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Combatting Multidrug Resistance in Bacteria: A Novel Approaches for Assessing Efflux Pumps

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Abstract

In order to defend themselves, bacteria contain efflux pumps that assist in evacuating harmful chemicals outside the cell. Antibiotic-active efflux is one of the main mechanisms of drug resistance in bacteria. Membrane transporters use a variety of unrelated compounds as substrates to mediate the efflux process. Because of this, the presence and activity of efflux pumps have a significant impact on the effectiveness of antibiotics, which contributes to bacteria developing drug resistance. Six samples were used in the investigation, and four were found to be E. coli, while the other two were Klebsiella pneumoniae. These isolates demonstrated efflux pump-mediated multidrug resistance. Notably, these bacteria with the Integron integrase 1 gene, called resistant strains, were resistant to 25 antibiotics. Including Aztreonam, Ampicillin/Sulbactam, Amoxyclav, Cefepime, Cefepime/Tazobactam, Ampicillin, and Cefotaxime, resistance was observed in both E. coli and Klebsiella pneumoniae. Given the complexity of drug efflux transporters, novel approaches are essential to combat multidrug resistance in bacteria effectively. A glucose-triggered rhodamine 6G (R6G) efflux assay from Sigma-Aldrich, USA, was used to test how well efflux pumps work.

Keywords: Efflux pump; Rhodamine 6G (R6G); Multi-drug resistance.

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1. Introduction

Antibiotic resistance is posing a growing threat to public health globally, making it increasingly challenging for us to effectively treat bacterial illnesses. To withstand the onslaught of antibiotics, bacteria have evolved several mechanisms, one of which, efflux pump-mediated resistance, has emerged as a key contributor to multidrug resistance (MDR) in both Gram-negative and Gram-positive pathogens [1, 4]. Antibiotic efflux is one of the most prevalent resistance mechanisms found in a wide variety of pathogenic bacteria [5, 6]. Bacterial efflux pumps perform as transporters and environmental stress defences, preventing multidrug resistance and influencing bacterial pathogenicity [7]. Overexpression of efflux pumps is critical in the development of antimicrobial resistance, including multidrug resistance. Multidrug efflux pumps work at the frontline to shield bacteria from antimicrobials by lowering drug intracellular concentrations. This protective barrier is made up of a group of transporter proteins that are found in the membrane and periplasm of bacterial cells. These proteins take things like antibiotics, organic solvents, poisonous heavy metals, and so on out of bacterial cells [8]. Overexpression of transporters can result in the co-selection of antimicrobial resistance traits as well as an impact on bacterial pathogenicity via biofilms and quorum sensing [1, 9, 10]

This study looks at whether efflux pumps are present and how well they work in clinically important bacterial isolates. This is done to find out how widespread and important MDR caused by efflux pumps is. This work focuses on the multidrug resistance of E. coli and Klebsiella pneumoniae, two bacteria that have received a lot of attention due to their clinical importance and growing resistance profiles [11].

Novel approaches are necessary to effectively treat efflux pump-mediated MDR in the face of these challenges. It is essential to research cutting-edge strategies to combat multidrug resistance, safeguard the efficacy of antibiotics, and enhance therapeutic outcomes due to the complexity of drug efflux transporters [12]. Energy is necessary for these pumps to operate, and ATP hydrolysis or, in some cases, glucose metabolism can provide this energy [13]. A glucose-triggered rhodamine 6G (R6G) efflux assay is used in this study to find out how efflux pumps work and how they help bacteria become resistant to antibiotics.

In addition to emphasising the critical role efflux pumps play in antibiotic resistance, this study also emphasises the urgent need for novel approaches to successfully combat multidrug resistance. Understanding the intricate processes of resistance in E. coli and K. pneumoniae will enable us to support current initiatives to preserve the effectiveness of antibiotics and ensure successful therapeutic outcomes.

2. Materials and Methods

2.1. Isolation and culture of bacteria

In this study, we used 6 samples, of which 2 were *Klebsiella pneumoniae* and 4 were E. coli. These pathogenic bacterial samples were obtained from Rajshahi Medical College's laboratory and were grown on Luria Broth (LB) agar plates. The samples were obtained using a sterile loop and maintained overnight at 37°C in an incubator. The next day, all bacterium colonies were identified and cultured on Extended Spectrum Beta-Lactamase (ESBL) media, which was kept at 37°C overnight for screening.

