrent infections[2]. This

study focused on the prevalence of biofilm producers among Gram-positive and Gram-negative organisms by phenotypic analysis and to assess the prevalence of extended-spectrum β-lactamase (*bla*$_{TEM}$, *bla*$_{CTX-M}$) gene among biofilm producing organism. This study emphasizes that routine screening of biofilm along with antimicrobial resistance genes which plays an important role in improving therapeutic and diagnostic approaches.

METHODOLOGY

Study Design and Duration

This retrospective study was carried out over a period of September 2024 - September 2025 at ACS Medical college and hospital, chennai. Ethical approval was procured by the institutional ethics committee of ACS Medical College and Hospital. No personal identifiers, and patient confidentiality were maintained throughout the study.

Collection and Processing of Clinical Samples

The 200 clinical specimens from both inpatient and outpatient departments, includes urine, pus, wound swabs, sputum, blood, catheter tips, was collected by standard aseptic techniques. The samples were processed according to standard microbiological procedures.

Isolation and Identification of Bacterial Isolates

The obtained clinical specimens were inoculated on blood agar and MacConkey agar except urine specimens, which were plated on Cysteine Lactose Deficient Medium as per standard bacteriological procedures. The culture plates were incubated at 37°C for 24–48 hours. Bacterial isolates were identified based on Colony morphology, Gram staining, Standard biochemical tests. Isolates were categorized as gram-positive and gram-negative organisms[3].

Inclusion criteria

Male and female patients of all age groups of various infections.
Non-duplicate bacterial isolates obtained from clinical samples.
Only clinically significant isolates were considered.

Exclusion criteria

Patients who were on antibiotic therapy or had a history of antibiotic intake within one week prior to sample collection.
Duplicate isolates from the same patient.
Commensal flora and contaminants.

Detection of Biofilm Production

Tube adherence method

Isolated colonies were inoculated into trypticase soy broth and incubated for 24 hours at 35 °C. The supernatant was discarded and the tube was washed with phosphate-buffer saline. The tube was treated with 0.1% crystal violet for staining and further washed with water and dried. The visible lining at the bottom and on the walls of tube was considered positive[4].

Phenotypic Detection of ESBL Production

The obtained gram-negative isolates were processed for ESBL screening by Kirby-Bauer disk diffusion method according to CLSI guidelines. This was performed using aztreonam 30µg, ceftriaxone 30µg, cefotaxime 30µg, cefoxitin 30µg, ceftazidime 30µg. They were further confirmed by the combined disk method using ceftazidime with and without clavulanic acid. An increase in zone diameter of ≥5 mm in the presence of clavulanic acid was considered ESBL positive as per CLSI guidelines[5].

Molecular Detection of ESBL Genes

DNA Extraction

Phenotypically confirmed ESBL-producing isolates, were further processed for the extraction of genomic DNA by using the boiling lysis method.

PCR Amplification

The molecular analysis of gram -negative organism along with quality control was employed by conventional PCR. The *bla*$_{TEM}$, *bla*$_{CTX-M}$ gene specific primers were used for the genotypic detection and listed in table 1. 25µL reaction was employed for the gene detection. The PCR was performed using a thermal cycler and cycling condition was depicted in the below table2 2 ,3. The obtained PCR products were visualized using UV illumination[6].

RESULTS

Table 1: primers used for PCR

bla$_{TEM}$	Forward Primer: 5′-CATTTYCGTGTCGCCCTTATTC-3′ Reverse Primer: 5′-CGTTCATCCATAGTTGCCTGAC-3′
bla$_{CTX-M}$	Forward Primer: 5′-TTAGGAARTGTGCCGCTGYA-3′ Reverse Primer: 5′-CGATATCGTTGGTGGTRCCCAT-3′

Table 2: Cycling conditions for $bla_{TEM,}$ amplification

Step	Temperature	Time
Initiation	95°C	5 minutes
Denaturation	94°C	30 secs
Annealing	58°C	45 secs
Extension	72°C	1 minute
Elongation	7°C	10 minutes

Table 3: Cycling conditions for bla_{CTX-M} amplification

Step	Temperature	Time
Initiation	95°C	5 minutes
Denaturation	94°C	30 secs
Annealing	62°C	40 secs
Extension	72°C	1 minute
Elongation	7°C	10 minutes

Statistical analyses

Data were analyzed using SPSS version 22.0 and chi-square test.

