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Efflux Pump Activity as a Determinant of Multidrug Resistance in Acute Otitis Media-Associated Gram-Negative Pathogens

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ABSTRACT: Background: Antibiotic resistance presents a significant health concern, complicating the treatment of common infections. Acute Otitis Media (AOM) is increasingly difficult to manage due to the rise of multidrug-resistant (MDR) pathogens. This study aimed to identify the bacterial etiologies of AOM and characterize their resistance patterns, with a particular focus on the role of efflux pumps in MDR.

Methods: Samples were retrieved from AOM patients admitted to intensive care unit (ICU) and surgical wards of Al-Haboubi Hospital, Nasiriyah, Iraq. Gram staining and colony morphology were exploited for characterization of bacterial isolates. Kirby-Bauer disc diffusion method was used to assess resistance against common antibiotic agents, while Ethidium Bromide cartwheel assay was utilized for efflux pump activity and associated phenotypes.

Results: A total of 150 (100 from AOM patients and 50 healthy controls) samples were analyzed, which identified the following pathogens: *Pseudomonas aeruginosa*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, and *Escherichia coli*. The hospital-acquired pathogens showed substantially higher MDR levels compared to community-acquired strains. Likewise, the efflux pump activity was amplified in hospital-associated pathogens. Statistical analysis revealed a strong association between efflux pump activity and antibiotic susceptibility, particularly in *Klebsiella pneumoniae* and *Pseudomonas aeruginosa* isolates.

Conclusion: This study found high MDR levels in pathogens isolated from AOM patients, likely governed by active efflux mechanisms. Targeting the efflux pump activity may be a potential solution for overcoming MDR.

INTRODUCTION

Acute otitis media (AOM) is one of the most common infection encountered clinically, particularly in young patients. Although the use of antibiotics is the primary treatment of AOM, their substantial and often inappropriate use had triggered a marked increase in antimicrobial resistance (AMR). Indeed, studies have shown an alarmingly high AMR in bacterial strains retrieved from AOM patients (Hullegie et al. 2021). Consequently, the AMR potentially compromise treatment outcomes,

leading to limited quality of life and increased burden of healthcare system (Blaskovich and Cooper 2025; R. T. Khan et al. 2024). Altogether, these findings accentuate the need for a deeper comprehension of AMR mechanisms in AOM patients and beyond (Elshobary et al. 2025).

Classically, the bacterial etiology of AOM has been dominated by typical community-acquired organisms such as *Haemophilus influenzae*, *Streptococcus pneumoniae*, and *Moraxella catarrhalis* (Hu et al. 2021; Schilder and Damoiseaux 2021). However, recent pathogenesis studies have revealed an elevated prevalence of Gram-negative bacteria in both community and hospital settings, including *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*, *Acinetobacter baumannii*, and *Escherichia coli* (Gavrilovici et al. 2022; Nawaz et al. 2023b). These pathogenesis are of particular concern due to their intrinsic resistance profiles and their frequent association with multidrug-resistant (MDR) phenotypes (Lia et al. 2021; Niño-vega, Ortiz-ramírez, and López-romero 2025; Zhang et al. 2025).

Active efflux pumps have emerged as a primary contributor to MDR in Gram-negative pathogens, among other diverse mechanisms. This particular secretion systems of bacteria allow the removal of a wide variety of different antibiotics from cells. In effect, they decrease the amount of antibiotic in bacteria, which reduces the effectiveness of the antibiotic. Some efflux systems that belong to the resistance-nodulation-division (RND) family, most notably the MexAB-OprM, AcrAB-TolC, MexB-OprM, MexCD-OprJ, and MexEF-OprN systems, are critically important in the removal of antibiotics through active transport (Detweile and Ala 2025; Routh et al. 2011). These systems also help the bacteria respond to stress from outside sources, in addition to their role in creating MDR. Although efflux pumps have been studied primarily in experimental animal models, and therefore, there is a wealth of structural information about their function, the clinical implications of these studies have yet to be fully explored concerning the development of MDR among pathogens responsible for AOM (Ojha et al. 2025; Zack, Sorenson, and Joshi 2024). The vast majority of clinical studies on MDR infections have been performed on patients with invasive disease or special patient populations. However, there are relatively few studies that correlate the increased activity of efflux with MDR phenotypes for AOM isolates (El-sayed, Messiha, and Zafer 2026; Mather et al. 2019). One potential future research avenue will be the objective comparison of efflux activity between nosocomial and community-generated AOM isolates, particularly in less developed countries, although these data do not currently exist.

