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# Comparative and Correlative Analysis of Biofilm Formation and Antimicrobial Resistance Traits towards Extended Spectrum β-Lactamase (ESBL) and Metallo-β-Lactamase (MBL) Producing Pathogenic Bacteria among the Clinical Isolates

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## Abstract

**Background: Pathogenic bacteria mostly cause emerging infectious diseases, including community-acquired, nosocomial, and opportunistic infections. The recent emergence of biofilms and the production of beta-lactamases strains have led to the widespread number of drug-resistant bacterial pathogens associated with infectious illness and death.**

**Materials and Methods: This study was carried out to detect the ability of biofilm formation by gram-negative and gram-positive pathogenic isolates collected from clinical environments and determine their multi-drug resistant traits along with the detection of Extended-spectrum beta-lactamases (ESBLs) and Metallo-beta-lactamases (MBLs) production.**

**Results: Among the 15 pathogenic isolates, *A. baumannii* (85%) were ESBL positive and (65%) MBL positive; *P. mirabilis* was ESBL and MBL positive (65% and 90%), respectively. The uropathogenic bacterial isolates both *K. pneumoniae* and *E. coli* were found to be ESBL positive (60%) and MBL positive (40%); ESBL positive (75%) and MBL positive (60%), respectively. Whereas the pathogenic *P. aeruginosa* isolates were found to be ESBL and MBL positive (100%). In contrast, among the three gram-positive *S. aureus* isolates, two isolates were found to be ESBL positive (85%), and one isolate was found to be MBL positive (35%). The association between ESBL and MBL production and biofilm formation was statistically significant (P value=0.001). Besides, most active drugs, penicillin G, kanamycin, ciprofloxacin, ceftazidime, azithromycin, and erythromycin, were resistant against all pathogenic isolates, whereas pathogens were significantly sensitive against Cotrimoxazole, Erythromycin, Streptomycin, and Ceftriaxone.**

**Conclusion: This experiment's findings indicate the emergence and rapid spread of such multidrug-resistant pathogens are of great concern. Early detection of ESBL and MBL-producing pathogens is of paramount clinical significance; therefore, strict infection control practices and therapeutic guidance for confirmed infection can be rapidly initiated.**

Keywords: **Antimicrobial resistance • Biofilm formation** • Drug resistance • Extended-Spectrum β- lactamase (ESBL)

## Introduction

Gram-negative bacilli and Gram-positive cocci have emerged to be an important cause of common causative infectious diseases. Extracellular matrices of polysaccharides, proteins, enzymes, and nucleic acids are firmly aggregated with bacteria, forming biofilm anchorage to any surfaces irreversibly [1]. Though attachment is a complex process regulated by diverse characteristics of the growth medium, substratum, and cell surface; however, microorganisms eventually attach to surfaces and develop biofilms. Antibiotic resistance confers by this extracellular matrix through the processes of chromosomally encoded resistant gene expression, reduction of survival and growth rate, restriction of antibiotics, and even counteracting the host immunity. Multi-drug resistant strains of gram-negative bacilli are synergistically increased by the biofilm formation and beta-lactamases production of bacterial isolates. They are highly responsible for implicating chronicity, persistence, and relapse of infections leading to high morbidity and mortality; thus, posing a severe health crisis. Before detecting dissemination by beta-lactamases (ESBL and MBL) producing strains, beta-lactams were the most promising drugs to reduce bacterial infections by inhibiting their growth. "Extended-spectrum beta-lactamases (ESBLs) may be defined as plasmid-mediated enzymes that hydrolyze oxyimino-cephalosporins (ceftriaxone, cefotaxime, and ceftazidime) and monobactams (aztreonam) but not cephamycins or carbapenems. They are inhibited in vitro by clavulanate". EDTA is a metal chelating agent that inhibits carbapenem hydrolyzing enzymes like Metallo-beta-lactamases (MBLs). Antibiotic resistance is one of the major health issues over the world, which is globally recognized as a term of drug-resistant that is induced by pathogens. Overuse and abuse of antibiotics are the most important driving forces in developing antibiotic resistance by pathogenic bacteria. More than 100 000 tons of antibiotics are used by culture and clinical industries. Industrial aquaculture is the fastest growing industry worldwide, and China is the largest aquaculture producer. Thus, the urgency of developing a new therapeutic strategy for controlling aquatic infectious diseases, whereas antibiotics play a significant role in inhibiting microbial growth. A group of pathogenic bacteria commonly known as ESKAPE has drawn special attention by producing biofilm over the last two decades. Recently researchers identified several nosocomial infections mainly caused by *P. aeruginosa* and *S. aureus*, which are already shown resistant to third-generation antibiotics. Even so, the ability of biofilm formation and their resistance to antibiotics are also reported earlier. Additionally, scientists observed multidrug-resistant *Escherichia coli* from non-clinical sources. Because of the genetic flexibility and adaptability of *E. coli*, they can develop AR mechanisms by changing their environments. Therefore, the correlation between biofilm formation and antibiotic resistance needs to be further studied for gram-positive and gram-negative bacteria from various samples, including clinical and non-clinical sources. MDR bacteria's emergence is a growing concern for public and animal health day by day, particularly in Bangladesh due to antibiotics' frequent uses. Moreover, nearly 90% of veterinary medicines commonly used in animal farming are excreted from the animal faeces. These animal wastes are further used as fertilizer in agriculture, containing antimicrobial residues and resistant bacteria. For instance, poultry wastes are directly used in fish culture, and crop production in Bangladesh, and approximately 50% and 10% of total poultry wastes are used, respectively. The prevalence of MDR bacteria in raw milk and associated public health problems are well documented as well. Besides, transforming pathogens can emerge the public health threats or transforming their resistance genes to other bacterial genera, and the human also a severe threat for health issues. This study aimed to quantitatively evaluate biofilm formation among

bacterial species isolated from two hospitals' clinical sample. In addition, the susceptibilities of gram-positive and gram-negative isolates to antimicrobials were assessed by examining the effect of different antimicrobials on pathogenic bacteria. This study also carried out the comparative and correlative analysis of biofilm formation and antibiotic resistance traits among the isolates. It investigated their ESBL and MBL producing ability along with the synthesis of biofilm in vitro.

## Materials and Methods

### Sample Collection and Isolation of Bacterial Strains

The Department of Microbiology conducted this cross-sectional study at Stamford University of Bangladesh, and all experiments were carried out in the aforementioned department (January–March 2020). We collected fifteen non-repetitive gram-negative and gram-positive bacterial pathogens (*Acinetobacter spp., Proteus spp., Klebsiella spp., Escherichia coli*, *Pseudomonas spp*., and *Staphylococcus spp.,* respectively) from different clinical specimens, including blood, urine, pus, CSF, endotracheal tube, tracheal aspirate, fluids, lesion swab, genital swab, catheter tips, and sputum. The samples were transferred to the microbiology lab for routine culture and sensitivity testing after collecting from various inpatients wards and outpatient departments of Dhaka Medical College Hospital and Bangabandhu Sheikh Mujib Medical University (PG Hospital).

