Since 2002

Interrelationship of Extended Spectrum Beta-Lactamase Producers and Biofilm Formation among the Gram-Negative Bacteria from Tabuk, KSA

Mohammad Zubair[1]* , Ibrahim Mohammad[2]

[1]*Department of Medical Microbiology, Faculty of Medicine, University of Tabuk, Tabuk, Saudi Arabia;* [2]*Chattahoochee High School, Johns Creek, GA, USA*

Abstract

AIM: The present study investigates the production of extended-spectrum beta-lactamases (ESBL) and the formation of biofilm among different bacterial pathogens.

METHODS: The study conducted prospective analysis on bacteria isolates (Gram-negative) from patients who have diagnosed with infections with bacteria between October 2020 and January 2022.

RESULTS: The results showed that there were 53 biofilm producers in Escherichia coli. In contrast, Pseudomonas aeruginosa was observed to have the highest percentage, with 32/40 (80%) isolates being biofilm producers. The least number of isolates were Morganella morganii (n = 2) with two (100%) biofilm producers. The resistance in the biofilm positive isolates was high compared with biofilm negative. About 88% of phenotypic ESBL-positive isolates were biofilm producers, and 97% of cefotaxime-resistant biofilm-positive isolates were genotypic positive for CTX-M, TEM, and SHV genes.

CONCLUSION: The present study has shown that protection against antibiotics through mucus production is possible due to bacteria's reduced metabolic activity and diffusion of antibiotics across the biofilm matrix. In this study, all the bacterial strains of *E. coli* and *Klebsiella pneumoniae* were reported to be MDR and competent for establishing biofilm.

Introduction

The therapy deficiency and interfered illness control are remarkably assisted with the production of *Enterobacteriaceae* (*Klebsiella pneumoniae* and *Escherichia coli*) through extended-spectrum beta-lactamases (ESBLs) in human and veterinary medicine [1]. Various ESBL genotypes in *E. coli* are present in the environment, livestock, wild animals, and poultry [2]; however, the formation of biofilm by the organisms produces some of the cell surface constituents and increases endurance to multiple antimicrobials for promoting their availability in different communities [3]. The development of biofilm is known as a complicated procedure [4]. A biofilm develops when specific microorganisms follow the surface of some objects in a moist environment and commence to reproduce. For instance, colonies are cells attached to a surface, which allow *E. coli* to bind and colonize through several surface determinants [5]. The colonies largely comprise polysaccharides as it synthesizes a matrix around the biofilm [6]. The removal of planktonic cells results in the progression of circ and colonizes different surfaces. Hydrophobic interactions play important roles in adhering to and forming biofilm [1].

Firmly lodged bacterial aggregates are present on the biofilms in extracellular matrices of polysaccharides, nucleic acids, enzymes, and proteins to facilitate irreversible anchoring to surfaces [7]. The matrix confers antibiotic resistance through different processes such as expressing chromosomally encoded resistant genes, reducing growth rate, restriction of antibiotics, and counteracting host immunity [8]. Extensive dissemination of multi-drug resistant (MDR) strains of Gram-negative bacilli is contributed through the formation of biofilm and production of beta-lactamases.

Almost all MDR, *K. pneumoniae* possess ESBL enzymes [9]. This highlights the association of Gram-negative pathogens with severe infections such as urinary tract infection, pneumonia, septic shock, wound infection, septicemia, and intra-abdominal infection [10]. It is reported that ESBL producing *K. pneumoniae* (ESBL-*K. pneumoniae*) is mostly acquired in hospital settings, for instance, in the intensive care unit, and

had been associated with increased morbidity and mortality. Therefore, the increased resistance of ESBL *K. pneumoniae* has grabbed the attention of many researchers, considering the wide range of beta-lactam antibiotics used as antibacterial agents [11].

There is an obvious need for studies focusing on the speedy and precise detection of ESBL-producing bacteria. Significant knowledge about the antibiogram of bacterial isolates and the formation of biofilm is important to render reliable empirical antibiotic therapy to patients [12]. The conventional methods are reliable and economical for routine screening and diagnosis, regardless of numerous molecular detection techniques [13]. This present study focuses on the production of ESβL and the formation of biofilm among different bacterial pathogens. This research aimed to employ a polymerase chain reaction (PCR) test to identify ESBL-producing genes in a subset of Gram-negative clinical bacteria in Kingdom of Saudi Arabia (KSA) that is currently scarce.

Methodology

Bacterial isolates

In the 1.5-year study period between October 2020 and January 2022 from two civilian hospital patients in Tabuk, Saudi Arabia, a prospective analysis of bacteria isolated (Gram-negative) with illnesses was conducted. Each specimen's culture and smear were examined for noteworthy microbiological characteristics using established methods [14]. Utilizing culture traits and common biochemical tests, bacteria isolates were identified up to the species level [15]. Utilizing the Clinical and Laboratory Standards Institute (CLSI) serum standards, the Kirby–Bauer disk diffusion method was used to conduct *in vitro* susceptibility testing [15].

