Evaluation of Antibiotic Sensitivity, Biofilm Formation Ability, and Prevalence of Extended-Spectrum Beta-Lactamase (ESBLs) in Clinically Isolates *Escherichia coli* and *Klebsiella pneumoniae*

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ABSTRACT

Background & Objective: Extended-spectrum beta-lactamases (ESBLs) are enzymes in bacteria that resist many antibiotics. Detection of ESBLs production is important as it's a marker of colonization and potential transfer to other patients. We studied the antibiotic susceptibility, biofilm formation capacity, and prevalence of ESBLs of opportunistic bacteria including *K. pneumoniae* and *E. coli*. The isolates capable of biofilm formation were analyzed among 100 *E. coli and* 104 *K. pneumoniae* isolates.

Materials & Methods: This process involved collecting and identifying bacterial samples, testing antibiotic susceptibility, detecting ESBLs phenotypes, and multidrug-resistance (MDR) isolates, assessing biofilm formation capability, and evaluating results through statistical analysis.

Results: The susceptibility tests for discs were performed following the guidelines outlined by the Clinical Laboratory Standards Institute in 2023 (CLSI). *K. pneumoniae* exhibits inherent resistance to ampicillin, while 80 (80%) strains of *E. coli* have been reported to be resistant to ampicillin. Additionally, 50 *K. pneumoniae* isolates and 41 *E. coli* isolates were found capable of forming a biofilm. Seven of *E. coli* (17.07%), and seven of *K. pneumoniae* (14%) isolates could form a mighty biofilm. It was observed that the strongest resistance in the isolates that formed strong biofilm was related to tetracycline with 5 (7.2%) resistance in *K. pneumonia* and 7 (7%) resistance in *E. coli*. Furthermore, 47 (47%) of *E. coli*, and 21 (20.2%) of *K. pneumoniae* isolates were classified as ESBLs producers, and 52 (50%) *K. pneumoniae* and 72 (72%) *E. coli* isolates were classified as MDR.

Conclusion: Considering the role of biofilm in the transfer of genes, appropriate health policies, and the correct administration of effective antibiotics can help in prevention.

Keywords: *Escherichia coli*, *Klebsiella pneumoniae*, Antibiotic-resistant, Biofilm ESBLs, MDR

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Introduction

Antibiotics have played a vital role in healthcare worldwide and have made significant contributions to the containment of a wide range of infections. However, the rise of antimicrobial-resistant strains can be directly linked to the inappropriate use of antibiotics. Currently, the primary causes of this resistance are the bacteria *Klebsiella pneumoniae* (*K. pneumoniae*) and *Escherichia coli* (*E. coli*). The increased occurrence of antimicrobial resistance (AMR) reduces the effectiveness of drugs, resulting in

costly and challenging treatment for patients. AMR is linked to high mortality rates, particularly among patients in intensive care units (ICUs) **(1, 2)**. Ultimately, the emergence of drug-resistant strains could potentially transport us back to the era before the discovery of antibiotics **(1)**. These microorganisms are widely discussed globally and are commonly found in the human digestive system as part of the normal microbial flora. Additionally, they are significant pathogens in humans. *E. coli is* the primary cause of most Gram-negative bloodstream and urinary tract infections (UTIs) in humans. It often colonizes the endocervix and vagina of women, leading to complications such as puerperal infections, neonatal sepsis, and Intra-amniotic infections in pregnant women **(1, 3)**. On the other hand, *Klebsiella* species can cause various medical complications, including pneumonia, UTIs, bloodstream infections, and sepsis **(2)**. Polymyxins like Polymyxin E (colistin) are essential antibiotics for treating infections from multidrug-resistant Gram-negative bacteria, often used as a last resort **(4)**. The global rise of antibiotic resistance is causing deep concern among experts. The pharmaceutical industry's slow progress in addressing this issue is bringing us dangerously close to a global disaster. Biofilm formation is a critical factor in both disease development and AMR **(5)**. This study is aimed at exploring the prevalence of AMR, general Spectrum ESBLs production, biofilm formation, and resistance patterns in clinical isolates of *K. pneumoniae* and *E. coli*.

Materials and Methods

Exploration and sample collection of bacterial isolates:

In this cross-sectional study conducted in the northwestern region of Iran between September 2021 and March 2022, samples of patients admitted to the hospital were gathered to detect isolates of *K. pneumoniae* and *E. coli* from urine, blood, sputum, and fecal matter. These isolates were derived from diverse patients suspected of exhibiting clinical infections and were admitted to various departments within the hospital. There was a lack of direct interaction with the patients, and all laboratory examinations were carried out on bacteria obtained from clinical samples that were transported to the laboratory. Blood specimens containing citrate anticoagulant were incubated and preserved in Tryptic Soy Broth (TSB) at 37°C. Isolate identification was initially conducted using established biochemical microbiological techniques and media, including MacConkey agar, gram staining, and a range of biochemical assays such as indole, sulfide mobility (SIM), citrate, methyl red (MR), Voges Proskauer (VP), triple sugar iron (TSI), Deoxyribonuclease (DNase), Urease, Gelatinase, Lysine decarboxylase (LDC), Oxidase, and Catalase. Standard biochemical assays such as VP and non-motility were integral in elucidating the biochemical attributes of K. pneumoniae isolates **(6-8)**.