RESULTS:

Distribution clinical isolates:

200 non-duplicate isolates were obtained from various clinical specimens. Among the obtained isolates, gram-negative bacteria 120(60%) were significantly higher than gram-positive bacteria 80 (40%). The assessment is shown in table 4.

Prevalence of biofilm production

Among 80 gram-positive organism 41(51%) were biofilm producers, and 39 (49%) were non- biofilm producers. From the 120 isolated gram-negative organism 90 (75%) isolates expressed biofilm and 30 (25%) were non-biofilm producers. The biofilm producing organism is significantly higher among gram negative organism. The difference is statistically significant ($\chi^2 = 8.87$, $p < 0.05$) depicted in table 5.

Table 4: Distribution of clinical isolates

Bacterial isolates	Number (n)	Percentage %
Gram negative bacteria	120	60
Gram positive bacteria	80	40
TOTAL	200	

Table 5: Prevalence of biofilm production

Bacterial groups	Biofilm producers n(%)	Non- biofilm producers n(%)
Gram positive bacteria	41 (51%)	39 (48.7%)
Gram negative bacteria	90 (75%)	30 (25%)

Phenotypic Detection of ESBL by Disc Diffusion Method

Phenotypic screening by Kirby Bauer disc diffusion method for 120 obtained gram-negative isolates expressed ESBL 68(56.6%) and non-ESBL 52(43.3%)

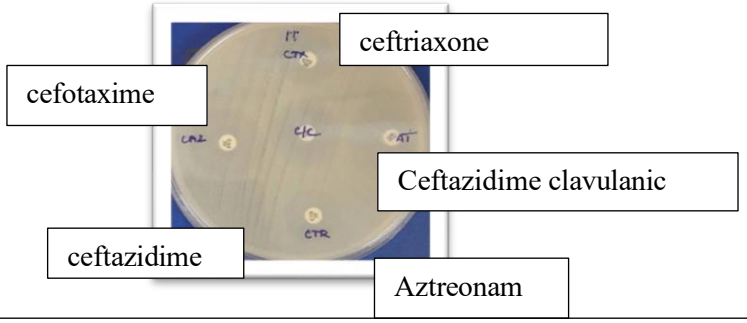

Figure 1: Disc diffusion test showing positive ESBL result

	Biofilm producing isolates n (%)	Non-biofilm producing isolates n (%)
ESBL positive	58(64.4)	10(33.3)
ESBL negative	32(35.6)	20(66.7)

Genotyping for ESBL genes:

Among 58 biofilm-producing ESBL isolates, 10 (17%) expressed bla_{TEM} and 44 (75.8%) expressed $bla_{CTX\text{-}M}$. Among 10 non-biofilm-producing ESBL isolates, 4(40%) expressed $bla_{CTX\text{-}M}$ and none expressed bla_{TEM} gene. Extended spectrum β-lactamase (ESBL) producing genes are expressed significantly high among biofilm producing organisms (figure 2,3). This shows significant association between biofilm production and ESBL production ($\chi^2 \approx 12.96$, $p < 0.001$). Among biofilm producing ESBL isolates the prevalence of $bla_{CTX\text{-}M}$ is higher than bla_{TEM} and this difference is statistically significant ($p < 0.005$) and chi square is ≈5.29. The results are stated in table 8.