Following a 24-h incubation at 37° Celsius, the obtained samples were inoculated into nutrient agar plates. Forty unique isolates were chosen based on their appearance on isolation agar. Eosin methylene blue agar, cetrimide agar, and MacConkey agar were used to culture the isolates. 24-h incubations at 37° Celsius were performed on the plates. Biochemical tests, including catalase, oxidase, IMViC, TSI, the carbohydrate utilization test, and urease, were performed on the colonies exhibiting distinctive growth patterns in addition to the standard micromorphological tests of Gram staining, capsule staining, spore staining, and motility. Kirby–Bauer disk diffusion assays using antibiotic discs were performed on all the detected isolates to determine their susceptibilities to these drugs. To the closest millimeter, the diameter of the zone was noted. Kirby–Bauer disk diffusion isolates showing antibiotic resistance were chosen to identify ESBL producers. Bacteria (Gram-negative) were isolated and subcultured onto MacConkey agar (M008; Hi-Media Laboratories Pvt. Ltd., India) and were single species, pure, and non-duplicate organisms. The study was conducted after approval from the Institutional Ethical Research Committee (READ) (Approval No: READ-0132).

Antibacterial susceptibility testing

In vitro antibacterial susceptibility testing for each pure isolate was performed by Kirby–Bauer disk diffusion method against the third-generation disk of cephalosporins, (10 μg cefpodoxime, 30 μg ceftazidime, 30 μg cefotaxime, and 30 μg ceftriaxone [CE]), monobactams (30 μg aztreonam), aminoglycosides (30 μg amikacin, 10 μg tobramycin, and 10 μg gentamicin), fluoroquinolones (5 μg ciprofloxacin, 5 μg levofloxacin, 5 μg ofloxacin, and 5 μg gatifloxacin), and phenolics (30 μg chloramphenicol) using HiMedia susceptibility disks and interpreted according to the CLSI standards guidelines [15].

Selection of the ESBL producing strains

Standard methods were used to obtain a culture and smear from each. The CLSI guidelines state the disk diffusion method for testing antibiotic susceptibility can screen for ESBL activity if the zone of inhibition is ≤22 mm with 30 g ceftazidime disks and ≤27 mm with 30 g cefotaxime disks. These isolates were identified as potential ESBL producers and selected for the screening and confirmatory test of ESBL activity [15].

Phenotypic confirmatory test for ESBL production

According to CLSI guidelines, the disk test was carried out with overnight growth of the test isolates (0.5 McFarland standard) on Mueller–Hinton agar plates, with 25 mm distance between plain and clavulanate (10 g/disk) incorporated cefotaxime disk (30 g/disk). The difference between must shows an increase of ≥5 mm with clavulanate impregnated disk showing phenotypic confirmation of ESBL formation [15].

Quality control

A conventional non-ESBL-producing organism from the American Type Culture Collection (ATCC), *E. coli* ATCC 25922, and an ESBL-producing organism, *K. pneumoniae* ATCC 700603, were used to assess the antibacterial drugs' effectiveness.

Molecular detection and characterization

Molecular methods for β-lactamase detection

Detection of bla genes by PCR: Molecular detection of bla$_{CTX-M}$, bla$_{TEM}$, and bla$_{SHV}$ was performed

in cefotaxime-resistant isolates using PCR according to the methods described previously with minor modifications [16], [17]. The primers and cycling conditions for the detection of bla genes were the same as described by [16].

Biofilm production

This study has adopted the biofilm production assay from the study of Zubair *et al.* [18]. A quantitative determination was used to examine biofilm formation in 96 flat bottom plates. To be precise, TSB was used to prepare fresh bacterial suspensions from overnight cultures and balanced for OD_{600} of 0.1. Afterward, inoculation of 100 µL aliquots of bacterial suspension was processed into individual wells at 37°C for 48 h. 1X phosphate buffered saline was used to gently wash plates for 30 min at room temperature through 0.1% crystal violet (Sigma-Aldrich, St. Louis, MO). Removal of excess crystal violet was performed through washing, whereas subsequent $OD_{590\ nm}$ of the supernatant was used to quantify biofilm following the solubilization of CV in 95% ethanol. The test was performed in triplicate to determine the mean biofilm absorbance value for each clinical strain tested. As per the method proposed by Stepanović *et al*. [19], the classification of biofilm was based on the following categories: weak (OD_{590} 0.1 to ≤0.400), moderate (OD_{590} > 0.400), and strong (OD_{590} > 0.800). However, this study categorized positive isolates through moderate and strong classifications, while negative biofilm production was represented through weak non-biofilm.

Statistical analysis

SPSS software (version 25.0) was used to enter and analyze data. The actual number of ESBL-producing isolates was used for describing frequency outputs for categorical variables. Tables and figures were used to present the data. Continuous variables were presented in the form of mean and standard deviation.

Results

A total of 126 Gram-negative isolates were biofilm positive among 167 studied isolates. Out of 71 *E. coli* strains, 53 (74.6%) were biofilm producers. Similarly, 32 (80%) of the biofilm producers represented *Pseudomonas aeruginosa* (n = 40). The least number of isolates were *Morganella morganii* (n = 2) with two (100%) biofilm producers.