Reconfirmation of isolates by PCR:

The biochemically identified isolates of *K. pneumoniae* and *E. coli* were validated once again through the utilization of polymerase chain reaction (PCR) testing targeting the *16S rRNA* gene and a distinctive haemolysin encoding gene (*khe*), respectively. To carry out this task, specific primers were employed based on a prior publication **(9, 10)**.

Antibiotic susceptibility test:

The susceptibility of disc release was assessed through compliance with the protocols established by the CLSI 2023 **(11)**. The discs utilized for the examination contained Amikacin (30µg), Cefepime (30µg), Ceftriaxone (30µg), Ampicillin (25µg), Imipenem (10µg), Cefotaxime (30 µg), Ceftazidime (10µg), Ciprofloxacin (5µg), Gentamicin (10µg), Tetracycline (30µg) (Mast, UK). The examination was conducted utilizing Mueller Hinton Agar (Merck, Germany). Following the incubation process, ESBL-producing isolates were identified through the utilization of Cefpodoxime, Cefotaxime, Ceftriaxone, Ceftazidime, and Azetronam by the guidelines established by the CLSI 2023. After this, by the recommendations outlined by the Centers for Disease Control and Prevention (CDC) and the European Center for Disease Prevention and Control (ECDC), multi-drug resistance (MDR) isolates were interpreted **(12)**. For control, *K. pneumoniae* ATCC 13883 was employed in this stage.

Phenotypic confirmation test for isolates producing ESBLs:

ESBLs phenotypes were ascertained through the analysis of the antibiogram results, employing the reference article as a basis **(13)**. To validate the production of ESBLs in the cultured bacteria, phenotypic testing was conducted in adherence with the guidelines provided by the CLSI 2023 disk release. This testing required the utilization of cefotaxime (30µg) and Ceftazidime (30µg) (Mast, UK) in conjunction with Clavulanic acid. The incubation of the Mueller Hinton's culture medium took place for a duration of 24 h at a temperature of 37°C. An evident augmentation exceeding 5 mm in size of the Clavulanic acid-combined discs as opposed to the discs lacking Clavulanic acid functioned as an indicator of ESBLs production in the cultured bacterial population. *K. pneumoniae* ATCC 13883 was utilized as a reference strain at this phase as well **(14)**.

Measuring biofilm formation in the laboratory:

To assess the formation of biofilm by *K. pneumoniae* and *E. coli*, we implemented the procedures that had been previously published **(15, 16)**. At first, the colonies were inoculated into TSB and the optical density (OD 600) was standardized to 0.7. Following this, a diluted suspension at a ratio of 1:200 (OD 600 = 0.005) in TSB supplemented with 1% glucose (TSBg) was then inoculated into a polystyrene microtiter plate (Nunc, Roskilde, Denmark). The plate was then incubated at 37°C for 16 h, following which the supernatant cells and plankton were washed thrice with an additional phosphate-buffered saline (PBS). The wells were subsequently treated with 150 µl of 0.1% crystal violet, and any surplus dye was removed through two rounds of rinsing with PBS buffer. The absorbance of crystal violet in 160 µl of alcohol/acetic acid solvent was then measured (concentration 4:1) was measured at a wavelength of 595 nm. Each sample underwent triplicate testing and the capacity for biofilm formation was determined using the subsequent formula: Optical density cutoff value (ODc)= mean OD of negative control + 3 times the standard deviation (3xSD) of negative control. The control utilized was the 0.5 McFarland standard *K. pneumoniae* ATCC 13883 in TYCSB suspension (equivalent to 1.5 x 10^8 CFU/mL). Spectroscopy was conducted using an ELISA reader at a wavelength of 595 nm **(16)**.