### Ethical Issue

Permission was taken from the administrative authority of Dhaka Medical College Hospital, Bangladesh, and Bangabandhu Sheikh Mujib Medical University Hospital (PG Hospital) Bangladesh. Informed written consent from the patient or legal guardian was taken prior to collecting samples. The DMC and PG Hospital ethics committee specially approved this study.

### Morphological, Cultural, and Biochemical Tests for the Identification of the Isolates

Nutrient agar plates were used for subculturing bacterial isolates and collected pure colonies, which were further inoculated on Mac-Conkey agar (MAC, TM Media, India), Eosin Methylene Blue Agar (EMB, HiMedia, India), Cetrimide Agar (CEA, HiMedia, India), and Mannitol Salt Agar (MSA, Scharlau, Spain) media. Standard microbiological procedures were used to characterize the isolates. Specially colony morphology on different culture media, Gram staining, pigmentation, odour, microscopic analysis for differentiating into gram-positive and gram-negative genera, and a series of biochemical tests (TSI, MR-VP, catalase test, oxidase reaction, carbohydrate fermentation, indole production, and motility test) ware performed.

### Biofilm Biomass Assay

Congo red agar (CRA), tube method ™, and microtiter-Plate method were used to detect the biofilm formation ability of isolates. The statistical analysis of CRA and TM was done using TCP as the reference method.

### Congo red Agar Method

A combined mixer of brain heart infusion broth, sucrose, congo red dye, and agar (HiMedia) in 1 L distilled water were used to prepare congo red agar media. The isolates were plated on CRA medium and incubated aerobically at 37 ºC for 24 h. The black-coloured colony's observation was considered as biofilm positive and red-coloured colony as negative. Tube Adherence Method (quantitative biofilm production assay) Biofilm production was detected by a quantitative assay proposed by researchers in previous studies. Briefly, a loopful of the isolate was inoculated into a glass tube containing 5 mL of Tryptic Soy Broth (TSB, HiMedia, India) and incubated at 37ºC for 48 hours. The tubes were decanted and dried after incubation and stained with 0.1% crystal violet (CV). Subsequently, the tubes were gently washed and placed upside down for drying. The visible lining of the wall and bottom of the tube by a film was considered as positive. Further, the optical density (OD) of the stained adherent bacterial biomass was measured at 595 nm (OD595) with a UV-VIS spectrophotometer (Shimadzu, Australia). Sterile TSB tubes were used as a negative control.

### Microtiter-Plate Method (quantitative biofilm production assay)

Researcher previously used 96-well microtiter plates to monitor the formation of biofilm of bacterial iso. Generally, after growing in TSB at 37°C for 36 hours, nonadherent bacterial cells were removed by an aspirator. However, the adherent cells were stained with 0.1% CV solution for 50 minutes, after which the CV solution was removed. Furthermore, the excess stain was rinsed off by dH2 O two times through gentle shaking for 20 minutes. Biofilm production was quantitatively measured at OD595 with Tecan GENios Microplate Reader (Tecan Group Ltd., Switzerland), and the wells containing TSB broth only served as the negative control.Moreover, the mean OD595 of the negative control was subtracted from the mean OD595 of inoculated wells to correct CV's background staining. The adherence ability was classified based on cut-off absorbance value, according to the previous study [2]. Finally, a microscopic portion of the adhered biofilms was visualized in bright field microscopy as well.

### Antibiotic Resistance and Multiple Antibiotic Resistance Value Index

Antibiotic susceptibility and resistance patterns towards fifteen clinically relevant antibiotics (included in fife classes) were examined by the Kirby-Bauer method according to the standard of Clinical and Laboratory Standards Institute [3]. The following antibiotic discs (HiMedia, India) were used. Penicillin G (P, 10 µg), tetracycline (TE, 30 µg), cotrimoxazole (COT, 25 µg), erythromycin (E, 15 µg), kanamycin (K, 30 µg), streptomycin (S, 10 µg), ciprofloxacin (CIP, 5 µg), ceftazidime (CAZ, 30 µg), azithromycin (AZM, 30 µg), nalidixic acid (NA, 30 µg), colistin (CL, 10 µg), polymyxin B (PB, 300 µg), doxycycline (DO, 30 µg), amoxicillin (AMX, 30 µg), cefotaxime-clavulanic acid (CTC; 40 µg) and ceftriaxone (CTR, 30 µg). Multi-drug resistance was defined as the acquired non-susceptibility to at least one agent in 3 or more antimicrobial categories [4]. The MAR index was calculated as the ratio of the number of antibiotics to which the isolate displayed resistance to the number of antibiotics to which the isolate was evaluated for susceptibility.

### Minimum inhibitory concentration (MIC)

Resistance to kanamycin and doxycycline in *S. aureus* and kanamycin-resistant *E. coli* and *K. pneumoniae* were confirmed by calculating the MIC of the antibiotics using the broth dilution method (Clinical and Laboratory Standards Institute, 2015). On the other hand, nalidixic acid resistance in *A. baumannii*, *P. mirabilis*, and *P. aeruginosa* were confirmed by calculating the MIC of the antibiotics using the broth dilution method. S. aureus isolates with MIC to kanamycin ≤2 µg/mL was considered susceptible, 4–8 µg/mL intermediate and ≥16 µg/mL resistant. For doxycycline-resistant *Staphylococcus aureus*, MIC to nalidixic acid ≤2 µg/mL was considered susceptible and ≥ 4 µg/mL considered resistant. For E. faecalis, MIC to doxycycline ≤4 µg/mL was considered susceptible, 8–16 µg/mL intermediate, and ≥32 µg/ mL resistant Clinical and Phenotypic identification of High Biofilm Forming Multidrug-Resistant Isolates with Comparison and association between ESBL and MBL producer. Standard biochemical and molecular techniques were used to identify and characterize biofilm formation and bacterial isolates' antibiotic resistance traits. Morphological and biochemical characteristics were evaluated following standard methods described in Benson’s Microbiological Applications Lab Manual for tentative identification Benson. Drug-resistant strains screened ESBL and MBL production by using the disc diffusion test. To increase in the zone of the diameter of ≥5-mm was considered ESBL positive. The multidrug-resistant strains were tested for MBL production by combined disc diffusion assay using the discs, one with added 10 µl of 0.5 EDTA. The increased zone of inhibition of >7 mm around the EDTA disc compared to the antibiotic disc's zone size was confirmed positive for MBL production.