2.2. Antibiotic Susceptibility Test

The antibiotic susceptibility test, also known as the disc diffusion test or Kirby-Bauer test, is a popular method for finding specific antibacterials for a given ailment. Mueller-Hinton agar is the preferred agar for this test. The antibiotic sensitivity of isolated bacteria was assessed using standard techniques [14].

Isolated bacterial strains were subjected to susceptibility testing against a range of antibiotics, which are Ampicillin, Amikacin, Amoxyclav, Ampicillin/Sulbactam, Azithromycin, Aztreonam, Bacitracin, Carbenicillin, Cefepime, Cefepime/Tazobactam, Cefotaxime, Chloramphenicol, Ciprofloxacin, Co-Trimoxazole, Lomefloxacin, Gatifloxacin, Imipenem, Levofloxacin, Rifampicin, Tetracycline, Vancomycin, Neomycin, Norfloxacin, Nitrofurantoin, and Netilmicin. Those antibiotics were assessed to determine their effectiveness against the isolated bacterial strains.

2.3. Determination of Efflux Pump Activity

As described in [15], the glucose-induced efflux of rhodamine 6G (R6G) (Sigma-Aldrich, USA) assay was used to measure how active ABC-type drug transporters were in bacterial cells.

The bacterial colony was transferred into 10 ml of LB liquid medium using a sterile inoculation loop from an agar plate. In an incubation shaker at 150 rpm, the culture was incubated at 37 °C for 20 h, and the cell number was determined using a hemocytometer. From the overnight culture, we added 1 ml (a total of 5 x 108 cells) into 100 ml of fresh LB liquid medium. We had incubated the cells in an incubation shaker at 150 rpm for 1.5–2 h at 37 °C until they reached the exponential phase (1 x 107 cells/ml). The cell density was monitored by counting the cell number in a hemocytometer, then the cells were harvested using centrifugation at 3,000 x g for 5 min at laboratory temperature, and the supernatant was discarded. The cell pellet was washed twice with 50 ml of sterile distilled water (room temperature, RT), then with 50 ml of HEPES/NaOH assay buffer and vortex. The cells were collected by centrifugation (3000 x g, 5 min, RT). The cell pellet (total cell number 109) was resuspended in 50 ml of HEPES/NaOH assay buffer (RT) containing 2-deoxy-D-glucose (2 mM) and R6G (10 µM).

The cells were collected by centrifugation (3000 x g, 5 min, RT) at 150 rpm for 2 h at 28 °C. Then, the cells were collected by centrifuging them for 5 minutes at 3,000 x g at 4 °C. The pellet was washed with 50 ml of sterile distilled water (4 °C) and then with 50 ml of HEPES/NaOH assay buffer (4 °C). Resuspend the pellet to 108 cells/ml in 50 ml of HEPES/NaOH (4 °C) assay buffer. Transfer 5 ml of the cell suspension to a new 50 ml centrifuge tube and add 500 µl of glucose to a final concentration of 2 mM from a 20 mM stock solution. In the case of the negative control experiment, glucose is not supplemented in the culture medium. We put 400 µl of cell suspension into a microcentrifuge tube at 0, 10, 15, 20, and 30-minute intervals. We then harvested the cells by centrifuging them at 10,000 x g for 1 minute at 4 °C. Finally, we put 100 µl of the supernatant into a well of a microtiter plate.

The supernatant is enough for three technical replicates. We kept the samples on ice until the experiment was finished. The spectrofluorometer was used to measure the R6G fluorescence of the samples at laboratory

temperature (excitation wavelength 515 nm; emission wavelength 555 nm).

3. Results

3.1. Screening of isolated bacteria from people

There were two steps involved in the isolation and identification processes. First, LB agar, and then ESBL selective media for screening. The ESBL-selective medium was used to grow just the bacteria whose colonies are blue for *Klebsiella pneumoniae* and purple for *E. coli*. We used six samples in this study. Four of them were *E. coli*, which was purple, and two were *K. pneumoniae*, which was blue (Figure 1). Sequencing was performed on this subset for confirmation.