The study aimed to assess both the bacteriology of AOM in an Iraqi hospital and how this bacteriology exhibited varying degrees of susceptibility to multiple antibiotics. Specifically, the study aimed to integrate phenotypic susceptibility to antibiotics, efflux activity assays, molecular identification of efflux pump genes and correlate the phenotypes with genetic susceptibility data on MDR. Such mechanistic approach of the MDR may contribute to better understanding of clinical resistance in AOM patients, and thereby beneficial in developing appropriate regional strategies to combat efflux pump-mediated resistance in AOM patients.

MATERIALS AND METHODS

Study Setting and Ethical Considerations

Samples were obtained at Al-Hussein Teaching Hospital, Nasiriyah, Iraq; these samples were acquired from patients admitted to the intensive care unit (ICU), as well as to the surgical ward sections. These patients were suspected for bacterial infection, as indicated by their clinical and laboratory indices. Moreover, patients with no evidence of infection caused by resistant bacteria were treated as control group. Ethical approval was granted by the Al-Nahrain Specialist Laboratory, Dhi Qar Governorate, Iraq.

Bacterial Isolation and Identification

The bacterial specimen was obtained by aerobic culture at 37 °C using standard bacteriological medium (blood and MacConkey agar); bacterial identification of the various isolate organisms was determined using microbiological methods (i.e., Gram stain; colony morphology). The selected organism included *Escherichia coli*, *Acinetobacter baumannii*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*, and *Enterobacter spp.*

Antimicrobial Susceptibility Testing

To determine antibiotic resistance, the Kirby-Bauer disk diffusion method was utilized. Briefly, standard antibiotic disks containing the target antibiotic agents (i.e., ciprofloxacin, ceftriaxone, gentamicin and Imipenem) on agar plates was

inoculated with standard concentrations of bacteria. Afterwards, the sizes of inhibition zones (in mm) following incubation were measured. Pre-defined cutoff values were used to establish if sensitivity of the isolates were high, intermediate or resistant. A resistance to three or more antibiotics was called as MDR.

Assessment of Efflux Pump Activity

For assessment of efflux pump activity, the Ethidium Bromide Cartwheel Assay (EBCA) was performed. In this assay, the fluorescence pattern of each bacterial isolate after exposure to ethidium bromide was assessed. A weak fluorescent signal was expected from isolates that expel ethidium bromide from their inner compartment. Thus a reduced intracellular accumulation of the dye provides an evidence of efflux pump activity.

Statistical Analysis

For the statistical analyses we used MS Excel. The resistance rates between isolates from hospitals and control groups were compared using unpaired t-tests. Logistic regression was used to test for correlation between efflux pump activity and antibiotic resistance patterns.

RESULTS

This study enrolled 100 AOM patients and 50 healthy controls. Samples were collected from all these participants to assess the prevalence of bacterial pathogens and associated resistance profiles. Results demonstrated positivity of 106 samples for different types of Gram-negative bacteria. Specifically, *Klebsiella pneumoniae* was the most common organism ($n = 33$), followed by *Escherichia coli* ($n = 27$), *Pseudomonas aeruginosa* ($n = 26$), and *Acinetobacter baumannii* ($n = 20$), as summarized in Figure 1. Nosocomial isolates were relatively common among all species, with the ICU samples revealing the highest burden. Samples from patients admitted to ICU and surgical ward illustrated higher positivity for *K. pneumoniae* and *P. aeruginosa*, whereas *E. coli* was more frequently isolated from the control group. This may indicate a higher prevalence of *E. coli* in the community-associated infections.