## Results

### Morphological Characteristics

The morphological Characteristic has been identified under the microscope. Acinetobacter baumannii has the 0.9 to 1.6 and 1.5 to 2.5 micrometres size, pleomorphic aerobic bacillus. With the presence of polar fimbriae, it was twitching motility exhibit by their cells. India ink wet mounts are used to see encapsulated strains. Proteus mirabilis from the Enterobacteriaceae family occurs as normal intestinal flora of humans that widely distributed in nature. It has pili (fimbriae), which are associated with adhesive properties that may correlate with virulence. Also, their heterogeneity has been involved in horizontal gene transformation. The morphological structure of K. pneumoniae is singly and in pairs or short chains. The microscopic size of K. pneumoniae is 0.3 -1.0 micrcomputers to 0.6-6.0 micrometres. It has a non-motile capsular form. *E. coli* is the 1-3 x 0.4-0.7 µm in size and 0.6 to 0.7 µm in volume; and arranged singly or in pairs. It is motile due to peritrichous flagella. Being a motile organism (polar flagella), *Pseudomonas aeruginosa* characteristically produces water-soluble pigments that diffuse through the medium. All these bacterial species have rod-shaped and morphologically proved that all these are gram-negative. Conversely, *S. aureus'* microscopic view appears as grape-like clusters though it is characteristically facultatively anaerobic, Gram-positive coccus. The microscopic images are presented in figure 1.

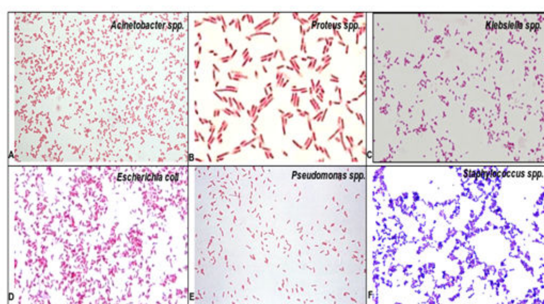


**Figure1:** Microscopic images of st of bacterial species including (A) *Acinetobacter baumannii*, (B) *Proteus mirabilis*, (C) *K. pneumoniae*, (D) *E. coli*, (E) *Pseudomonas aeruginosa*, and (F) *S. aureus* respectively.

### Cultural Characteristics

The *Acinetobacter baumannii* isolates produced 1 to 2 mm size colonies on Mac-Conkey agar. Colonies are non-pigmented, domed, and mucoid with smooth to pitted surfaces. These isolates can't be able to reduce nitrate or to grow anaerobically. *Proteus mirabilis* is non-fastidious bacteria with pale yellow colonies (NLF), moderate in size, moist, and low convex on Mac-Conkey agar. It also produced a fishy smell. *E. coli* isolates' colony characteristics appear to produced pink, round, entire, convex, and opaque colonies on MacConkey agar. In contrast, colonies with a green metallic sheen on EMB agar. On the other hand, *Klebsiella spp*. grew on EMB agar, but their colonies did not produce a green metallic sheen. However, *K. pneumoniae* colonies were dark pink, mucoid, and convex. Besides, P. aeruginosa isolates were produced "grape-like" or "fresh-tortilla" on Cetrimide agar media. It can be isolated in mixed cultures as clear colonies on MacConkey agar (as it does not ferment lactose), which will test positive for oxidase. Conversely, the *S. aureus* colonies on Mannitol Salt agar media are round, smooth, raised, and glistening. Colonies are circular, 2–3 mm in diameter, with a smooth, shiny surface; colonies also appear opaque and are often pigmented golden yellow. S. aureus usually forms grey to deep golden yellow colonies on Mannitol Salt agar media, often with hemolysis, when grown on blood agar plates (Figure 2).

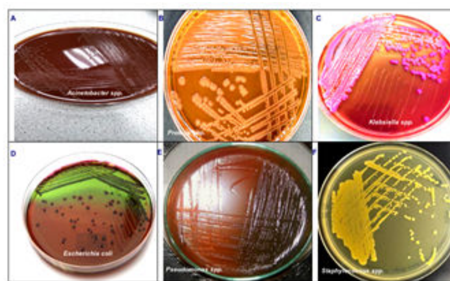

**Figure2:** Different culture media are used to grow *Acinetobacter spp*., *Proteus spp*., *Klebsiella spp., Escherichia coli*, *Pseudomonas spp*., and *Staphylococcus spp*. Images of *Acinetobacter spp*., *Proteus spp*., *Klebsiella spp*., *Escherichia coli*, *Pseudomonas spp*., and *Staphylococcus spp*., growing on (A) MacConkey agar and (B) Mac-Conkey agar (C) Mac-Conkey agar (D) EMB agar (E) Cetrimide agar and (F) Mannitol Salt agar respectively.

### Biochemical Characteristics

All these pathogenic isolates were biochemically characterized by a series of biochemical tests presented in Table 1.

| Isolate | TSI test | | | | MR-VP | MIU | Identified Organisms | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Slant | Butt | Gas | H2S | Citrate Utilization | MR | | VP | Motility | Indole | Urease | Catalase | Oxidase |
| AB 01 | A | A | - | - | + | - | - | - | - | - | ++ | - | *Acinetobacter baumannii* |
| AB 02 | A | A | - | - | + | - | - | - | - | - | ++ | - | *Acinetobacter baumannii* |
| AB 03 | A | A | - | - | ++ | - | - | - | - | - | + | - | *Acinetobacter baumannii* |
| PM 01 | A | A | + | + | + | + | - | ++ | + | - | + | - | *Proteus mirabilis* |

| | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| PM 02 | A | A | + | + | + | ++ | - | + | + | - | + | - | *Proteus mirabilis* |
| KP 01 | A | A | + | - | ++ | - | + | - | - | + | + | - | *K. pneumoniae* |
| KP 02 | A | A | + | - | + | - | + | - | - | + | ++ | - | *K. pneumoniae* |
| EC 01 | A | A | + | - | - | + | - | ++ | + | - | + | - | *E. coli* |
| EC 02 | A | A | + | - | - | + | - | + | + | - | + | - | *E. coli* |
| EC 03 | A | A | + | - | - | + | - | ++ | + | - | + | - | *E. coli* |
| PA 01 | A | A | - | - | + | - | - | + | - | - | + | + | *Pseudomonas aeruginosa* |
| PA 02 | A | A | - | - | + | - | - | + | - | - | ++ | ++ | *Pseudomonas aeruginosa* |
| SA 01 | A | A | - | - | + | + | + | - | - | + | ++ | - | *Staphylococcus aureus* |
| SA 02 | A | A | - | - | + | + | + | - | - | + | + | - | *Staphylococcus aureus* |
| SA 03 | A | A | - | - | ++ | ++ | + | - | - | + | ++ | - | *Staphylococcus aureus* |

**Table1:** Biochemically characterized of pathogenic isolates.

## The growth of bacterial isolates on (CRA) Congo red agar media

In this experiment, pathogenic bacterial isolates were inoculated on CRA plates to determine slime-producing ability in vitro. All these pathogenic isolates were produced gummy, black colored colony on CRA media with slime or extracellular matrix of biofilm (Figure 3).