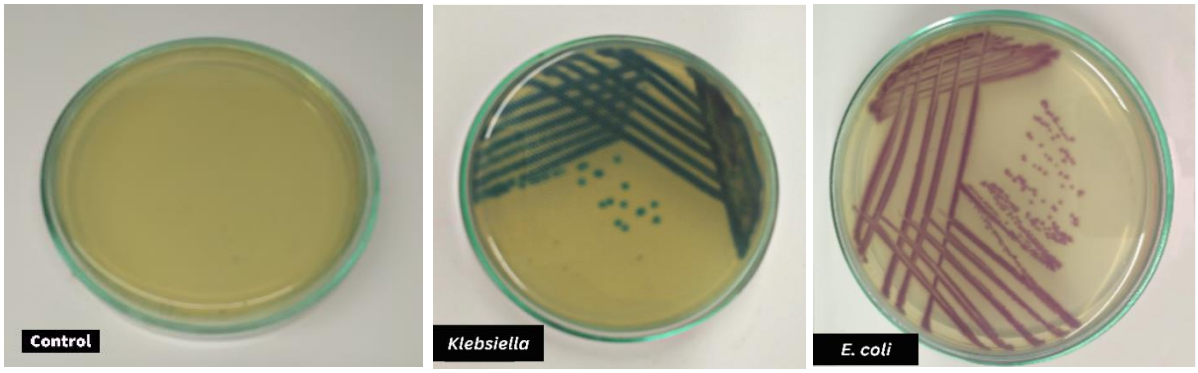


Figure 1: Bacterial samples on ESBL selective media show blue and pink colors indicating *Klebsiella pneumoniae* and *E. coli* respectively.

3.2. Antibiotic Susceptibility Profile

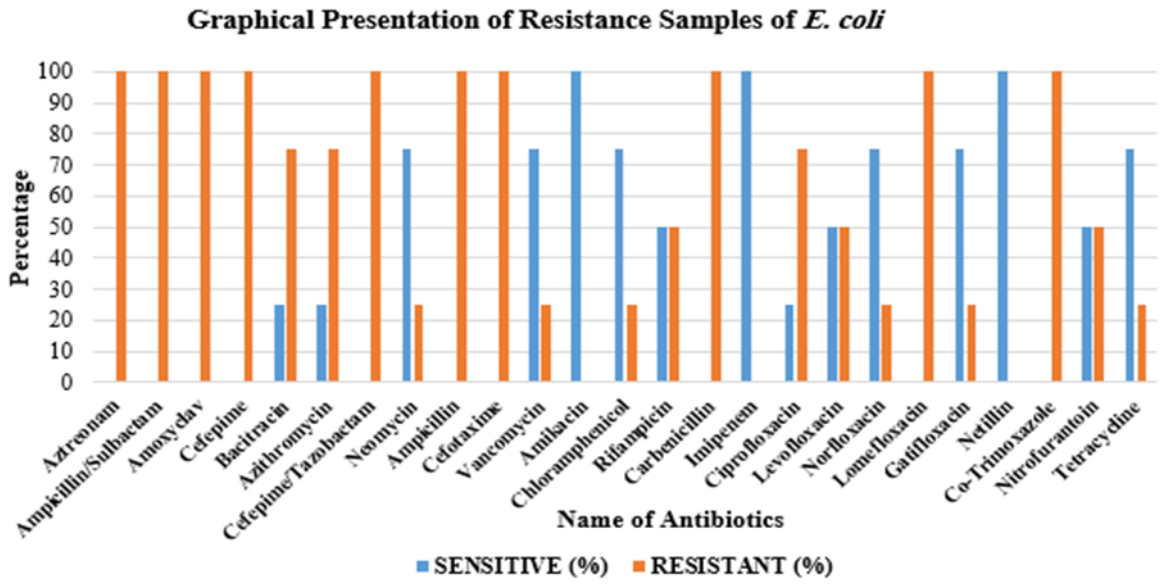


Figure 2.1: This graph represents the results of the antibiotic sensitivity tests performed among 25 antibiotics in *E. coli*. The *E. coli* was completely resistant against Aztreonam, Ampicillin/Sulbactam, Amoxyclav, Cefepime,

Cefepime/Tazobactam, Ampicillin, Cefotaxime, Vancomycin, Carbenicillin and Lomefloxacin.

