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Exploring Drug Resistance and Biofilm Formation in Gram-Negative Clinical Isolates

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ABSTRACT:
Background: Biofilms are clusters of microorganisms surrounded by a slimy matrix composed of extracellular polysaccharides known as polysaccharide intercellular adhesion (PIA). Bacterial species frequently associated with biofilm formation include Enterococcus faecalis, Staphylococcus aureus, Escherichia coli, Klebsiella pneumoniae, and Pseudomonas aeruginosa [1], [3]. This study was conducted to analyze the antibiotic resistance patterns and assess the capacity to create biofilms in clinical isolates of gram-negative bacteria. Methods: All clinical samples received in the laboratory for microbial culture during a one-year study period were incorporated into this research. We conducted antibiotic susceptibility testing, detected extended-spectrum beta-lactamase (ESBL) and metallo-beta-lactamase (MBL) presence in clinical isolates. Biofilm production was assessed using the Congo red agar method, Christenson's Test Tube method, and the Tissue culture plate method. Results: A total of 320 gram-negative isolates were identified in this study. The highest proportion consisted of Klebsiella pneumoniae (32.62%), followed by Escherichia coli (28.54%), Acinetobacter baumanii (16.41%), Pseudomonas aeruginosa (16.61%), and Citrobacter species (3.67%). The majority of the isolates exhibited resistance to ampicillin (93.47%), amoxiclave (86.46%), and ceftazidime (75%). Conclusion: There is a rising prevalence of multidrug-resistant bacteria that also form biofilms [4]. It is advisable to implement regular monitoring of multidrug resistance patterns and biofilm formation in clinical laboratories to provide guidance for appropriate antibiotic treatment.

I. INTRODUCTION

Biofilms consist of clusters of microorganisms enclosed within a matrix of extracellular polysaccharide, known as polysaccharide intercellular adhesion (PIA). They have been linked to various chronic and persistent infections. Biofilm formation represents an adaptive and protective growth strat- egy that allows bacteria to survive in hostile environments, such as the human host [5]. This growth mode also fa- cilitates their dispersal and colonization of new habitats, guided by chemical communication through quorum sensing. In many instances, chronic infections are accompanied by the development of biofilms. Over the past decade, therehas been a noticeable increase in bacteria acquiring the ability to form biofilms as a means of survival in challeng- ing environments where mechanical stress, desiccation, and exposure to biocides are prevalent threats. Bacteria capable of forming biofilms are frequently responsible for numerous nosocomial infections. According to several reports, more than 60% of infections acquired in hospitals are caused by organisms capable of producing biofilms. Biofilms are linked to various medical conditions, such as indwelling medical devices, catheters, urinary tract infections, dental plaque, upper respiratory tract infections, peritonitis, and urogenital infections. They

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often lead to chronic and per- sistent infections that are difficult to treat effectively. Both Gram-positive and Gram-negative bacteria possess the ability to create biofilms. Common bacterial species involved in this process include Enterococcus faecalis, Staphylococcus aureus, Staphylococcus epidermidis, Streptococcus viridans, Escherichia coli, Klebsiella pneumoniae, Proteus mirabilis, and Pseudomonas aeruginosa. Among these, Pseudomonas aeruginosa and Staphylococcus aureus are notable bacterial pathogens that have developed intricate mechanisms for eva- sion, counter-inhibition, and subjugation in their competition for space and nutrients. Organisms capable of produc- ing biofilms exhibit significantly higher antibiotic resistance compared to their planktonic counterparts, with resistance levels potentially increasing by up to a thousandfold [6]. This resistance primarily arises from the inability of antibiotics to penetrate the protective polysaccharide matrix that sur- rounds biofilms. Within the host, this matrix shields biofilm bacteria from innate immune defenses, such as opsonization and phagocytosis, as well as from antibiotic treatments [7].It is widely recognized that biofilms pose considerable chal- lenges in terms of eradication and often display resistance to systemic antibiotic therapies. In such cases, the removal of infected medical devices may become necessary. The present study aimed to identify antibiotic resistance patterns and assess the biofilm-forming ability of gram-negative clinical isolates.