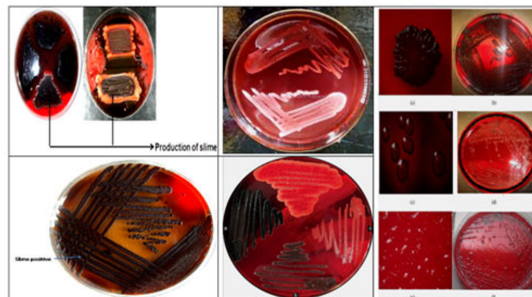


**Figure3:** Extracellular polysaccharide (Slime) formation by a) Acinetobacter baumannii; b) Proteus mirabilis; c) *K. pneumoniae*, d) *E. coli*; e) *Pseudomonas aeruginosa*; f) *S. aureus* on Congo Red Agar medium.

## Tube Adherence Method (quantitative biofilm production assay)

Among the 15 bacterial isolates, four isolates were weak biofilm formers, three isolates were moderate biofilters, and eight isolates produced biofilms with high power. All the pathogenic isolates were able to form biofilm in the tube adherence method that had been indicated by the measurement of absorbance at 560 nm (Figure 4 and figure 6).

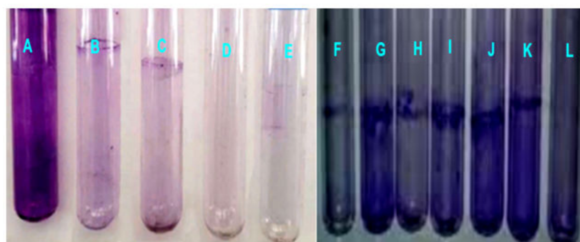


**Figure4**: Biofilm formation of pathogenic bacterial isolates were investigated by the tube adherence test. The figure is presenting Strong-biofilm-producing isolate (A, G, J, K), Weak-biofilm-producing isolate (B, C, H, I, L), Moderate-biofilm-producing isolate (D, F), and Negative control (E).

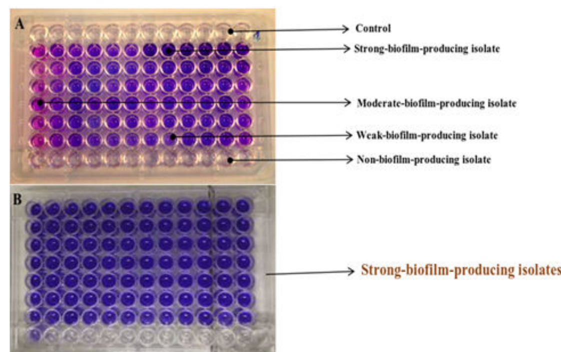


**Figure5:** Positive with strong, moderate, weak, and non-biofilm producing pathogenic isolates are indicating by the 96-well microtiter plate method (A & B). ESBL-producing pathogenic bacterial isolates were indicated by deep blue color portions (B), whereas on-ESBL-producing pathogenic bacterial isolates were indicated by neutral colors like white.

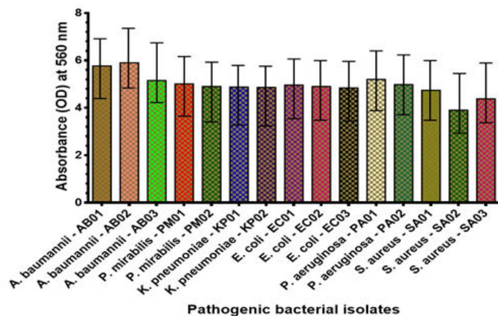


**Figure6:** Biofilm formation assay with the absorbance of OD in a 96-well microtiter plate.

## Biofilm Formation by Pathogenic Bacterial Isolates in 96-Well Microtiter Plate

The 96-well microtiter plate method was used to investigate the biofilm formation of pathogenic bacteria. All the isolates were monitored by comparing with the negative control wells, forming the biofilm (Figure 5). Further, these biofilm-forming isolates were monitored by measuring OD absorbance at 560 nm (Figure 6).All the pathogenic isolates were able to form a biofilm in the 96-well microtiter plate that had been indicated by the measurement of absorbance at 560 nm. The absorbance of gram-negative bacterial isolates including; *A. baumannii* - AB01, A. *baumannii* - AB02, *A. baumannii* - AB03, *P. mirabilis* - PM01*, P. mirabilis* - PM02, *K. pneumoniae* - KP01, *K. pneumoniae* - KP02, *E. coli* - EC01, *E. coli* - EC02, *E. coli* - EC03, *P. aeruginosa* - PA01, *P. aeruginosa* - PA02 were 5.77, 5.9, 5.15, 5.01, 4.9, 4.88, 4.86, 4.96, 4.9, 4.84, 5.2 and 4.98 nm respectively; whereas the absorbance of gram-positive bacterial isolates including *S. aureus* - SA01, *S. aureus* - SA02 and *S. aureus* - SA03 were 4.74, 3.9 and 4.38 nm respectively.

## Multi-Drug Resistant Traits of Pathogenic Isolates

The *Acinetobacter spp.*, *Proteus spp.*, *Klebsiella spp., Escherichia coli, Pseudomonas spp.,* and *Staphylococcus spp.* Isolates were tested for antimicrobial resistance and susceptibility against common active antibiotics by the disc diffusion method. Isolates did not produce any inhibition zone around the antibiotic discs were assumed to be resistant to those antibiotics. In contrast, those isolates produced clear zones of inhibition around the antibiotic discs and were considered susceptible to those antibiotics (Figure 7 and Table 2).