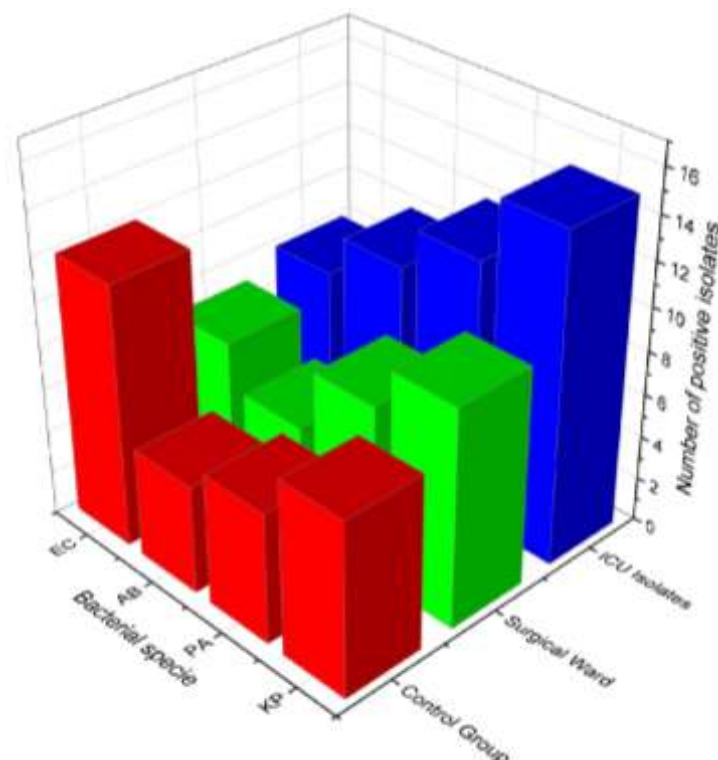


Figure 1: Positivity of samples for Gram-negative bacteria. AB: *Acinetobacter baumannii*, KP: *Klebsiella pneumoniae*, EC: *Escherichia coli*, PA: *Pseudomonas aeruginosa*

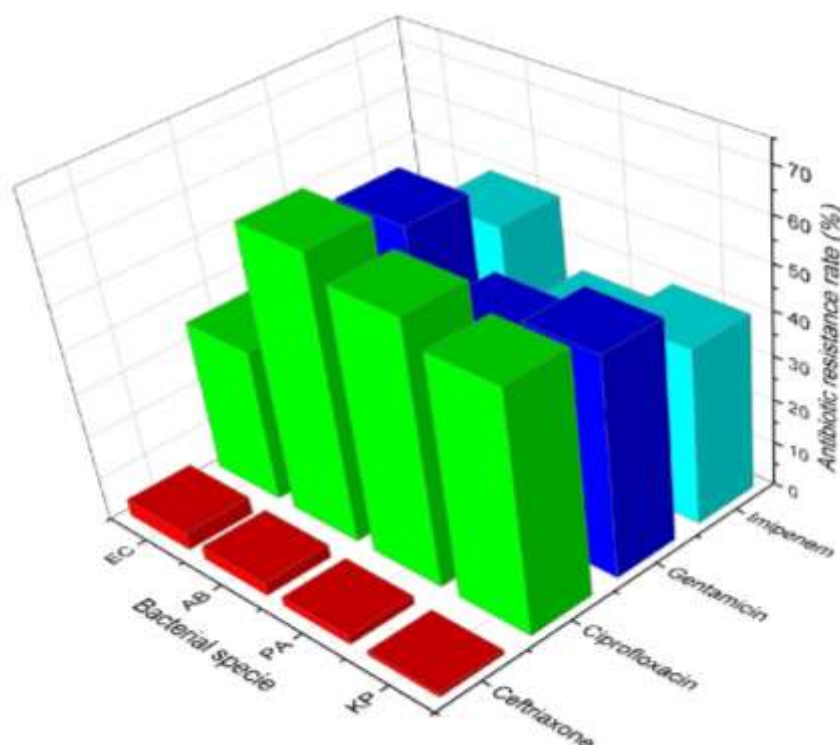


Figure 2: Antibiotic resistance rates (in percent) for the isolated samples. AB: *Acinetobacter baumannii*, KP: *Klebsiella pneumoniae*, EC: *Escherichia coli*, PA: *Pseudomonas aeruginosa*

Following separation of the bacteria-positive samples, their antibacterial susceptibility was tested against a set of common antibiotic agents (Figure 2). The results showed higher antibiotic susceptibility in nosocomial isolates. Specifically, *A. baumannii* had the most resistance to ceftriaxone (70%), followed by *K. pneumoniae* (60%) and *P. aeruginosa* (50%) (Figure 2). For both *A. baumannii* and *P. aeruginosa*, resistance to ciprofloxacin was 60%, while 55% in *K. pneumoniae*. Furthermore, gentamicin showed a variable resistance across the set of bacterial species; *E. coli* revealed 30% while *A. baumannii* showed 60% resistance to gentamicin (Figure 2). Finally, imipenem retained a relatively higher antibiotic activity, and showed resistance rates of 35–50% among the nosocomial isolates. For completeness, resistance to a minimum of three antibiotic agents/ classes fulfilled the criteria for MDR. Unpaired t-test showed that resistance to ciprofloxacin was significantly higher ($p > 0.001$) in nosocomial isolates compared to control samples. Likewise, resistance to imipenem was also significantly higher in nosocomial samples ($p = 0.00018$).