Results

According to the CLSI 2023 guidelines, susceptibility tests were conducted on discs that contained Gentamicin, Amikacin, Imipenem, Tetracycline, Ceftazidime, Cefepime, Ceftriaxone, Cefotaxime, Ciprofloxacin, and Ampicillin for disc release.Detailed data was shown in Table 1. *K. pneumoniae* is intrinsically resistant to ampicillin, while 80 (80%) strains of *E. coli* have been reported to be resistant to ampicillin. Additionally, 41 *E. coli* isolates and 50 *K. pneumoniae* isolates were found capable of forming a biofilm. 7 (17.07%) *E. coli* and 7 (14%) *K. pneumoniae* isolates formed a strong biofilm. It was observed that the strongest resistance in the isolates that formed strong biofilms was related to tetracycline with 7 (7%) resistance in *E. coli* and 5 (7.2%) resistance in *K. pneumonia*.Detailed data was reported in Table 2. Furthermore, 47 (47%) of *E. coli*, and 21 (20.2%) of *K. pneumoniae* isolates were classified as ESBLs producers, and 72 (72%) *E. coli*, and 52 (50%) *K. pneumoniae* isolates were classified as MDR (Figure 1).

Table 1. Antibiotic susceptibility pattern of isolated strains

	K. pneumoniae			*E. coli*		
	Susceptible	**Intermediate**	**Resistant**	**Susceptible**	**Intermediate**	**Resistant**
Cefepime	42 (40.38%)	10 (9.61%)	52 (50%)	57 (57%)	0	43 (43%)
Ampicillin		Intrinsic resistance		18 (18%)	2 (2%)	80 (80%)
Amikacin	59 (56.7%)	8 (7.6%)	37 (35.5%)	96 (96%)	3 (3%)	1 (1%)
Ceftriaxone	38 (36.5%)	3 (2.8%)	63 (60.5%)	45 (45%)	0	55 (55%)
Ceftazidime	44 (42.3%)	2 (1.92%)	58 (55.7%)	51 (51%)	14 (14%)	35 (35%)
Imipenem	57 (54.8%)	10 (9.6%)	37 (35.5%)	98 (98%)	1 (1%)	1 (1%)
Cefotaxime	28 (26.9%)	1 (0.9%)	75 (72.11%)	41 (41%)	1 (1%)	58 (58%)
Tetracycline	45 (43.2%)	4 (3.84%)	55 (52.8%)	45 (45%)	0	55 (55%)
Ciprofloxacin	45 (43.2%)	3 (2.8%)	56 (53.8%)	40 (40%)	-	60 (60%)
Gentamycin	55 (52.8%)	-	49 (47.1%)	81 (81%)	4 (4%)	15 (15%)

Table 2. Frequencies of a resistant pattern of utilized antibiotic based on the biofilm formation capability

	K. pneumoniae			*E. coli*		
	Weak biofilm	**Moderate biofilm**	**Strong biofilm**	**Weak biofilm**	**Moderate biofilm**	**Strong biofilm**
Cefepime	10 (9.7%)	8 (7.76%)	7(6.79%)	4 (4%)	12 (12%)	7 (7%)
Ampicillin	26 (25.2%)	15 (14.56%)	7 (6.79%)	15 (15%)	15 (15%)	6 (6%)
Amikacin	11 (10.67%)	5 (5.85%)	3 (2.91%)	0	1 (1%)	0
Ceftriaxone	14 (13.59%)	10 (9.7%)	7 (6.79%)	10 (10%)	12 (12%)	7 (7%)
Ceftazidime	12 (11.65%)	9 (8.73%)	7 (6.79%)	2 (2%)	8 (8%)	7 (7%)
Imipenem	7 (6.79%)	3 (2.91%)	6 (5.82%)	0	1 (1%)	0
Cefotaxime	17 (16.5%)	11 (10.67%)	7 (6.79%)	10 (10%)	12 (12%)	7 (7%)
Tetracycline	15 (14.56%)	8 (7.76%)	5 (7.28%)	11 (11%)	12 (12%)	7 (7%)
Ciprofloxacin	13 (12.61%)	8 (7.76%)	7 (6.79%)	12 (12%)	12 (12%)	3 (3%)
Gentamycin	1 (0/97%)	8 (7.76%)	6 (5.82%)	2 (2%)	6 (6%)	2 (2%)