| **Antibiogram profile** | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | **A. baumannii** | | | **P. mirabilis** | | **K. Pneumoniae** | | **E. coli** | | | **P. aeruginosa** | | **S. aureus** | | |
| Antibiotics | AB01 | AB02 | AB03 | PM01 | PM02 | KP01 | KP02 | EC01 | EC02 | EC03 | PA01 | PA02 | SA01 | SA02 | SA03 |
| Penicillin G | R | R | R | R | R | S | S | R | R | R | R | R | S | S | S |
| Tetracycline | S | R | S | S | S | S | I | S | S | I | I | R | S | S | I |
| Cotrimoxazole | S | R | I | S | S | S | S | S | S | S | S | S | S | S | S |
| Erythromycin | S | S | S | R | R | R | R | S | S | S | R | R | S | S | S |
| Kanamycin | R | R | R | R | R | S | S | S | S | S | S | S | I | R | R |
| Streptomycin | S | S | I | R | R | S | R | R | R | I | R | R | S | S | S |
| Ciprofloxacin | S | S | I | R | S | R | R | I | I | S | I | S | R | I | I |
| Ceftazidime | S | S | R | S | R | R | R | R | R | R | R | R | R | R | R |
| Azithromycin | S | R | R | R | R | R | R | S | S | S | R | R | R | R | R |
| Nalidixic acid | I | R | R | R | I | I | R | S | S | I | S | S | S | S | S |
| Colistin | S | R | R | R | R | R | I | S | R | R | S | S | I | R | R |
| Polymyxin B | S | S | R | I | S | R | R | R | R | R | S | S | S | S | I |

| | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Doxycycline | I | R | R | R | R | S | I | S | S | R | S | S | I | S | I |
| Amoxicillin | S | R | I | S | I | S | S | S | S | S | S | I | S | I | I |
| Cefotaxime-clavulanic acid | S | S | S | S | S | S | S | S | S | S | S | S | S | S | S |
| Ceftriaxone | S | I | I | S | I | S | I | S | S | I | S | I | S | S | R |

**Table2:** Antibiogram profiling of pathogenic isolates.

The multi-drug resistant traits obtained in this experiment showed that the most active drugs Erythromycin, Streptomycin, Nalidixic acid, and Azithromycin to which all the isolates were resistant to followed by Penicillin G (100% resistant), Kanamycin (100% resistant), Ciprofloxacin (45% resistant), Ceftazidime (55% resistant), Azithromycin (60% resistant), Nalidixic acid, Colistin and Doxycycline (75% resistant) and Amoxicillin (65% resistant). All the isolates showed 100 % sensitivity to Cotrimoxazole and Erythromycin. Next to Streptomycin, Ciprofloxacin, Polymyxin B, Cefotaxime-clavulanic acid, Ceftriaxone, and Ceftriaxone (90%, 55%, 55%, 50%, and 85% sensitive) respectively (Figure 8).

## Minimum Inhibitory Concentration (MIC)

The minimum inhibitory concentration was measured by the disc diffusion method with ug/mL scale. MIC rate of *A. baumannii*, *P. mirabilis*, *K. pneumoniae*, *E. coli*, *P. aeruginosa* and *S. aureus* strains were susceptible to resistance kanamycin, doxycycline, and nalidixic acid. The minimal rate of concentration was graphically presented in figure 9.

## Identification of High Biofilm Forming Multidrug-Resistant Isolates

The isolates were further subjected to phenotypic determination of ESBL and MBL production using antibiotic discs containing third-generation cephalosporins with and without clavulanic acid. Clavulanic acid is a penicillin-type antibiotic used to treat a wide variety of bacterial infections. Combining other third-generation antibiotics and clavulanic acid is used to treat certain infections caused by bacteria, including urinary tract infections, wound infections, intestinal infection, etc. It works by preventing pathogenic bacteria from destroying amoxicillin, kanamycin, cephalosporin, and other third-line drugs. Among the three A. baumannii isolates, all are found to be ESBL positive (85%), and two isolates were MBL positive (65%); both P. mirabilis were ESBL and MBL positive (65% and 90%), respectively. Besides, uropathogenic bacterial isolates both *K. pneumoniae* and *E. coli* were found to be ESBL positive (60%) and MBL positive (40%), and ESBL positive (75%) and MBL positive (60%). Whereas the pathogenic P. aeruginosa isolates were found to be ESBL and MBL positive (100%). In contrast, among the three gram-negative S. aureus isolates, two isolates were found to be ESBL positive (85%); and one isolate was found to be MBL positive (35%) (Figure 10). The ESBL and MBL positive isolates were also multidrug-resistant (Figure 11 A, B, C, D, E, F). All the isolates were showed variable degrees of resistance against commonly used antibiotics. Isolates of Acinetobacter baumannii (A) and Proteus mirabilis (B) were showed 100 % resistance against penicillin G (P, 10 µg) and kanamycin (K, 30 µg). *Uropathogens K.* pneumoniae (C) isolates were 100 % resistant against ciprofloxacin (CIP, 5 µg), ceftazidime (CAZ, 30 µg) and azithromycin (AZM, 30 µg); and *E. coli* (D) were 100 % resistant against penicillin G (P, 10 µg) and ceftazidime (CAZ, 30 µg). Pseudomonas aeruginosa (E) isoltes were 100% resistant against penicillin G (P, 10 µg), erythromycin (E, 15 µg), ceftazidime (CAZ, 30 µg) and azithromycin (AZM, 30 µg). And surprisingly, gram-positive pathogenic isolates Staphylococcus aureus (F) were showed 100% resistance against ceftazidime (CAZ, 30 µg) and azithromycin (AZM, 30 µg). Also, noticeably streptomycin (S, 10 µg) were showed 80% and 95% resistance to Pseudomonas aeruginosa and Proteus mirabilis, respectively. 90% resistance of erythromycin (E, 15 µg) was also examined to Proteus mirabilis. 90% resistance of doxycycline (DO, 30 µg) showed against Proteus mirabilis. 80% resistance of Polymyxin B (PB, 300 µg) was observed to both *K. pneumoniae* and *E. coli.* On the other hand, antibiotic susceptibility tests also performed in this experiment, and patterns were also shown in the (Figure 10). *Acinetobacter baumannii* was sensitive against tetracycline (TE, 30 µg), erythromycin (E, 15 µg), streptomycin (S, 10 µg), and cefotaxime-clavulanic acid (CTC; 40 µg). Proteus mirabilis was sensitive against tetracycline (TE, 30 µg), erythromycin (E, 15 µg), amoxicillin (AMX, 30 µg), and cefotaxime-clavulanic acid (CTC; 40 µg). *K. pneumoniae* was sensitive against Penicillin G (P, 10 µg), cotrimoxazole (COT, 25 µg), kanamycin (K, 30 µg), amoxicillin (AMX, 30 µg), and cefotaxime-clavulanic acid (CTC; 40 µg). *E. coli* was sensitive against cotrimoxazole (COT, 25 µg), erythromycin (E, 15 µg), kanamycin (K, 30 µg), azithromycin (AZM, 30 µg), amoxicillin (AMX, 30 µg) and cefotaxime-clavulanic acid (CTC; 40 µg). Pseudomonas aeruginosa was sensitive against cotrimoxazole (COT, 25 µg), kanamycin (K, 30 µg), nalidixic acid (NA, 30 µg), colistin (CL, 10 µg), polymyxin B (PB, 300 µg), doxycycline (DO, 30 µg) and cefotaxime-clavulanic acid (CTC; 40 µg). And gram-positive bacterial isolate Staphylococcus aureus was sensitive against Penicillin G (P, 10 µg), tetracycline (TE, 30 µg), cotrimoxazole (COT, 25 µg), erythromycin (E, 15 µg), streptomycin (S, 10 µg), nalidixic acid (NA, 30 µg), cefotaxime-clavulanic acid (CTC; 40 µg) and ceftriaxone (CTR, 30 µg). The presented data were statistically analyzed by showing standard errors considered as 5%. All experiments were carried out three times, and 95% accuracy was found.