Phenotypic ESBL detection

Table 1A presents the distribution of ESBL positive by phenotypic methods among Gram-negative bacteria. Among the tested isolates of ESBL screening positive and confirmatory positive, 60.4% and 61.3% of the tested isolates were represented as ESBL screening positive and ESBL confirmatory positive, respectively. Out of the ESBL-screened positive isolates, 43 (42.5%) were *E. coli* isolates, followed by *P. aeruginosa* (20.7%), *Klebsiella oxytoca* (12.8%). Likewise, out of ESBL-confirmed positive isolates, 61.3% of the screening-positive isolates were confirmed positive by a confirmatory test. Moreover, out of 101 ESBL screening-positive isolates, 88% were biofilm activity, and 87% showed positive biofilm activity in ESBL-confirmed positive isolates.

From the total Gram-negative, around 62% showed screening positivity of ESBL by disk diffusion method, in which 109 (65.2%) isolates were positive using ceftazidime and 101 (60.4%) for cefotaxime. In the confirmatory ESBL test, 67.8% were found positive by disk potential method using ceftazidime/ceftazidime + clavulanic acid 76 (75.2%), and 62 (61.3%) with cefotaxime/cefotaxime + clavulanic acid (Table 1B). The overall reduction in ESBL from screening to confirmatory test among the Gram-negative isolates was 34%.

Table 1: Screening test and confirmatory test results of extended-spectrum b-lactamase-producing Gram-negative bacilli, and number of biofilm producers from screening test and confirmatory test results of extended-spectrum b-lactamase-producing Gram-negative bacilli

A				
Name of bacterial isolates	ESBL study		Biofilm positive activity from ESBL	
	Screening positive n (%)	Confirmatory positive n (%)	Screening positive (n)	Confirmatory positive (n)
Escherichia coli	43 (42.5)	28 (65.1)	40	26
Pseudomonas aeruginosa	21 (20.7)	14 (66.6)	19	12
Klebsiella oxytoca	13 (12.8)	8 (61.5)	11	6
Klebsiella pneumonia	9 (8.9)	5 (55.5)	7	4
Proteus vulgaris	6 (5.9)	3 (50.0)	5	3
Proteus mirabilis	3 (2.9)	2 (66.6)	2	2
Acinetobacter spp.	5 (5.9)	2 (40)	4	1
Morganella morganii	1 (0.9)	0 (0)	1	-
Total	101 (60.4)	62 (61.3)	89 (88.1)	54 (87.0)
B				
Screening ESBL result	n = 167	Confirmatory ESBL	n = 167	Percentage reduction of ESBL from screening to confirmatory
Ceftazidime	109 (65.2%)	Ceftazidime + clavulanic acid	76 (45.5%)	30.27%
Cefotaxime	101 (60.4%)	Cefotaxime + clavulanic acid	62 (37.1)	38.61%
Average positivity	62.8%	Average positivity	41.3%	34.23%

Data are number (%). ESBL: Extended-spectrum beta-lactamases.

Antibiotic resistance pattern among biofilm positive and negative isolates

Figure 1 represents the antibiotic resistance pattern among biofilm positive and negative isolates. Here, only cefotaxime and ofloxacin were not significantly associated (p > 0.05). It was reported that overall antibiotic resistance was higher in biofilm positive isolates than in biofilm-negative isolates.
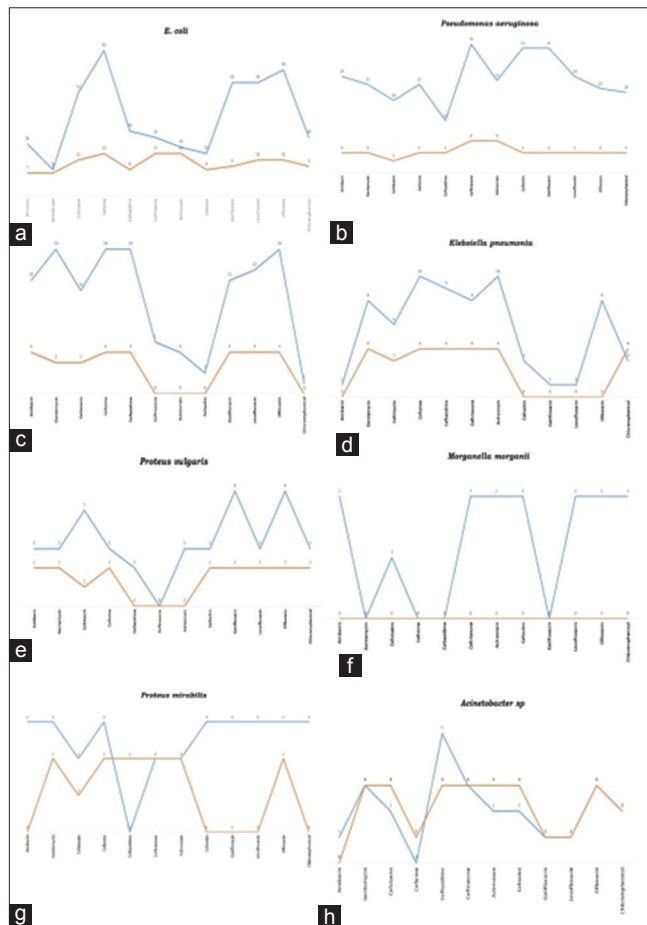
Figure 1: Comparative antibiotic resistance pattern among biofilm positive (blue) and biofilm negative (orange) (a) Escherichia coli, (b) Pseudomonas aeruginosa, (c) Klebsiella oxytoca, (d) Klebsiella pneumonia, (e) Proteus vulgaris, (f) Morganella morganii, (g) Proteus mirabilis, and (h) Acinitobacter spp. isolates. Data show high resistance among biofilm positive isolates