After evaluating the antibiotic resistance, the EBCA was used to assess the efflux pump activity (Figure 3). The results demonstrated a higher efflux pump activity for the nosocomial isolates compared to the control isolates. Specifically, a positive efflux phenotype was detected in 80% of hospital-derived isolates compared with 25% of control isolates (Figure 3). Moreover, molecular analysis of the bacterial isolates showed 70% of genes in nosocomial isolates, whereas only 15% genes in control isolates which were involved in efflux pump activities. The difference in efflux pump genes was significant between the two groups ($p < 0.0001$). Moreover, these results support the markedly higher antibiotic resistance in the hospital-acquired pathogens. This was confirmed with logistic regression analysis. Specifically, a strong association was revealed between the presence of efflux pump gene and antibiotic resistance. Hospital isolates expressing efflux pump genes demonstrated 5.6-fold increased likelihood of antibiotic resistance (odds ratio = 5.6; 95% CI: 2.4–13.1; $p < 0.0001$).

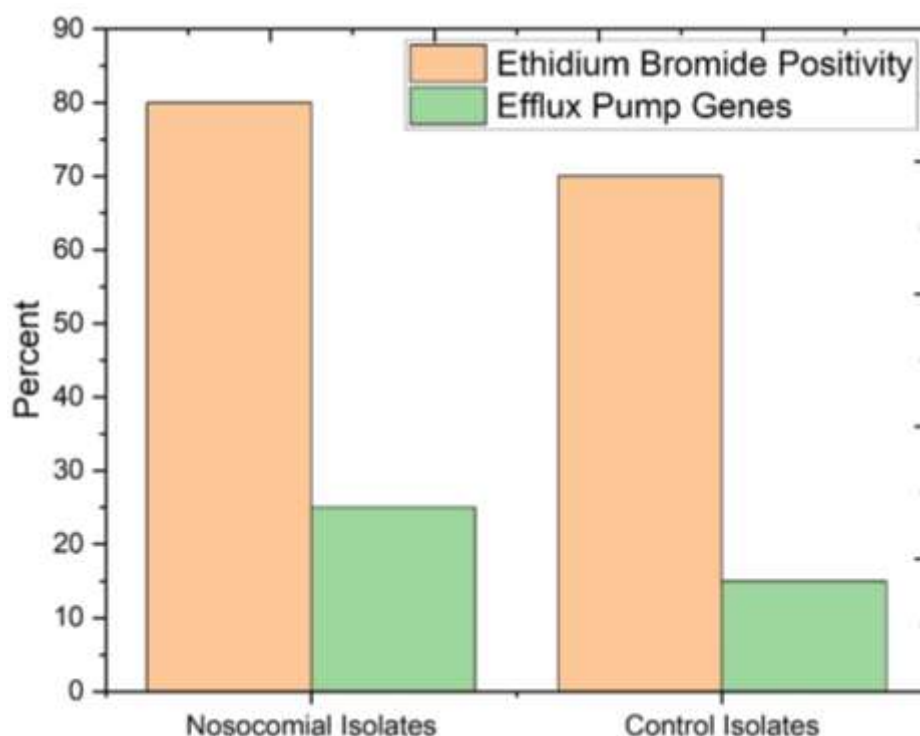


Figure 3: Efflux pump activity of the bacterial isolates and the associated genes

DISCUSSION

This study investigated the AMR profiles of bacterial pathogens isolated from AOM patients. The results demonstrated that the AOM was governed by several bacterial pathogens, including *Acinetobacter baumannii*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*, and *Escherichia coli*. This cohort of pathogens was consistently reported in AOM patients from other Arab countries (Kabra et al. 2021; Moghnieh et al. 2018). However, the typical bacterial strain seen in Europe and Western countries comprise of *Streptococcal*-dominated etiology (Sader et al. 2019). Such differences in bacterial profiles across different regions may indicate the explicit importance of local epidemiological data to govern antibiotic therapy effectively. Moreover, the present study also focused on exploring such differences in nosocomial versus community-derived infections. The results illustrated a significantly higher MDR burden among hospital-acquired isolates. This indicated the challenge to manage otologic infections of nosocomial origin.

This study demonstrated a higher prevalence of *K. pneumoniae*, *P. aeruginosa*, and *A. baumannii* in hospital-acquired isolates, which may be correlated to the opportunistic nature of these microbes (S. Chen, Liao, and Wang 2025). Since these pathogens are often assumed as harmless or sometimes dismissed as normal flora, these may, however, potentially cause severe infections, particularly in individuals presenting with compromised immune system (Samsing and Barnes 2024). On the other hand, these opportunistic pathogens also exhibit innate susceptibility mechanisms, and may acquire additional resistance under favorable antibiotic stimuli (Bianfang et al. 2025). The higher prevalence of such pathogens in samples retrieved from patients admitted to ICU and surgical ward may be attributed to longer stay at hospital, invasive (and even) multiple interventions, and extensive antibiotics utilization (J. Chen et al. 2023).