## Comparison and Association between ESBL And MBL Producing Isolates

There were a significant comparison and association of ESBL and MBl production as well as biofilm formation to antibiotic resistance. For all antibiotics, the comparison and association between ESBL, MBL production, and antibiotic resistance were noted to be statistically significant (P-value=0.001) (Table 3).

| Antibiotics | ESBL | MBL | Biofilm | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | Resistance (%) | | P-value | Resistance (%) | | P-value | Resistance (%) | | P-value |
| | Producers (%) | Non-producers (%) | | Producers (%) | Non-producers (%) | | Formers (%) | Non-formers (%) | |
| Penicillin G | 100 | 47.82 | 0.001 | 96.08 | 55.13 | 0.001 | 66.5 | 53.85 | 0.026 |

| | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| Tetracycline | 26.19 | 35.22 | 0.132 | 100 | 19.77 | 0.001 | 39.09 | 22.22 | 0.002 |
| Cotrimoxazole | 69.04 | 46.52 | 0.013 | 94.12 | 37.64 | 0.001 | 54.31 | 34.19 | 0.001 |
| Erythromycin | 66.67 | 34.38 | 0.126 | 80 | 25 | 0.001 | 42.86 | 30 | 0.267 |
| Kanamycin | 72.62 | 45.65 | 0.001 | 90.19 | 45.62 | 0.001 | 57.87 | 44.44 | 0.021 |
| Streptomycin | 47.82 | 100 | 96.08 | 0.001 | 55.13 | 0.001 | 53.85 | 66.5 | 0.026 |
| Ciprofloxacin | 58.33 | 42.6 | 0.013 | 94.12 | 37.64 | 0.001 | 54.31 | 34.19 | 0.001 |
| Ceftazidime | 46.52 | 69.04 | 0.013 | 94.12 | 37.64 | 0.001 | 54.31 | 34.19 | 0.001 |
| Azithromycin | 41.67 | 35.21 | 0.295 | 82.35 | 28.13 | 0.001 | 42.64 | 27.35 | 0.007 |
| Nalidixic acid | 16.67 | 9.38 | 0.241 | 20 | 7.14 | 0.079 | 10.71 | 10 | 0.33 |
| Colistin | 41.67 | 35.21 | 0.295 | 82.35 | 28.13 | 0.001 | 42.64 | 27.35 | 0.007 |
| Polymyxin B | 34.38 | 66.67 | 0.126 | 25 | 80 | 0.001 | 42.86 | 30 | 0.267 |
| Doxycycline | 0 | 0 | | 0 | 0 | 0 | 0 | 0 | 0 |
| Amoxicillin | 84.52 | 43.48 | 0.001 | 94.12 | 46.77 | 0.001 | 59.39 | 46.15 | 0.023 |
| Cefotaxime-clavulanic acid | 22.61 | 30.43 | 0.174 | 84.31 | 17.49 | 0.001 | 34.52 | 17.95 | 0.002 |
| Ceftriaxone | 35.71 | 27.39 | 0.153 | 66.67 | 22.43 | 0.001 | 32.49 | 24.79 | 0.148 |

**Table3:** Showing association between antibiotics resistance, ESBL production, MBL production, and biofilm production.

## Quality Control

*Acinetobacter baumannii* ATCC 17978, Proteus mirabilis ATCC 7002, *K. pneumoniae* ATCC 13884, *E. coli* ATCC 25922, *Pseudomonas aeruginosa* ATCC 27853, and *Staphylococcus aureus* ATCC 6538P were used for quality control of the biochemical tests, MIC, and antimicrobial discs. For biofilm formation, *Escherichia coli* ATCC 25922 was used as a positive control, and *E. coli* ATCC 35218 was used as a negative control.

## Statistical Analysis

All experiments were performed in triplicates, and the obtained data were presented as mean ± standard deviation (SD) where appropriate. Pearson's correlation analysis was used to estimate the relationship between biofilm formation and antibiotic resistance of pathogenic isolates. Also, intergroup differences were calculated by a standard technique such as a one-way analysis of variance (ANOVA). Microsoft ExcelTM2013 was used to perform the statistical analysis and considered their significant when the P-value was less than 0.01, 0.05, and 0.001. Graphical illustrations were prepared by using Origin Pro, version 8.0 (OriginLab Corporation, USA) as well.