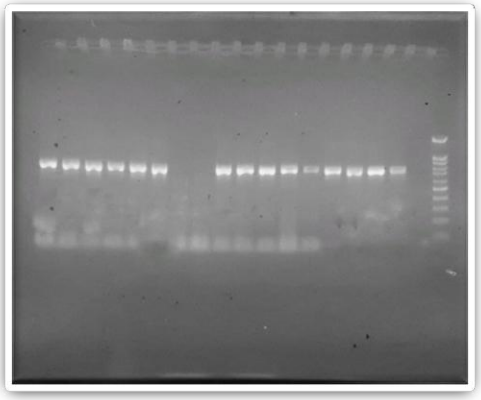

Figure 2: PCR amplification of $bla_{CTX\text{-}M}$ after 1.5% agar electrophoresis (PC- positive control, NC- negative control, L-

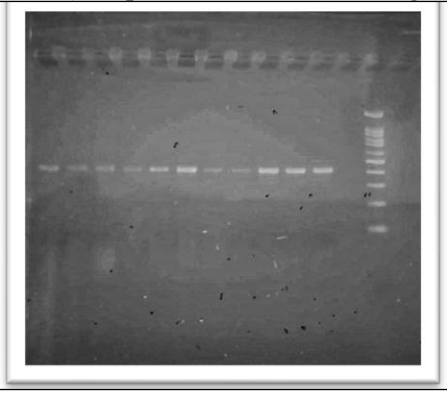

Figure 3: PCR amplification of bla_{TEM} after 1.5% agar electrophoresis. PC- positive control, NC- negative control, L-ladder (1000kb)

Table 8: Association between Biofilm Production and ESBL gene:

Biofilm status	*bla*$_{TEM}$ n (%)	*bla*$_{CTX-M}$ n (%)	*Coexistence (bla*$_{TEM,}$ *bla*$_{CTX-M}$*)* n (%)
Biofilm producing isolates	10 (17%)	44 (75.8%)	4 (7%)
Non-biofilm producing isolates	4(7%)	4(7%)	0

DISCUSSION

Recurrent infection among the clinical setup is majorly contributed by biofilm formation and extended spectrum β-lactamase (ESBL) production. This study evaluated the prevalence of biofilm production among clinical isolates and assessed the association of ESBL gene carriage among biofilm producing Gram-negative bacteria. Our study shows that high prevalence of extended spectrum β-lactamase (ESBL) production among biofilm producing gram negative organism and showed statistical significance.

Hasan et al., stated that the correlation between biofilm forming and anti-microbial susceptibility patterns in ESBL and non ESBL producing uropathogenic bacteria. This observation correlates with the finding of the present study, where prevalence of biofilm formation was observed high among ESBL positive isolates. Biofilm formation is the most important virulence factor that synergizes with ESBL- mediated resistance. This complicates the clinical management. Furthermore, biofilm producing isolates showed increased resistance to the multiple antibiotic classes7.

A systematic meta-analysis by keikha and karbalaei stated that ESBL producing bacteria are significantly high among biofilm producing organism than non-biofilm producing organisms. This indicates a strong association of biofilm associated virulence and antimicrobial resistance globally. The findings of the present study are in agreement with the meta-analysis. The analysis demonstrated that more than half of ESBL positive isolates exhibited to strong biofilm forming ability. The author analyzed data from multiple studies of different geographical locations including wide range of gram-negative bacteria. Additionally, review suggested that biofilm facilitate horizontal gene transfer, which fastens the dissemination of ESBL genes such as blaTEM, blaCTX-M within clinical settings and concluded that coexistence of ESBL AND biofilm production will lead to major clinical challenge8.

In the study conducted in tertiary care center in Palakkad, kerala shows close agreement with the present study stating that predominance of blaCTX-M genes than other genes (blaTEM, bla-SHV) among ESBL producing Klebsiella pneumonia isolates. Similarly, in our study blaCTX-M frequency was higher than blaTEM in gram negative isolates. In Palakkad study they focused on molecular characterization of ESBL genes, our results provide additional insight that biofilm formation plays an important virulence associated with resistance determinants. However, all these findings emphasize the urgent need to integrate a surveillance approach to combat antimicrobial resistance6.