Twenty-five different antibiotics were used to test the antibiotic susceptibility of six samples. All of the samples of *E. coli* are resistant to Ampicillin, Ampicillin/Sulbactam, Amoxyclav, Cefepime, Cefepime/Tazobactam, Co-Trimoxazole, Carbenicillin, Lomefloxacin, Azthrenam, and Cefotaxime. In *Klebsiella pneumoniae*, all are resistant to Ampicillin, Amoxyclav, Ampicillin/Sulbactam, Cefepime, Aztreonam, Cefepime/Tazobactam, Cefotaxime, and Nitrofurantoin. Ampicillin, Amoxiclav, Ampicilin/Salbactam, Aztrnam, Cefepime, Cefepime/Tazobactam, and Cefotaxime are all resistant to either strain. The results are shown in Figures 2.1 and 2.2 as a summary.

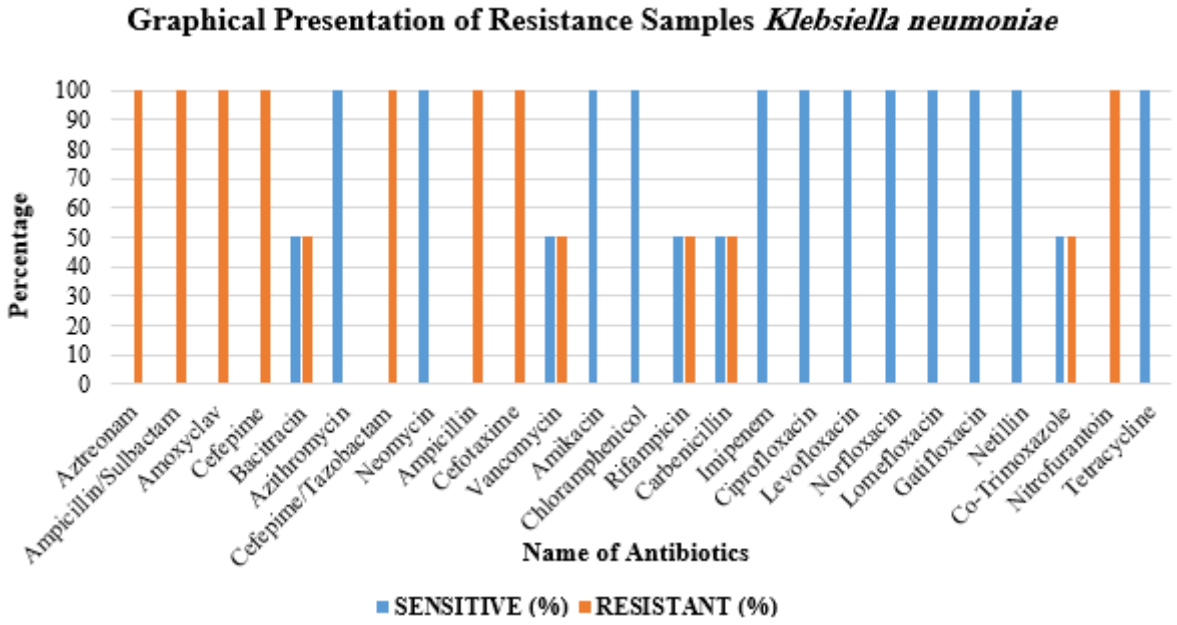


Figure 2.2: This graph represents the results the antibiotic sensitivity tests performed among 25 antibiotics against *Klebsiella pneumoniae*. The Klebsiella pneumoniae completely resistant against Aztreonam, Ampicillin/Sulbactam, Amoxyclav, Cefepime, Cefepime/Tazobactam, Ampicillin, Cefotaxime and Nitrofurantoin.