## Discussion

Biofilm production by pathogenic *Acinetobacter baumannii*, *Proteus mirabilis, K. pneumoniae, E. coli*, *Pseudomonas aeruginosa*, and *Staphylococcus aureus* may play an important role in the nosocomial infection with various kind of emerging disease. Besides, biofilm has the potency of colonization by ESBL and MBL production and could be a rapidly contributing multi-drug resistance factor worldwide. In this current study, the fifteen different types of gram-negative and gram-positive pathogenic bacterial strains were collected from various clinical samples in different hospital environments. All the bacterial strains were able to produce biofilm; notably, gram-negative isolates*; A. baumannii* - AB02, *P. mirabilis* - PM01, and *P. mirabilis* - PM01 were higher biofilm producer *in-vitro*, whereas the gram-negative strain isolate *S. aureus* - SA01 was able to produce high biofilm by an *in-vitro* process such as 96-well microtiter plate method. The spread of multidrug-resistant bacterial infections and emerging diseases mainly caused by the incongruous use of antibiotics. Antibiotics are usually used for treating infectious diseases, e.g., UTI, respiratory tract infection, gastrointestinal infections, etc but misuses of antibiotics have raised the emergence of diseases as death rates over the last two decades. The efficacy of many antibacterial agents has been confined to reduce significant therapeutic properties. This experiment was undertaken to investigate the Extended-Spectrum β-Lactamase (ESBL) and *Metallo-β-Lactamase* (MBL), producing pathogenic strains and antibiotic-resistant traits of biofilm-forming pathogens isolated from clinical samples. Fifteen pathogenic bacterial isolates, including Gram-negative and Gram-positive pathogenic, were introduced in this study. Among the all tested isolates, *A. baumannii* - AB01*, A. baumannii* - AB02, *A. baumannii* - AB03, *P. mirabilis* - PM01, *P. mirabilis* - PM02*, K. pneumoniae* - KP01, *K. pneumoniae* - KP02, *E. coli* - EC01, *E. coli* - EC02, *E. coli* - EC03, *P. aeruginosa* - PA01, *P. aeruginosa* - PA02, *S. aureus* - SA01, *S. aureus* - SA02 and *S. aureus* - SA03 were showed resistance to several numbers of antibiotics, and most of them are cephalosporins. Some effective antibiotics (Cotrimoxazole, Erythromycin, Streptomycin, and Ceftriaxone) significantly showed sensitivity against all these pathogens. In a previous study, among the two hundred bacteria isolated from clinical samples, researchers found *E. coli* and *Staphylococcus spp*. were the least resistant bacteria. The antimicrobial resistant pattern obtained in this study showed that Amoxicillin, Azithromycin, and Kanamycin followed the most active drugs Erythromycin, Streptomycin, Nalidixic acid, and azithromycin. Drug resistance traits were shown by the isolates towards third-generation cephalosporin antibiotics and even fourth-generation antibiotics, including Nalidixic acid, Colistin, and Doxycycline. Cotrimoxazole, Cefotaxime-clavulanic acid, and erythromycin were the most active antibiotics whether researchers found 92.6% sensitivity to Imipenem. Being highly resistant to pathogenic isolates, cefotaxime, ceftazidime, and cefuroxime were suggested to avoid. Surprisingly researchers supported this suggestion by obtaining their experimental results and strictly advised to avoid these antibiotics without taking any precision. Therefore, the treatments become more challenging for infectious diseases caused by multidrug-resistant bacteria due to increasing medical costs and mortality and morbidity rates. *Acinetobacter baumannii* were susceptible to tetracycline (TE, 30 µg), erythromycin (E, 15 µg), streptomycin (S, 10 µg) and cefotaxime-clavulanic acid (CTC; 40 µg) and *Proteus mirabilis* was sensitive against tetracycline (TE, 30 µg), erythromycin (E, 15 µg), amoxicillin (AMX, 30 µg) and cefotaxime-clavulanic acid (CTC; 40 µg). Conversely, *Acinetobacter baumannii*, and Proteus mirabilis were shown

to be resistant against penicillin G (P, 10 µg) and kanamycin (K, 30 µg). In another study, strong resistance of *Acinetobacter spp*. was found against penicillins (*ticarcillin, piperacillin*, and carbenicillin) with resistance rates higher than 40%. *K.pneumoniae* and *E. coli* was sensitive against *Penicillin G* (P, 10 µg), cotrimoxazole (COT, 25 µg), amoxicillin (AMX, 30 µg), cefotaxime-clavulanic acid (CTC; 40 µg), erythromycin (E, 15 µg), kanamycin (K, 30 µg), and azithromycin (AZM, 30 µg) but both isolates were found to resistant against ciprofloxacin (CIP, 5 µg) and ceftazidime (CAZ, 30 µg). In another study, 100% resistance of *E. coli* were found for chloramphenicol and amoxicillin and clavulanic acid, more than 80% for gentamicin, cefotaxime, ceftazidime, cotrimoxazole, and piperacillin/tazobactam, and 70% for amikacin (Ponnusamy, Natarajan, & Sevanan, 2012) and *E. coli* was susceptible to all antibiotics apart from amoxicillin but resistant to cotrimoxazole, ciprofloxacin, and cephalosporins. Another emerging pathogen; e.g., Pseudomonas aeruginosa and Staphylococcus aureus were susceptible to moxazole (COT, 25 µg), kanamycin (K, 30 µg), nalidixic acid (NA, 30 µg), colistin (CL, 10 µg), polymyxin B (PB, 300 µg), doxycycline (DO, 30 µg), cefotaxime-clavulanic acid (CTC; 40 µg); and Penicillin G (P, 10 µg), tetracycline (TE, 30 µg), cotrimoxazole (COT, 25 µg), erythromycin (E, 15 µg), streptomycin (S, 10 µg), nalidixic acid (NA, 30 µg), cefotaxime-clavulanic acid (CTC; 40 µg) ceftriaxone (CTR, 30 µg) whereas, both pathogens were resistant to ceftazidime (CAZ, 30 µg) and azithromycin (AZM, 30 µg). The previous study showed *Pseudomonas spp*. and *Staphylococcus spp*. were resistant to P, TE, COT, E, NA, K, S, CAZ, and AMX and susceptible to CL, DO, PB, CIP, AZM, and CTR.

The antibiotic susceptibility patterns further confirmed by MIC rates which indicate the exact inhibition rate of selected antibiotics against pathogenic isolates. Significant inhibition rate of kanamycin, doxycycline and nalidixic acid against *A. baumannii, P. mirabilis, K. pneumoniae*, *E. coli*, *P. aeruginosa and S. aureus* were followed by MIC 45 ug/mL, MIC 70 ug/mL, MIC 45 ug/mL; MIC 60 ug/mL, MIC 55 ug/mL, MIC 25 ug/mL; MIC 85 ug/mL, MIC 60 ug/mL, MIC 55 ug/mL; MIC 35 ug/mL, MIC 20 ug/mL, MIC 25 ug/mL; MIC 35 ug/mL, MIC 35 ug/mL, MIC 45 ug/mL; MIC 75 ug/mL, MIC 50 ug/mL, MIC 45 ug/mL; and MIC 55 ug/mL, MIC 45 ug/mL, MIC 15 ug/mL respectively. This study comparatively quite similar to the previous study where researchers concluded their decision by presenting their experimental data as similar as 41% against gentamicin (MIC ≥ 16 µg/mL); 27% against amikacin (MIC ≥ 64 µg/mL); 32% against streptomycin (MIC ≥ 16 µg/mL); 59% against cefepime (MIC ≥ 16 µg/mL); 13% against ceftazidime (MIC ≥ 32 µg/mL); 28% against imipenem (MIC ≥ 8 µg/mL); 41% against ticarcillin (MIC ≥ 128 µg/mL); 43% against piperacillin (MIC ≥ 128 µg/mL); 56% against carbenicillin (MIC ≥ 64 µg/mL); 63% against trimethoprim-sulfamethoxazole (MIC ≥ 76 µg/mL); and 27% against tetracycline (MIC ≥ 16 µg/mL).