II. MATERALS AND METHODS

This study is a prospective investigation conducted within a hospital setting, and it received approval from the Institu- tional Ethical Committee. During a one-year study period, we collected various clinical samples such as urine, pus, blood, sputum, and swabs (including wound, throat, vagi- nal, tracheal, endotracheal, or any device-related swabs) for microbial culture analysis. Every sample collection adhered strictly to established protocols and maintained stringent aseptic precautions. These samples underwent processing using standardized procedures for the isolation and identi- fication of pathogens. Antibiotic susceptibility testing was conducted using the Kirby-Bauer disc diffusion method on Muller Hilton Agar [8]. The isolated organisms were cat- egorized into multidrug-resistant (MDR) organisms based on their resistance profiles to various antibiotics. Addition- ally, we performed the detection of extended-spectrum beta-lactamases (ESBL) and metallo-beta-lactamases (MBL) in clinical isolates.For the phenotypic detection of ESBL, we followed the Clinical and Laboratory Standards Institute (CLSI) confirmatory method. Initially, ESBL production was assessed using Ceftazidime (30 μg) and Cefotaxime (30 μg) discs both individually and in combination with Clavulanic acid (10 μg) [9]. Interpretation: A positive result for extended-spectrum beta-lactamase (ESBL) production is indicated when there is a 5 mm or greater increase in the zone diameter for ceftazidime or cefotaxime when tested in combination with clavulanic acid compared to their respec- tive zones when tested alone. This increase suggests that the presence of clavulanic acid has effectively inhibited the ESBL activity, resulting in a larger zone of inhibition [10]. To detect metallo-beta-lactamases (MBL), various methods were employed, including the Imipenem-EDTA combined disc test, the Double disc synergy test using Imipenem and EDTA, and the EDTA disc potentiation using ceftazidime and cefepime. These techniques rely on the capacity of metal chelators like EDTA and thiol-based compounds to inhibit MBL activity. In the case of the phenotypic Imipenem- EDTA combined disc test (IMP EDTA CDT), the test or- ganism is first inoculated on a Mueller-Hinton Agar (MHA) plate. Subsequently, two discs are placed on the MHA plate: one containing 10 μg of imipenem and the other being an imipenem-EDTA combined disc with 10 μg of imipenem and 720 μg of EDTA. A positive result for MBL is confirmed when there is a significant increase of more than 7 mm in the inhibition zone surrounding the imipenem-EDTA disc in comparison to the zone around the imipenem disc alone. This method effectively identifies MBL-producing strains based on their susceptibility to imipenem in the presence of EDTA, which inhibits MBL activity. Biofilm production was evaluated through the utilization of three distinct meth- ods: the Congo red agar method, Christenson's Test Tube method, and the Tissue culture plate method. These diverse approaches collectively facilitated a thorough assessment of biofilm formation, each contributing valuable insights into the adhesive properties and biofilm-forming capacities of the microorganisms under investigation [11].

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- **Congo red method:** In the Congo red agar method, colonies were inoculated onto agar plates and then incu- bated at 36 degrees Celsius for a duration of 24 hours. Colonies that exhibited a black coloration and possessed a dry, metallic consistency were identified as positive indicators of slime production.
- **Test tube method:** colonies were inoculated into Brain Heart Infusion broth enriched with 1% sucrose. Fol- lowing an overnight incubation period at 36 degrees Celsius, the tubes were carefully decanted, washed three times with phosphate-buffered saline, and subsequently stained with 0.2% crystal violet. The presence of a discernible violet film adhering to the inner walls and bottom of the tube was regarded as a positive indicationof biofilm formation.
- **Tissue Culture Plate Method:** colonies obtained from fresh agar plates were first inoculated in a culture medium (Brain Heart Infusion broth with 1% sucrose) and incubated for 16 hours at 36°C. This culture was then diluted at a ratio of 1:100 using fresh medium. Next, 200 μl of this diluted broth was added to each well of a 96-well microtiter plate. The tissue culture plates were incubated for 16 and 24 hours at 36°C. Following incubation, the contents of each well were gently removed by tapping the plates, and the wells were washed four times with 0.2 mL of phosphate-buffered saline (PBS, pH 7.2) to eliminate any free-floating 'planktonic' bacteria. The biofilms formed by adherent 'sessile' organisms in the plates were then fixed using sodium acetate (2%) and stained with crystal violet (0.1% w/v). The plates were allowed to dry,and the optical density (OD) of the stained adherent bacteria was measured using a micro ELISA auto reader(Thermo LabSystems) at a wavelength of 450 nm (OD 450 nm). These OD values served as an index for quantifying bacteria adhering to surfaces and forming biofilms.