3.3. Determination of Efflux Pump Activity

It was an ATP-binding cassette transporter that is energy-dependent and mediates R6G efflux in isolates. Firstly, it was tested with 20 mM glucose (+glucose) and then with 2 mM 2-deoxy-D-glucose (-glucose) to find out what kind of activity the ABC transmembrane transporter does best. (+glucose) significantly increased ABC-type efflux activity in both *E. coli* and *Klebsiella pneumonia*. RLU is an abbreviation for relative luminescence units (Figure 3).

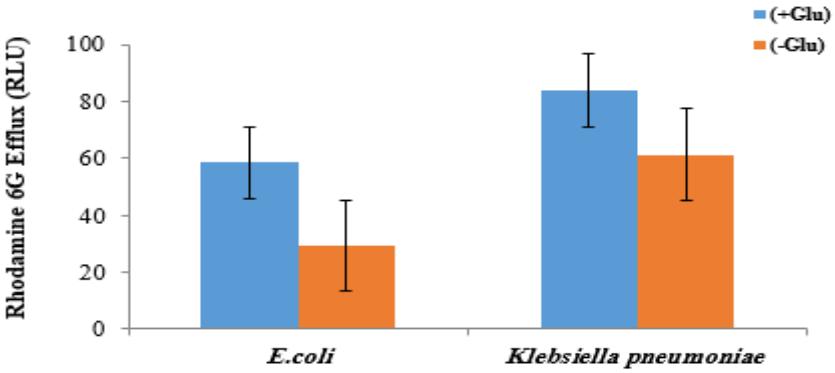
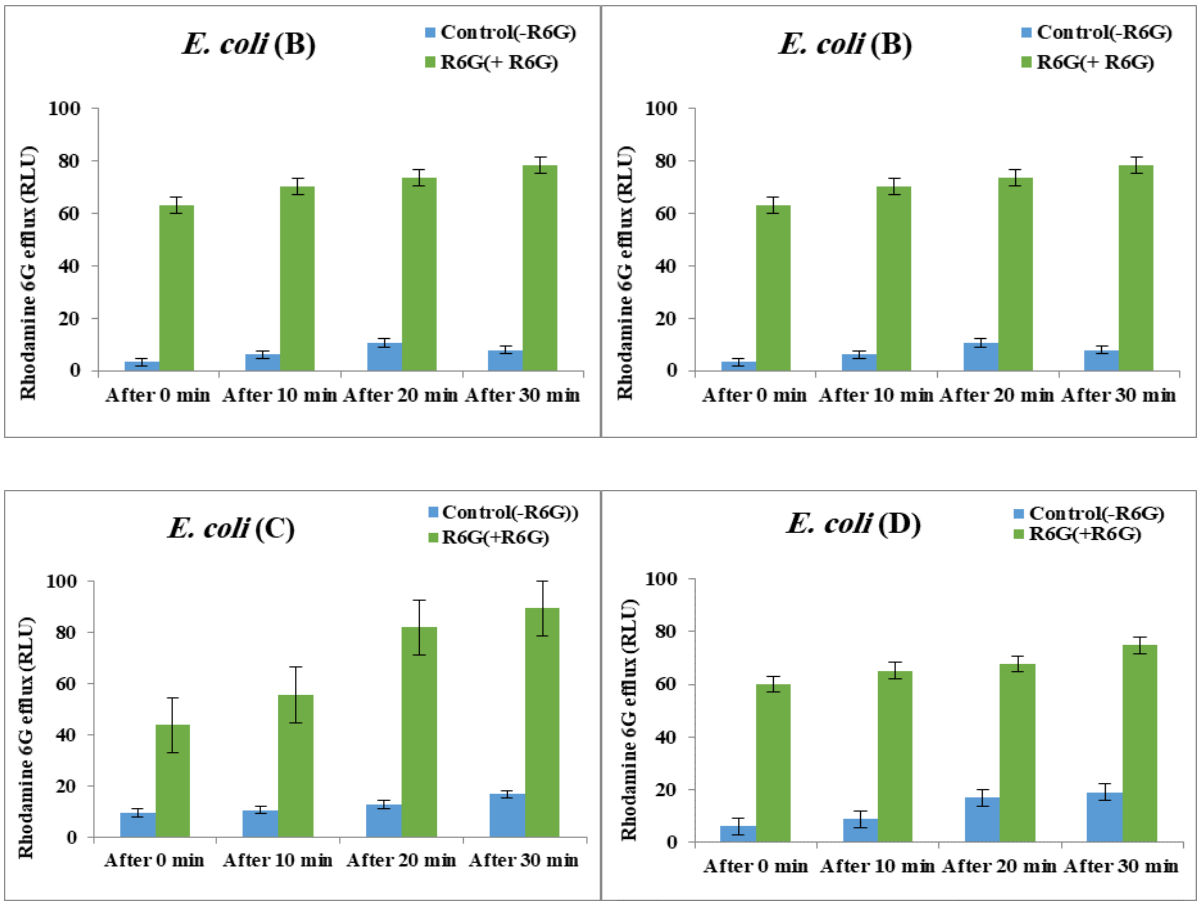