Genotypic ESBL detection and bla gene distribution

The frequency of bla genes is shown in Table 2a and b. Only cefotaxime-resistant isolates (n = 101) were subjected to Class A (CTX-M, TEM, and

SHV) ESBLs study. Eighty-seven (86.1%) cefotaxime resistant isolates were found positive for bla genes, in which CTX-M was found to be the most prevalent ESBL noticed in 76 (87.3%), followed by SHV [41 (47.1%)] and TEM beta-lactamases were reported in 31(35.6%) isolates. The genotype distribution analysis (Table 2B) reveals that 23 (26.4%) strains were having all three genes (CTX-M + TEM + SHV), 12 (13.7%) strains (CTX-M + SHV), 5 (5.7%) strains (CTX-M + TEM), and 2 (2.2%) strains (TEM + SHV). The majority of the tested strain were having CTX-M alone, 36 (41.3%) strains. Organism wise, ESBL gene distribution was depicted in Table A1.

Biofilm analysis

The results have shown a total of 167 isolates, out of which 126 isolates were biofilm producers (Table 3). The overall antibiotic resistance was higher in biofilm positive isolates compared with negative isolates (Figure 1). In addition, 88% of the ESBL-positive (phenotypic-screening) isolates were biofilm producers, and 88% of ESBL-positive (phenotypic-confirmatory) isolates were biofilm producers (Table 1A and B). Among the ESBL genotypic (87) positive isolates, the majority of them show biofilm activity 74 (85%) (Table 2c). Seventy-six positive biofilm isolates were resistant to cefotaxime antibiotic; the average positivity of the ESBL gene in biofilm positive (cefotaxime resistant) isolates was 97% (Table 4).

Table 3: Distribution of isolates

Name of isolates	Number (%)	Biofilm producers (%)
Escherichia coli	71 (42.5)	53 (74.6)
Pseudomonas aeruginosa	40 (24)	32 (80)
Klebsiella oxytoca	18 (10.8)	14 (77.8)
Klebsiella pneumonia	15 (9)	11 (73.3)
Proteus vulgaris	8 (4.8)	6 (75)
Proteus mirabilis	5 (3)	3 (60)
Acinetobacter spp.	8 (4.8)	5 (62.5)
Morganella morganii	2 (1.2)	2 (100)
Total	167	126

Data are number (%).

Discussion

A total of 167 different types of isolates were analyzed. *E. coli* accounted for the largest population of Gram-negative bacteria, whereas *M. morganii* accounted for the least. Most of them tested positive for the presence of biofilm. According to the result

Table 2: (a) bla (CTX-M, TEM, SHV) gene positivity result, (b) bla (CTX-M, SHV, TEM) gene combination distribution among the total gram negative bacterial isolates, and (c) Biofilm positivity among bla (CTX-M, SHV, TEM) gene combination distribution

(a) bla gene	n = 87	(b) bla gene distribution	n = 87	(c) Biofilm positivity n=74 (85.0)
bla average positivity	86.1%	All three genes (CTX – M+TEM+SHV)	23 (26.4)	20 (27.0)
CTX-M	76 (87.3%)	Two genes (CTX – M+TEM)	5 (5.7)	4 (5.4)
TEM	31 (35.6%)	Two genes (CTX – M+SHV)	12 (13.7)	9 (12.1)
SHV	41 (47.1%)	Two genes (TEM+SHV)	2 (2.2)	1 (1.3)
		CTX-M alone	36 (41.3)	33 (44.5)
		SHV alone	4 (4.5)	3 (4.0)
		TEM alone	5 (5.7)	4 (5.4)

Data are number (%).

Table 4: bla-gene distribution among the gram negative bacterial

Name of bacterial isolates	Total no. of isolates tested	CTX-M alone	bla_{TEM} alone	bla_{shv} alone	$bla_{ctx-m+b}la_{TEM}$	$bla_{ctx-m+b}la_{SHV}$	$bla_{shv+b}la_{TEM}$	$bla_{shv+b}la_{TEM\_}bla_{CTX-M}$
Escherichia coli	43	18	2	1	2	9	2	4
Pseudomonas aeruginosa	21	9	1	2	1	3	00	0
Klebsiella oxytoca	13	1	0	0	1	0	0	11
Klebsiella pneumonia	9	0	0	0	0	0	0	8
Proteus vulgaris	6	3	1	1	0	0	0	0
Proteus mirabilis	3	2	1	0	0	0	0	0
Acinetobacter spp.	5	2	0	0	1	0	0	0
Morganella morganii	1	1	0	0	0	0	0	0
Total	101	36	5	4	5	12	2	23
Total biofilm positive activity	74	33	4	3	4	9	1	20

Data represented in numbers.

of the study, E. coli was highly resistant to cefixime but very susceptible to cefoxitin. Carbapenem and cephalosporin antibiotics have little effect on *K. pneumonia*. Since more antibiotics are being used to treat urinary tract infections (UTIs), there has been a rise in the antibacterial resistance of *Enterobacteriaceae*, particularly the primary uropathogens *E. coli* and *K. pneumoniae* [20].