III. RESULTS

Throughout the study period, a total of 4,600 distinct clinical samples were received in the laboratory for aerobic bacte- rial culture and subsequent sensitivity testing. Among these, 320 non-repetitive gram-negative bacteria were selected and included in the study for further analysis and investigation.

Figure 1 presents the demographic profile of the samples in our study. The highest proportion of microorganisms was isolated from blood cultures, accounting for 28.35% of the total, followed closely by samples obtained from medical de-vices at 28.29%, and urine samples at 23.65%. The majority of the samples were collected from patients admitted to the hospital (90.42%), residing in rural areas (75.02%), and were from female patients (63.10%). Regarding age distribution, the largest proportion of samples came from individuals agedover 19 years, making up 37.17% of the total, followed by the age group under 1 month, representing 30.50%. Out of the 320 gram-negative bacterial (GNB) isolates included in the study, the most prevalent was Klebsiella pneumoniae, accounting for 32.62% of the total isolates, followed by Escherichia coli at 28.54%11. Other significant GNB isolates included Acinetobacter baumanii (16.41%), Pseudomonas aeruginosa (16.61%), and Citrobacter species (3.67%)

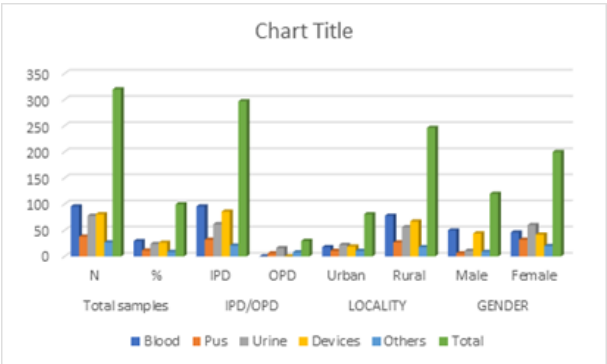

FIG 1: Showing demographic profile of samples

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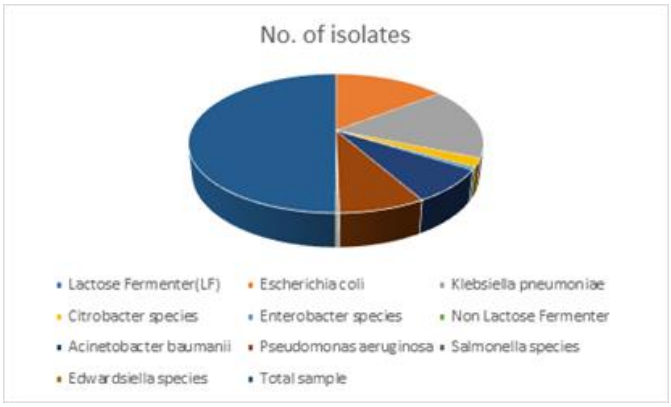

FIGURE 2: Distribution of Gram negative bacterial isolates

The antibiotic resistance patterns observed among the gram-negative bacterial (GNB) isolates. The majority of these isolates demonstrated high resistance rates, with the maximum resistance observed against ampicillin (93.07%), followed closely by amoxiclav (87.26%), ceftazidime (72%), and ciprofloxacin (73.25%). On the other hand, these GNB isolates exhibited sensitivity to meropenem (70.34%), piperacillin-tazobactam (66.20%), and cefepime (57.05%) [13], [14]. It is noteworthy that Klebsiella pneumoniae, Acinetobacter baumannii, and Pseudomonas aeruginosa were identified as multi-drug resistant, indicating their resistance to multiple classes of antibiotics, which is a concerning trendin healthcare settings.