A high resistance to commonly used antimicrobial agents is speculated to be associated with ESBL and MBL production. ESBL and MBL - producing pathogenic isolates is a well-recognized problem that makes them resistant to cephalosporins. The isolates included in this experiment were susceptible to cefotaxime-clavulanic acid, which belongs to the β-lactam antibiotic in the third-generation cephalosporins class Clavulanic acid is a β-lactam drug that functions as a mechanism-based β-lactamase inhibitor. Our previous study proved that uropathogenic microbes *E. coli* and K. pneumoniae were susceptible to Imipenem, which belongs to the carbapenem group of antibiotics. Carbapenems are considered the treatment of choice for serious infections caused by ESBL-producing and AmpC β-lactamase-producing *Enterobacteriaceae*.

A variety of gram-negative rod-shaped bacteria and gram-positive grape-like clusters with cocci-shaped bacteria produce Extended Spectrum β-Lactamase (ESBL) and Metallo-β-Lactamase (MBL). According to our study *Acinetobacter baumannii*, *Proteus mirabilis*, and *Pseudomonas aeruginosa* were most likely to produced ESBL and MBL among these isolates. Only *Klebsiella pneumoniae* was produced ESBL, and other isolates were less ESBL producers. Because of the excessive use and misuse of broad-spectrum antibiotics, including cephalosporins, there has been a rapidly rising prevalence of ESBL and MBL production among the Gram-Negative and Gram-Positive pathogenic isolates as a result of selection pressure. The genes encoding ESBL and MBL enzymes are generally plasmid-mediated. The same plasmid can also confer additional resistance determinants to aminoglycosides and fluoroquinolones. Furthermore, many of the ESBL and MBL isolates express cross-resistance to non-β-lactam antibiotics such as ciprofloxacin, aminoglycosides, nitrofurantoin, and TMP-SMX, posing an additional therapeutic challenge to both clinicians and clinical microbiologists. According to many reports, delay inapposite therapy and a concomitant increase in treatment failure rates and comorbidity are being caused by these enzymes.

In the present experiment, slime production was detected by three different methods. Black coloured colonies were produced on Congo red agar medium by all pathogenic isolates that indicate extracellular slime material production.

Researcher previously showed more rapid, more sensitive, and reproducible method is the Congo Red method, which has the advantage of the colonies remaining viable on the medium. A similar study also proved that uropathogens could produce biofilm near about 73%, considering their environmental situation. A significant proportion of biofilm-forming organisms was isolated from *E. coli,* whereas reported a similar biofilm production (67.5%) in *E. coli* . In another study conducted by it was found that among 100 *E. coli* strains, 72 strains were able to form a biofilm found that ESBL-producing strains highly form a biofilm compared with non-ESBL producers. No such correlation was observed in this experiment between ESBL producing strains and non-ESBL-producing strains, but this study has confirmed a significant correlation between ESBL and MBL producers (biofilm-forming pathogenic strains). All these isolates were able to form a biofilm by three different methods employed in this study. We already showed that the tube method (TM) was 83.2% sensitive to produce biofilm rather than the other two methods. The Accuracy was 95.89%, which was proved statistically (Table 4). This finding supports the previous study, where the researcher concluded that the tube method (TM) was 93.63% Accurate to produce biofilm [5].

| **Experimental Methods of Biofilm Formation** | **Sensitivity** | **Specificity** | **PPV (positive predictive value)** | **NPV (negative predictive value.)** | **Accuracy** |
|---|---|---|---|---|---|
| Congo red agar (CRA) | 0.8 | 0.975 | 0.958 | 0.925 | 0.946 |
| 96 well plate method | 0.819 | 0.98 | 0.955 | 0.928 | 0.9496 |
| Tube method (TM) | 0.832 | 0.986 | 0.959 | 0.937 | 0.9589 |

Table4: Statistical analysis of Congo red agar, 96-well plate method and tube method using tissue culture plate method as gold standard.

It has been stated that resistance toward commonly used antibiotics and biofilm formation is closely related to resistance, which increases the risk of developing chronic infections. Therefore, the infectious diseases caused by the biofilm-producing multidrug-resistant *Acinetobacter baumannii, Proteus mirabilis*, *K. pneumoniae*, *E. coli*, *Pseudomonas aeruginosa*, and *Staphylococcus aureus* pose a severe threat to the health status of the public and thus requires close monitoring and detailed investigation.

## Conclusion

Biofilms are structured communities of bacterial cells enclosed in a self-produced polymeric matrix capable of adhering to an inert or living surface. Production of slime protects against antibiotics, possibly due to the decreased metabolic activity of bacteria and decreased antibiotic diffusion through the biofilm matrix. The most worrisome finding is the high prevalence of multidrug-resistant pathogens. Most of the clinical

isolates were found as strong biofilm producers and resistant to multiple antibiotics. Clinical environments infested with these bacteria may increase morbidity and mortality since treatment against biofilm-forming MDR pathogens is far more complicated. In this study, all the bacterial isolates of Acinetobacter baumannii, *Proteus mirabilis*, *K. pneumoniae, E. coli, Pseudomonas aeruginosa,* and *Staphylococcus aureus* were found to be multidrug-resistant and able to form a biofilm, and their correlation between ESBL, MBL, and biofilm-producing isolates was determined along with the multiple drug resistance patterns of *A. baumannii*, *P. mirabilis*, *K. pneumoniae*, *E. coli*, *P. aeruginosa* and *S. aureus*. Moreover, out of fifteen isolates, both were able to produce ESBL and MBL. The small sample size prevented me from achieving an idea about the real scenario. Treatment failure and morbidity are often associated with multidrug-resistant, especially ESBL producing bacterial infection. Improper use, misuse, and easy availability of antibiotics without prescription should be strictly regulated to spread the pathogens' multidrug-resistant traits.

## Limitations

This study could not turn into the next level, such as PCR for strain detection and 16srRNA sequencing for molecular phylogeny, lacking molecular lab facilities. The lack of confirmation of biofilm, ESBL, and MBL production using molecular technologies are the drawbacks of this study. Also, the detection of multi-drug resistant genes among these isolates will be suggested for future experiments.

## Conflict of interest

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

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## Author Contributions

This work was carried out in collaboration between all authors. All the authors have accepted responsibility for the entire content of this submitted manuscript and approved the submission. Authors A. S.performed the experiments. AS M.F.R.S., and H.A. conceived the study and designed the experimental procedures. A.S. designed and planned the studies. A.S. also acted for all correspondences. A.S.; H.A. and A.R.M. participated in the manuscript draft and have thoroughly checked and revised the manuscript for necessary changes in format, grammar, and English standards. All authors read and approved the final version of the manuscript. All authors read and approved the manuscript.

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