The strains of *K. pneumoniae* resistant to 3rd generation cephalosporins were 56% resistant to cefotaxime and 47% resistant to ceftazidime. In India, it has been found that *K. pneumoniae* is resistant to ceftazidime at a very high rate of 84% [21]. The fact that these E. coli strains are resistant to third-generation cephalosporins might be because they have a plasmid-ESBL [22], [23]. Studies that were done in Gabon in the past showed that there were a lot of bacterial strains that made ESBLs [24], [25]. Furthermore, the Global Antimicrobial Resistance Surveillance Report found that *E. coli* and *K. pneumoniae* were resistant to C3Gs in five out of six (5/6) regions and six out of six (6/6) regions, respectively [26]. Imipenem and ertapenem, two types of carbapenems, worked well on *E. coli* and *K. pneumoniae* strains with resistance rates of 2% and 4%, which backed up what Leopold *et al*. found. [27]

The options for treating infections caused by these organisms are limited due to the increased prevalence of MDR *Enterobacteriaceae*. Moreover, a good rationale for using older antibiotics is provided due to the lack of new antibiotics. For instance, osfomycin can retain some activity against MDR bacteria [20]. Because resistance to carbapenems and other broad-spectrum beta-lactams are developing and new antibiotics are scarce, it is critical to investigate the possibility of combination treatment to boost the antibacterial properties of current medicines. For carbapenemase-producing *Enterobacteriaceae*, colistin-tigecycline and various combinations, including an aminoglycoside, a carbapenem, colistin, fosfomycin, rifampin, or tigecycline have been recommended [28], [29], [30], [31]. Based on the retrospective study, it has been advised to employ carbapenem-containing combinations for these bacteria if the carbapenem minimum inhibitory concentration (MIC) is 4 mg/L [28].

P. aeruginosa was highly resistant to CE. Recently, resistance was exhibited by MDR *P. aeruginosa* against 15 antibiotics. *P. aeruginosa* is among the notorious pathogen [20]. *P. aeruginosa* is also known to cause a wide variety of other infections, encompassing all organs of the human body, including soft-tissue infection in burns, open wounds, and post-surgery; urinary tract infection associated with the use of urinary catheter; foot infection in diabetics and individuals with impaired microvascular circulation; ear infection, especially otitis externa and chronic suppurative otitis media associated with tissue injury and water blockage; and keratitis associated with extended contact lens wear and contaminated contact lens [32], [33]. Similar to the present study, a previous study showed that most of the strains of *P. aeruginosa* were resistant to 30 μg/disk CE. Therefore, the doses are recommended in therapy [34].

K. oxytoca was highly resistant to cefixime. However, it contradicts the findings of a study conducted in Pakistan in 2013 [35]. Here, *K. oxytoca* had 57.7% sensitivity to cefixime. It could be due to the development of drug resistance in the bacteria against this drug. *Proteus vulgaris* was seen to be mostly resistant to gatifloxacin. The MIC value was seen to be 0.016–1 in *P. vulgaris* in relation to gatifloxacin in a previous study [36], indicating low potency. One of the previous studies determined the MIC values through agar dilution method based on the procedures outlined by the National Committee for Clinical Laboratory Standards [36]. Whereas, *Acinetobacter* spp. was highly resistant to ceftazidime, in accordance with the previous study [37]. There is a need to monitor the safe and proper usage of antibiotics. Their increased resistance might put us back in a pre-antibiotic era, where a simple bacterial infection could be life-threatening.

Antibiotics are commonly used due to the high rate of resistance speculated to be associated with ESBL production. One well-described problem is that ESBL-producing uropathogenic isolates confer resistance to cephalosporins. The isolates examined in this study were sensitive to imipenem, related to the carbapenem class of antibiotics. Carbapenems are undertaken as the treatment of selection for extremely significant infections resulted due to the production of ESBL and Amp C β-lactamase-producing *Enterobacteriaceae* [38]. ESBL is produced through different Gram-negative rod-shaped bacteria. In this regard, ESBL is made through *K. pneumoniae*, the ESBL-producing organism. There has been an instant elevating occurrence of ESBL production among cephalosporins due to the excessive use and misuse of broad-spectrum antibiotics among

Enterobacteriaceae resulted from selection pressure. Genes encoding ESBL enzymes are essentially plasmid-mediated. The same plasmid can also confer additional resistance contributors to aminoglycosides and fluoroquinolones. In addition, most ESBL isolates demonstrate cross-resistance to non-β-lactam antibiotics, including nitrofurantoin, TMP-SMX, ciprofloxacin, and aminoglycosides, stressing additional therapeutic challenges to both clinical microbiologists and clinicians. According to most of the reports, delays in opposite therapy and an associated elevation in the rates are caused by such enzymes [39].

Isolates of *E. coli* showed a substantial extent of biofilm-forming organisms, while a similar proportion of biofilm production has been reported in *E. coli* [40]. It was revealed that 72 strains were competent enough to establish a biofilm among 100 *E. coli* strains [41]. ESBL-producing strains establish a biofilm compared with non-ESBL producers [42]. No such association was revealed in this study as a biofilm was formed by two non-ESBL-producing strains such as RD7 and *E. coli* RD1. Resistance is closely associated with resistance toward biofilm formation and antibiotics, elevating the threat of forming chronic UTIs. Thereby, the biofilm-producing MDR *E. coli* and *K. pneumoniae* cause the UTIs to induce an adverse risk to the health status of the public and, therefore, need close monitoring and explicit examination.