Badran, A. A et al., conducted a study at Mansoura university, Egypt in which they have assessed the prevalence of ESBL gene among gram negative bacteria from community acquired and healthcare associated infections. Phenotypic analyses demonstrated that ESBL isolates are significantly high in health care associated infections and molecular characterization revealed that blaCTX-M was frequently detected. The author emphasizes that increase predominance of CTX-M dissemination highlighted the horizontal gene transfer via plasmids. The increase in ESBL genes represents serious public health concern. These findings are highly consistent with the present study, where blaCTX-M was higher than blaTEM ESBL gene, among biofilm forming isolates. This study when interpreted along with present study, the Mansoura study strengthens the blaCTX-M harboring harboring pathogens are widely disseminated9.

Jomehzadeh et al., investigated on Escherichia coli from urinary tract infections to study ESBL production, biofilm formation. Among 25 isolates atleast one targeted ESBL genes were expressed. Among ESBL positives blaCTX-M was higher than the blaTEM and blaSHV. Phenotypic biofilm testing showed that 42.82% were biofilm producers. The author also concluded that co-existence of ESBL

genes, MDR phenotype and biofilm production highlights the need for ongoing surveillance. This study supports our present investigation were 58% of ESBL gene are biofilm producers10.

The study from Thailand reported high prevalence of ESBL producing E.coli among clinical isolates, with a marked dominance of phylogroup B2 a lineage commonly associated with extraintestinal pathogenic E.coli. Majority of isolates carried blaCTX-M genes and phylogenetic analysis demonstrated that ESBL production was significantly associated with virulent phylogroups. These isolates exhibited high resistance rates to third generation cephalosporins, fluroquinolones and trimethoprim-sulfamethoxazole. This highlights that ESBL producing E.coli have stronger adaptive capacity, leading to dissemination in community and hospital settings. The present study underscores the importance of integrating phylogenetic profiling with resistance surveillance. Overall, the results, emphasizes the continuous molecular monitor, rational antibiotic use and infection control strategies11.

Lister JL et al., conducted a study which correlated with present study in which gram positive showed substantial biofilm formation, similar to certain literature that recognizes biofilm production as virulence factor among Staphylococcus, Enterococcus. Increased biofilm formation in such organisms has been linked to device associated and chronic infections12.

In summary, the coexistence of high biofilm formation rates and predominant blaCTX-M among ESBL producing isolates leads to challenges in antimicrobial therapy. The consistent surveillance of biofilm phenotypes and ESBL genotypes, particularly of blaCTX-M varients, is crucial for guiding treatment policies and infection control strategies both in India and globally.

CONCLUSION:

This study highlights the significant association between biofilm formation and extended-spectrum β-lactamase (blaTEM, blaCTX-M) gene among clinically isolated organisms. A high proportion of the obtained isolates shows biofilm production. The coexistence of biofilm with resistance mechanism, particularly extended-spectrum beta lactamase among gram negative isolates, emphasizes the complexity of clinical management.

The present study shows that biofilm formation plays a vital role as a virulence factor that contributes to therapeutic failure, recurrence of infections in clinical community. We highlight the significance of standard screening for biofilm formation along with antimicrobial susceptibility in laboratories. This plays an important role in controlling biofilm associated multidrug-resistance infections and improving patient outcomes.

Declarations:

Acknowledgment:
All the listed authors are thankfull to their respective universities/institution for supporting this study

Conflict of Interest
The authors declare that there is no conflict of interest.

Authors' Contribution
All authors listed in this study have made a substantial direct and intellectual contribution to the work and approved it for publication

Funding
None.

Ethics Statement
Ethical approval was procured by the institutional ethics committee of ACS Medical College and Hospital. No personal identifiers, and patient confidentiality were maintained throughout the study.

Data Availability
All dataset generated during the study are included in the manuscript.

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