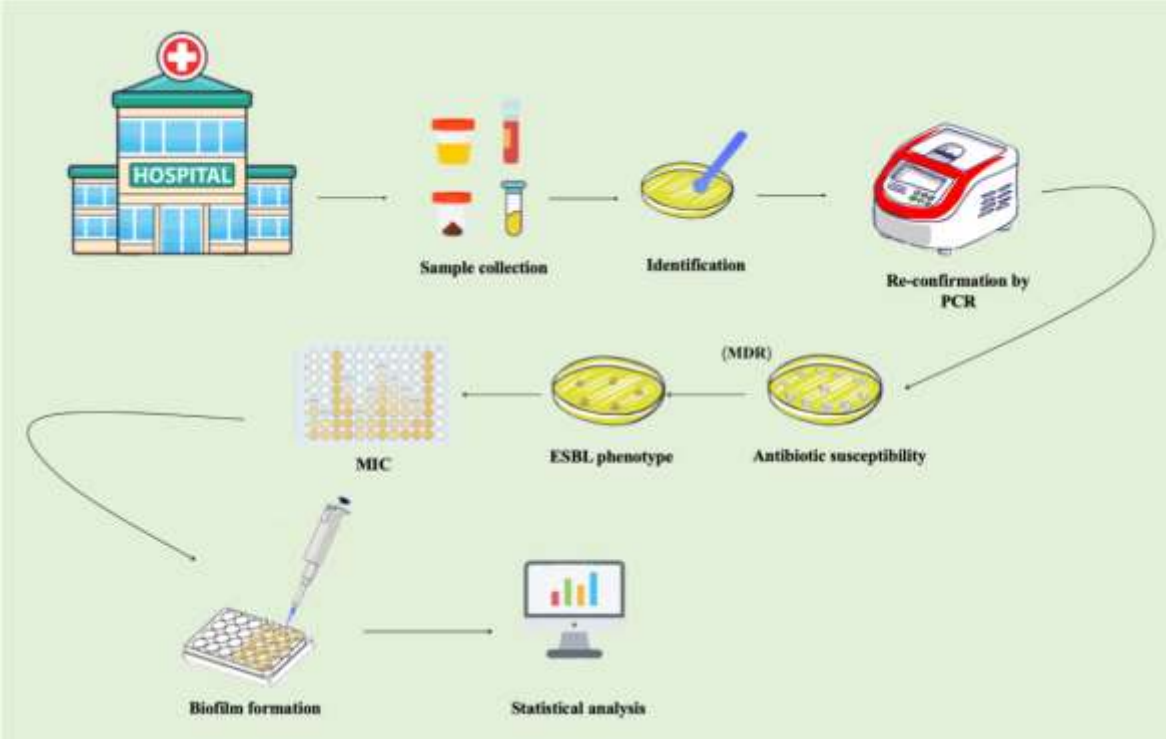


Figure 1. Graphical abstract

Discussion

Antibiotic-resistant strains present a considerable menace to humanity. When they acquire resistance to various therapies, their chances of survival diminish significantly. Pathogens that develop resistance to drugs often lead to treatment defeat and impose a substantial public and economic burden on the healthcare sector **(12)**. Over time, more strains become resistant to antimicrobial treatments. The identification and management of these strains may prevent catastrophic consequences resulting from the sluggish progress in the pharmaceutical industry **(17)**. Our investigation discovered that the prevalence of Ampicillin resistance was 80% in *E. coli*. Another inquiry conducted by Daoud *et al.* in 2021 studied the levels of resistance in *E. coli* isolates and reported that Amikacin exhibited the highest resistance at 98.6%, while Ampicillin displayed the lowest resistance at 39.1% **(17)**. Mostafavi *et al.* demonstrated that Trimethoprim/sulfamethoxazole (31.3%) and Imipenem (94.9%) were respectively ineffective and effective antibiotics against *E. coli* isolates in 2019 **(18)**. Variations in the outbreak of antibiotic resistance among patients with *K. pneumoniae* infections can be attributed to the specific treatment regimens employed in hospitals and the infection control strategies employed in the study areas. Our disc diffusion antibiogram findings indicate that Ampicillin exhibits the highest resistance ratio. These findings align with a study by Tanhaei *et al.* in 2020, which revealed that Ampicillin exhibited a 100% resistance rate among *K. pneumoniae* isolates, while Amikacin displayed the highest susceptibility rate at 90% **(19)**. According to a systematic review in 2018 on carbapenemase resistance in *K. pneumoniae* in Iran, aztreonam exhibited the highest rate of antibiotic resistance at 55%, while Amikacin exhibited the lowest rate at 23% **(20)**. A study conducted in Spain by Lopes *et al.* in 2020 demonstrated that *K. pneumoniae* isolates exhibited a relatively low rate of resistance to Amikacin (25%) compared to ciprofloxacin (76%) **(21)**. It is of paramount importance to consider epidemiological data obtained before prescribing antibiotics. MDR *Enterobacteriaceae* isolates have the potential to cause multiple infections in both animals and humans. The formation of biofilm constitutes a regulatory factor in bacterial species that facilitates the adhesion of bacterial cells to biological materials, thereby assisting microorganisms in evading the host's immune defense outbreak **(22, 23)**.

Conclusion

The contemplation of the biofilm's function in gene transfer necessitates the implementation of suitable health policies and the accurate administration of efficacious antibiotics, which can be instrumental in the prevention of this phenomenon.

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Conflict of Interest

The authors declare that they have no conflict of interest.

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Ethics Approval and consent to participate

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Authors' Contributions

All authors read and approved the manuscript. Contributions of the authors in this study were as follows: M Sh: Bacterial isolation, writing, Laboratory experiment, Sample collection, H S: Laboratory experiment, data analysis and, curation, B M: Conceptualization, supervision, methodology, final editing, revision.

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