The present study reported that 86.1% of cefotaxime resistant isolates were found positive for bla genes, in which CTX-M was found to be the most prevalent ESBL noticed in 76 (87.3%), followed by SHV (41 [47.1%]) and TEM beta-lactamases were reported in 31 (35.6%) isolates. Marthie *et al*. reported that ESBL genes in this study were 87%, with the bla_{TEM} gene only detected in 24% and the bla_{SHV} gene in 4% [43]. The genotype distribution analysis reveals that 26.4% strains had all three genes (CTX-M + TEM + SHV), 13.7% of strains (CTX-M + SHV), 5.7% of strains (CTX-M + TEM), and 2 (2.2%) strains (TEM + SHV). Most of the tested strains had CTX-M alone, 36 (41.3%) strains. Marthie *et al*. reported TEM + SHV was detected in 12%, TEM + CTX-M detected in 12%, and TEM + SHV + CTX-M was detected in 36% of the isolates [43].

The study has a limitation that it did not use molecular techniques due to easy accessibility to the advanced laboratory in Tabuk, KSA, due to expensive instruments availability. However, despite this limitation, up to our knowledge, this is the first study conducted to examine biofilm formation and ESBL procedures among Gram-negative bacteria in infected patients.

Conclusion

This study effectively found drug resistance patterns in Gram-negative bacteria. Biofilms consist of structured bacterial cells encapsulated in self-made polymer matrices, which can follow living or inert surfaces. Protection against antibiotics through mucus production is possible due to reduced metabolic activity of bacteria and reduced diffusion of antibiotics across the biofilm matrix. In this study, all the bacterial strains of *E. coli* and *K. pneumonia* were reported to be MDR and competent for establishing Biofilm. This study's implication will help clinicians select the drug of choice to treat these bacteria. For researchers, it has the implication that it will help in future studies to understand, in which drugs were sensitive before, as many antibiotics are now going toward resistant patterns. Morbidity and treatment failure is usually related to MDR, specifically ESBL-producing bacterial infection. Misuse, easy availability, and improper use of antibiotics must be strictly controlled for spreading the MDR characteristics of the pathogens regardless of prescription. The findings have shown a relationship between high biofilm positivity among ESBL-positive strains.

Author Disclosure Statement

Mohammad Zubair, researched data, performed biofilm studies and molecular analysis, wrote the manuscript, and contributed to the discussion. Ibrahim Mohammad contributed to the results and discussion.

Acknowledgments

References

1. Yılmaz ES, Güvensen NC. *In vitro* biofilm formation in ESBL-producing *Escherichia coli* isolates from cage birds. Asian Pac J Trop Med. 2016;9(11):1069-74. https://doi.org/10.1016/j.apjtm.2016.10.003
    PMid:27890366

2. Hasan B, Islam K, Ahsan M, Hossain Z, Rashid M, Talukder B, *et al*. Fecal carriage of multi-drug resistant and extended-spectrum β-lactamases producing *E. coli* in household pigeons, Bangladesh. Vet Microbiol. 2014;168(1):221-4. https://doi.org/10.1016/j.vetmic.2013.09.033
    PMid:24290770

3. Kragh KN, Hutchison JB, Melaugh G, Rodesney C, Roberts AE,

Irie Y, *et al*. Role of multicellular aggregates in biofilm formation. mBio. 2016;7(2):e00237. https://doi.org/10.1128/mbio.00237-16
PMid:27006463

4. Penesyan A, Gillings M, Paulsen IT. Antibiotic discovery: Combatting bacterial resistance in cells and biofilm communities. Molecules. 2015;20:5286-98. https://doi.org/10.3390/molecules20045286
PMid:25812150

5. Valentini M, Filloux A. Biofilms and cyclic di-GMP (c-di-GMP) signaling: Lessons from *Pseudomonas aeruginosa* and other bacteria. J Biol Chem. 2016;291(24):12547-55. https://doi.org/10.1074/jbc.r115.711507
PMid:27129226

6. Hobley L, Harkins C, MacPhee CE, Stanley-Wall NR. Giving structure to the biofilm matrix: An overview of individual strategies and emerging common themes. FEMS Microbiol Rev. 2015;39(5):649-69. https://doi.org/10.1093/femsre/fuv015
PMid:25907113

7. Dumaru R, Baral R, Shrestha LB. Study of biofilm formation and antibiotic resistance pattern of gram-negative Bacilli among the clinical isolates at BPKIHS, Dharan. BMC Res Notes. 2019;12(1):38. https:/doi.org/10.1186/s13104-019-4084-8
PMid:30658694

8. Shrestha LB, Bhattarai NR, Khanal B. Comparative evaluation of methods for the detection of biofilm formation in coagulase-negative staphylococci and correlation with antibiogram. Infect Drug Resist. 2018;11:607-13. https://doi.org/10.2147/idr.s159764
PMid:29731649

9. Teklu DS, Negeri AA, Legese MH, Bedada TL, Woldemariam HK, Tullu KD. Extended-spectrum beta-lactamase production and multi-drug resistance among *Enterobacteriaceae* isolated in Addis Ababa, Ethiopia. Antimicrob Resist Infect Control. 2019;8:39. https://doi.org/10.1186/s13756-019-0488-4
PMid:30815254