Figure 3: An ATP-binding cassette transporter that is energy-dependent mediates R6G efflux in isolates. Firstly, it was tested with 20 mM glucose (+glucose) and then with 2 mM 2-deoxy-D-glucose (-glucose) to find out what kind of activity the ABC transmembrane transporter does best. (+glucose) significantly increased ABC-type efflux activity in both *E. coli* and *Klebsiella pneumonia*. RLU is an abbreviation for relative luminescence units.

In this experiment, the rate of rhodamine 6G (R6G) efflux in clinical isolates of bacteria increases with time (data were taken after 0, 10, 20, and 30 minutes). It was evaluated in the presence of 20 mM glucose with R6G (R6G) and without R6G (control). When compared to the control (no R6G), using R6G increased the amount of glucose-induced R6G efflux activity in E. coli (A, B, C, and D) and Klebsiella pneumoniae (E and F).



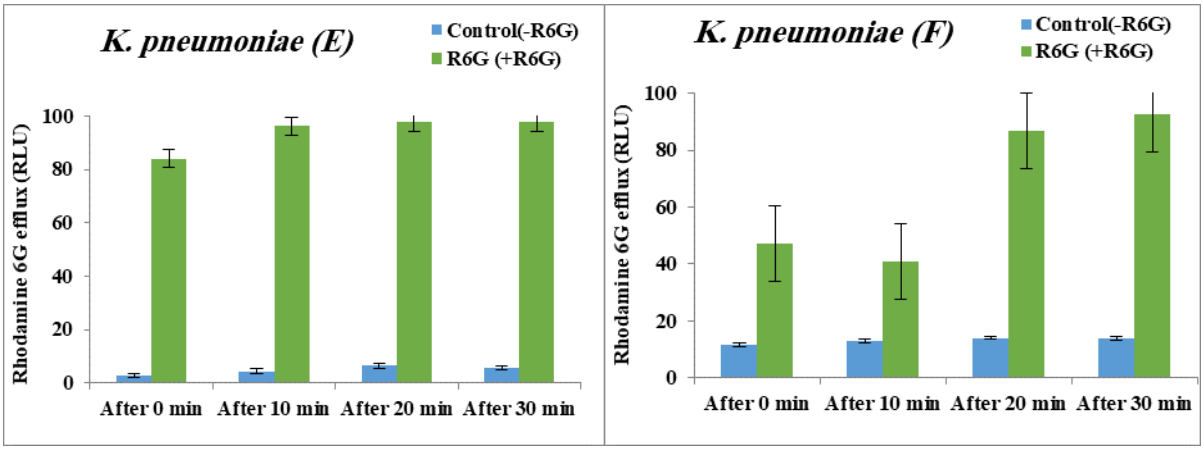


Figure 4: The graph represents the rate of rhodamine 6G (R6G) efflux in clinical isolates of bacteria increases with time interval. It was evaluated in the presence of 20 mM glucose with R6G (R6G) and without R6G (control). When compared to the control (no R6G), using R6G increased the amount of glucose-induced R6G efflux activity in *E. coli* (A, B, C, and D) and *Klebsiella pneumoniae* (E and F). Each experiment was repeated at least three times with similar results.