IV. DISCUSSION

In our current study, we identified a total of 320 gram-negative bacterial (GNB) isolates, with a notable predom- inance of Klebsiella pneumoniae and Escherichia coli. In- terestingly, previous studies conducted by Dumaru et al. and Fatima et al [15]. have also reported Escherichia coli as the predominant bacterial isolate. It's worth noting that variations in bacterial prevalence across studies may be at- tributed to differences in geographical locations and the types of populations under investigation. Furthermore, our study revealed that the highest number of positive isolates were detected in blood cultures, accounting for 29.35% of the total, which aligns with observations made in studies conducted by Khanal et al. and Roy et al [16]. These findings suggest a consistent trend in the prevalence of gram-negative bacterial isolates in blood cultures across different research efforts. In our present study, we observed that Klebsiella pneumoniae was predominantly isolated from blood cultures, representing 40.66% of the total isolates. These findings are in agreement with several other studies conducted by Negussie A et al., Jyoti et al., Vanitha RN et al. [17], and Nidhi Pal et al. [18], which also reported Klebsiella pneumoniae as a major isolate from blood samples. This concordance in results suggests a consistent trend in the prevalence of Klebsiella pneumoniae in blood cultures across multiple research studies. Similarly,in our study, Escherichia coli was found to be the predom- inant isolate in urine samples, accounting for 80.66% of the isolates. This finding is consistent with the observations made by Alanazi MQ et al. and Isaac Odongo et al., who also re- ported a high prevalence of Escherichia coli in urine samples in their respective studies. These consistent findings indicate that Escherichia coli is frequently associated with urinary tract infections across different research studies [19]. In our present study, the highest proportion of biofilm producers was observed among isolates obtained from medical devices, accounting for 25.67% of the total, closely followed by blood samples at 23.25%. Conversely, the lowest occurrence of biofilm producers was noted in urine samples (80.06%) and other sample types (82.33%). Among the 327 gram-negative bacterial (GNB) isolates tested, biofilm production was identified in 64 isolates (19.87%) using the Tissue Cul- ture Plate (TCP) method, 38 isolates (11.62%) through the Congo Red Agar (CRA) method, and 23 isolates (7.03%) via the Tube method. These findings align with the results of a study conducted by Pragyan et al. [20], where they reported similar trends. In their study, out of 200 isolates, 45.6% produced biofilm using the TCP method, 39.3% were detected as biofilm producers by the Tube adherence method, and

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11% showed biofilm production using the Congo Red Agar method. Importantly, the Tissue Culture Plate (TCP) method was found to be the most sensitive in detecting biofilm production, followed by the Tube method (TM) and Congo Red Agar method (CRA), which is in agreement with the observations in our study. Indeed, Pseudomonas aeruginosa is notorious in healthcare settings as a hospital- acquired, drug-resistant pathogen. It is well-known for its propensity to cause chronic infections, largely due to its ability to form robust biofilms. These biofilms make Pseu-domonas aeruginosa particularly challenging to eradicate and contribute to its resistance against various antibiotics [21]. Similarly, Acinetobacter baumannii has emerged as a troublesome pathogen, especially in intensive care units (ICUs). In recent years, it has gained notoriety as a hospital superbug due to its propensity to develop multidrug-resistant (MDR) and pandrug-resistant (PDR) profiles. Acinetobacter baumannii possesses a remarkable capacity to acquire resis-tance determinant genes, including those encoding enzymes like extended-spectrum beta-lactamases (ESBL) and metallo-beta-lactamases (MBL) [22]. This ability to acquire such resistance determinants makes it exceptionally resistant to most higher-order antibiotics, posing a significant challenge to infection control and treatment efforts in healthcare set- tings.

V. CONCLUSION

The rising prevalence of multidrug-resistant and biofilm- forming organisms presents a concerning glimpse into the emergence of hospital superbugs in our current era. To ef- fectively address this growing threat, it is imperative to rec- ommend routine monitoring of multidrug resistance patterns and biofilm detection in clinical laboratories. Additionally, strict adherence to institutional antibiotic policies, coupled with the proper implementation and continuous monitoring of hospital infection control and prevention activities, is es- sential [23], [24]. These measures are critical for containing the spread of superbugs, safeguarding patient health, and pre-serving the effectiveness of antibiotics in healthcare settings.

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CONFLICTS OF INTEREST

The authors declared no conflict of interest.

AUTHORS' CONTRIBUTIONS

All authors equally contributed to preparing this article.

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