10. De Oliveira DM, Forde BM, Kidd TJ, Harris PN, Schembri MA, Beatson SA, *et al*. Antimicrobial resistance in ESKAPE pathogens. Clin Microbiol Rev. 2019;33(3):e00181-19. https://doi.org/10.1128/CMR.00181-19
PMid:32404435

11. Zhao Y, Zhang X, Torres VV, Liu H, Rocker A, Zhang Y, *et al*. An outbreak of carbapenem-resistant and hypervirulent *Klebsiella pneumoniae* in an intensive care unit of a major teaching hospital in Wenzhou, China. Front Public Health. 2019;7:229. https://doi.org/10.3389/fpubh.2019.00229
PMid:31552210

12. Tanwar J, Das S, Fatima Z, Hameed S. Multidrug resistance: An emerging crisis. Interdiscip Perspect Infect Dis. 2014;2014:541340. https://doi.org/10.1155/2014/541340
PMid:25140175

13. Mohamed A, Rajaa AM, Khalid Z, Fouad M, Naima R. Comparison of three methods for the detection of biofilm formation by clinical isolates of *Staphylococcus aureus* isolated in Casablanca. Int J Sci Res. 2016;5:1156-9. https://doi.org/10.21275/ART20162319

14. Bharathi MJ, Ramakrishnan R, Shivakumar C, Meenakshi R, Lionalraj D. Etiology and antibacterial susceptibility pattern of community-acquired bacterial ocular infections in a tertiary eye care hospital in south India. Indian J Ophthalmol. 2010;58(6):497-507. https://doi.org/10.4103/0301-4738.71678
PMid:20952834

15. Hemachandran K, Bharathi S, Radhakrishnan M, Balagurunathan R. Studies on extended beta lactamase producing, biofilm forming clinical bacterial pathogens and it's *invitro* inhibition by actinobacterial extracts. J Appl Pharm Sci.

2011;1:210-3.

16. Shahid M, Ensor VM, Hawkey PM. Emergence and dissemination of *Enterobacteriaceae* with plasmid-mediated CMY-6 and CTX-M-15 beta-lactamases in community in North India. World J Microbiol Biotechnol. 2009;25:1439-46. https:/doi.org/10.1007/s11274-009-0032-4

17. Shahid M, Malik A, Adil M, Jahan N, Malik R. Comparison of beta-lactamase genes in clinical and food bacterial isolates in India. J Infect Dev Ctries. 2009;3(8):593-8. https://doi.org/10.3855/jidc.550
PMid:19801801

18. Zubair M, Malik A, Ahmad J, Rizvi M, Farooqui KJ, Rizvi MW. A study of biofilm production by gram-negative organisms isolated from diabetic foot ulcer patients. Biol Med. 2011;3(2):147-57.

19. Stepanović S, Vuković D, Hola V, Bonaventura GD, Djukić S, Ćirković I, *et al*. Quantification of biofilm in microtiter plates: An overview of testing conditions and practical recommendations for assessment of biofilm production by staphylococci. APMIS. 2007;115(8):891-9. https://doi.org/10.1111/j.1600-0463.2007.apm_630.x
PMid:17696944

20. Mazzariol A, Bazaj A, Cornaglia G. Multi-drug-resistant Gram-negative bacteria causing urinary tract infections: A review. J Chemother. 2017;29(Sup 1):2-9. https://doi.org/10.1080/1120009x.2017.1380395
PMid:29271736

21. Singh NP, Goyal R, Manchanda V, Das S, Kaur I, Talwar V. Changing trends in bacteriology of burns in the burns unit, Delhi, India. Burns. 2003;29(2):129-32. https://doi.org/10.1016/S0305-4179(02)00249-8
PMid:12615458

22. SPILF. Diagnostic et Antibiothérapie des Infections Urinaires Bactériennes Communautaires de L'adulte. Paris: SPILF; 2015. p.1-43.

23. Behzadi P, Urban E, Matuz M, Benko R, Gajdacs M. The role of gram-negative bacteria in urinary tract infections: Current concepts and therapeutic options. Adv Exp Med Biol. 2020;1323:35-69. https://doi.org/10.1007/5584_2020_566
PMid:32596751

24. Schaumburg F, Alabi A, Kokou C. High burden of extended-spectrum beta-lactamase-producing *Enterobacteriaceae* in Gabon. J Antimicrob Chemother. 2013;68(9):2140-3. https:/doi.org/10.1093/jac/dkt164
PMid:23645586

25. Yala JF, Mabika R, Bisseye C. Phenotypic and genotypic characterization of extended-spectrum-beta-lactamases producing-*Enterobacteriaceae* (ESBLE) in patients attending Omar Bongo Ondimba military hospital at Libreville (Gabon). J Mol Microbiol Biotechnol. 2016;4(6):944-9.

26. World Health Organization. Antimicrobial Resistance: Global Report on Surveillance. Geneva: World Health Organization; 2014.