4. Discussion

The findings of this study shed light on the critical role of efflux pumps in bacterial drug resistance and emphasised the worrying incidence of multidrug-resistant strains, notably among E. coli and Klebsiella pneumoniae. Efflux pumps are powerful defence mechanisms that remove antibiotics and other toxic substances from within bacterial cells, lowering intracellular drug concentrations and making antibiotics less effective [2]. The use of a glucose-triggered rhodamine 6G (R6G) efflux assay from Sigma-Aldrich, USA, gives strong evidence that these pumps work in the tested bacterial strains.

It is quite concerning to see that all of the isolates showed efflux pump-mediated multidrug resistance, with two of them being Klebsiella pneumoniae and the other four being E. coli. Multidrug-resistant bacteria significantly restrict treatment choices because of their resistance to various classes of antibiotics [11]. In this study, 25 different antibiotics were used. Ampicillin, Amoxiclav, Ampicilin/Salbactam, Aztrnam, Cefepime, Cefepime/Tazobactam, and Cefotaxime were found to not work against the multidrug-resistant bacteria. This widespread resistance highlights the critical need to treat multidrug resistance in these bacterial species [16].

The Integron integrase1 gene is a crucial genetic component of multidrug-resistant bacteria's resistance [17]. Integron is a genetic component that makes it easier for resistance genes to be acquired and expressed, and they frequently play a crucial role in the emergence of multidrug resistance. The resistance was demonstrated by the existence of the intI 1 gene, which was tested using standard protocols in our earlier study [14]. The identification of this gene in the isolates suggests that horizontal gene transfer may have played a significant role in shaping the resistance phenotype [18]. To fully comprehend the mechanisms underlying multidrug resistance, additional research into the genetic determinants of resistance, including the function of mobile genetic elements, is necessary.

Resistance to a wide range of antibiotics, including several routinely used therapeutic drugs, has serious consequences for antibiotic therapy. It presents a difficult challenge for doctors treating infections caused by these multidrug-resistant bacteria, not only complicating treatment but also increasing the chance of treatment failure, leading to extended sickness and potentially deadly results [19]. Antibiotic resistance is a critical clinical and public health issue.

Given the intricacy of drug efflux transporters, this demonstrates the need for new strategies to combat bacterial multidrug resistance. Research into efflux pump inhibitors, novel antibiotic classes, and combination therapy holds promise for reducing this serious issue [1, 20]. Furthermore, researching the genetic basis of resistance and the possibility of gene silencing or modification tactics may lead to novel solutions to multidrug resistance [21,23].

Finally, this work emphasizes the importance of efflux pumps in bacterial drug resistance and the occurrence of multidrug-resistant strains, notably among E. coli and Klebsiella pneumoniae. These results underline the significance of ongoing research in this area and highlight the urgent need for creative approaches to deal with multidrug resistance in bacteria. To maintain antibiotic effectiveness and guarantee successful clinical treatment outcomes, antibiotic resistance must be effectively combated.

5. Conflicts of interest

No conflict of interest from the authors regarding the publication of this manuscript.

6. Author's contribution

The study procedure was conceptualized and designed.: Ariful Haque and Arnaba Saha Chaity; Carried out experiments: Arnaba Saha Chaity, Dipa Roy, Md. Mahmudul Hasan Maruf; Analysis and interpretation of data: Arnaba Saha Chaity, Dipa Roy, Md. Mahmudul Hasan Maruf; Manuscript preparation: Dipa roy; Finalizing the manuscript: Ariful Haque; Critical review of the manuscript for significant intellectual substance : All authors; Technical, material, or administrative assistance: Institute of Biological Sciences, Rajshahi, Bangladesh; Supervision: Ariful Haque; Data visualization: Ariful Haque and Dipa Roy.

7. Ethical approval

Ethical approval was taken from the Institutional Animal, Medical Ethics, Biosafety and Biosecurity Committee (IAMEBBC) of the Institute of Biological Sciences, University of Rajshahi. Memo No: 37 (21)/320/ IAMEBBC/IBSc.

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