27. Leopold SJ, van Leth F, Tarekegn H, Schultsz C. Antimicrobial drug resistance among clinically relevant bacterial isolates in sub-Saharan Africa: A systematic review. J Antimicrob Chemother. 2014;69(9):2337-53. https://doi.org/10.1093/jac/dku176
PMid:24879668

28. Daikos GL, Markogiannakis A. Carbapenemase-producing *Klebsiella pneumoniae*: (When) might we still consider treating with carbapenems? Clin Microbiol Infect. 2011;17(8):1135-41. https://doi.org/10.1111/j.1469-0691.2011.03553.x
PMid:21635663

29. Michalopoulos A, Virtzili S, Rafailidis P, Chalevelakis G, Damala M, Falagas ME. Intravenous fosfomycin for the

treatment of nosocomial infections caused by carbapenem-resistant *Klebsiella pneumoniae* in critically ill patients: A prospective evaluation. Clin Microbiol Infect. 2010;16(2):184-6. https://doi.org/10.1111/j.1469-0691.2009.02921.x

PMid:19694767

30. Hirsch EB, Tam VH. Detection and treatment options for *Klebsiella pneumoniae* carbapenemases (KPCs): An emerging cause of multidrug-resistant infection. J Antimicrob Chemother. 2010;65(6):1119-25. https://doi.org/10.1093/jac/dkq108

PMid:20378670

31. Qureshi ZA, Paterson DL, Potoski BA, Kilayko MC, Sandovsky G, Sordillo E, *et al*. Treatment outcome of bacteremia due to KPC-producing *Klebsiella pneumoniae*: Superiority of combination antimicrobial regimens. Antimicrob Agents Chemother. 2012;56(4):2108-13. https://doi.org/10.1128/AAC.06268-11

PMid:22252816

32. Gellatly SL, Hancock RE. *Pseudomonas aeruginosa*: New insights into pathogenesis and host defenses. Pathog Dis. 2013;67(3):159-73. https://doi.org/10.1111/2049-632X.12033

PMid:23620179

33. Sahu MC, Patnaik R, Padhy RN. *In vitro* combinational efficacy of ceftriaxone and leaf extract of *Combretum albidum* G. Don against multidrug-resistant *Pseudomonas aeruginosa* and host-toxicity testing with lymphocytes from human cord blood. J Acute Med. 2014;4:26-37. https://doi.org/10.1016/j.jacme.2014.01.004

34. AFT Pharmaceuticals Side Effects of Ceftriaxone. 2011. Available from: https://www.medsafe.govt.nz/Profs/Datasheet/c/ceftriaxoneaftinj.pdf [Last accessed on 2021 Sep 24].

35. Abdullah FE, Mushtaq A, Irshad M, Rauf H, Afzal N, Rasheed A. Current efficacy of antibiotics against *Klebsiella* isolates from urine samples-a multi-centric experience in Karachi. Pak J Pharm Sci. 2013;26(1):11-5.

PMid:23261722

36. Fung-Tomc J, Minassian B, Kolek B, Washo T, Huczko E, Bonner D. *In vitro* antibacterial spectrum of a new broad-spectrum 8-methoxy fluoroquinolone, Gatifloxacin. J Antimicrob Chemother. 2000;45:437-46. https://doi.org/10.1093/jac/45.4.437

PMid:10747819

37. National Committee for Clinical Laboratory Standards. Methods for Dilution Antimicrobial Susceptibility Tests for Bacteria that Grow Aerobically.4th ed. Approved Standard M7-A4. Villanova, PA: NCCLS; 1997.

38. Vahaboglu H, Budak F, Kasap M, Gacar G, Torol S, Karadenizli A, *et al*. High prevalence of OXA-51-type class D β-lactamases among ceftazidime-resistant clinical isolates of *Acinetobacter* spp.: Co-existence with OXA-58 in multiple centres. J Antimicrob Chemother. 2006;58:537-42. https://doi.org/10.1093/jac/dkl273

PMid:16816400

39. Colodner R, Rock W, Chazan B, Sakran W. Risk factors for developing extended-spectrum beta-lactamase-producing bacteria in non-hospitalized patients. Eur J Clin Microbiol Infect Dis. 2004;23(3):163-7. https://doi.org/10.1016/j.ijantimicag.2004.06.001

PMid:14986159

40. Pfaller M, Davenport D, Bale M, Barrett M, Koontz F, Massanari RM. Development of the quantitative micro-test for slime production by coagulase-negative staphylococci. Eur J Clin Microbiol Infect Dis. 1998;7(1):30-3. https://doi.org/10.1007/bf01962167

PMid:3132372

41. Sharma M, Yadav S, Chaudhary U. Biofilm production in uropathogenic *Escherichia coli*. Indian J Pathol Microbiol. 2009;52(2):294. https://doi.org/10.4103/0377-4929.48960

PMid:19332956

42. Poovendran P, Vidhya N, Murugan S. Antimicrobial susceptibility pattern of ESBL and non-ESBL producing uropathogenic *Escherichia coli* (UPEC) and their correlation with biofilm formation. Intl J Microbiol Res. 2013;4:56-63. https://doi.org/10.5829/idosi.ijmr.2013.4.1.7123

43. Ehlers MM, Veldsman C, Makgotlho EP, Dove MG, Hoosen AA, Kock MM. Detection of bla_{SHV}, bla_{TEM} and bla_{CTX-M} antibiotic resistance genes in randomly selected bacterial pathogens from the Steve Biko Academic Hospital. FEMS Immunol Med Microbiol. 2009;56(3):191-6. https://doi.org/10.1111/j.1574-695X

PMid:19453751