Results of this study are in accordance with previous studies. For instance, analysis of pathogens involved in ear infections at Al-Kindy Teaching Hospital, Baghdad showed that *Pseudomonas aeruginosa* was involved in 51% cases (Al-Hamamy, Salih, and Al-Mudallal 2021). Similar results have also been reported from other major cities of Iraq, including Busra (Alkhulaifi and Mohammed 2023) and Mosul (Abdulrahman Ahmed et al. 2024). Likewise, assessment of microbial infections in samples retrieved from hospitalized patients and community individuals of the neighboring countries demonstrated consistent pathogenic profiles. To exemplify, swab samples from Hail Maternity Hospital, Saudi Arabia showed positivity for different bacterial strains including *K. pneumoniae* (32.48%), *E. coli* (19.74%) and *P. aeruginosa* (8.9%) (M. A. Khan et al. 2018). Moreover,

Pseudomonas aeruginosa was the most common microbe in clinical isolates of Egypt (Helmy et al. 2023; Salem et al. 2025). In Jordanian population, the leading cause of otitis was *Pseudomonas aeruginosa*, followed by *Staphylococcus epidermis* and *Staphylococcus aureus* (Aljaafreha, Tawalbeh, and Shehabi 2019; Battikhi and Ammar 2004). In addition to Arab countries, epidemiology assessment of bloodstream infections in Thailand showed that one-quarter of infections exhibited MDR, and one-third of *Escherichia coli* were not susceptible to ceftriaxone (Chotiprasitsakul et al. 2023).

This study revealed a high resistance of the bacterial isolates retrieved from Iraqi individuals to commonly used antibiotic agents; these included the third-generation fluoroquinolones (i.e., ciprofloxacin), cephalosporins (i.e., ceftriaxone), and aminoglycosides (i.e., gentamicin). Moreover, carbapenems (i.e., imipenem) showed retention of a relatively better activity compared with other antibiotic agents, the observed resistance was, nevertheless, clinically concerning, particularly among *A. baumannii* and *K. pneumonia* (Figure 2). These findings are coherent with the global trends, where the efficacy of last-line antibiotic agents is diminishing (Nawaz et al. 2023a; Sánchez Arlegui et al. 2024). This suggests the need for strengthening infection control measures and a prudent use of antimicrobial agents (Chang Pitter et al. 2022).

The results from this study supports the hypothesis of efflux pump-mediated AMR (Figure 3). In particular, the hospital-acquired pathogens, as compared with community isolates, illustrated higher activity of efflux pump as well as amplified expression of associated genes. These results support the hypothesis that higher expression of genes involved in efflux pump (and thereby the efflux activity) may primarily govern the MDR in these pathogens, as suggested by statistical analyses (i.e., unpaired t-tests and logistic regression). Although not directly shown by this study, the efflux mechanism may also confer cross-resistance to multiple antibiotic classes (Gaurav et al. 2023). This may contribute to compromising therapeutic efficacy of antibiotic agents.

Studies aimed to explore AMR and associated variables offers direct clinical significance. It has been documented by several studies that AMR is typically higher in nosocomial isolates, as compared to community isolates (Bean, Krahe, and Wareham 2008; Cerini et al. 2023). This trend has been attributed to multiple factors, including amplified selectivity of nosocomial pathogens for antibiotic agents, longer stay of patients at hospital, undergoing invasive interventions, and overall higher resistance of bacterial strains grown in healthcare systems. In addition, assessment of higher MDR in hospital-acquired bacteria has also been correlated with several cofactors, such as old age, substantial use of antibiotics and inappropriate empiric therapy (Kumar et al. 2024). Moreover, another major difference is the resistance of community infections to older antibiotic agents, while innate MDR of the nosocomial pathogens. It has been shown that multi-model approach in treatment of infections caused by MDR pathogens is more effective in decreasing the burden of such infections (Li et al. 2023).

A few limitations of this study may be highlighted. Although the sample size (i.e., 100 AOM patients and 50 controls) showed significant associations, it may not be sufficient enough to generalize findings of this study to other settings. Also, the analyses were only focused on specific genes associated with efflux pump activity. However, other potential resistance mechanisms (e.g., enzymatic degradation or target site modification) were not evaluated. Finally, this was a cross-sectional study, which inherently limits characterization of temporal trends in resistance development to antibiotic agents. That said, it is suggested that future studies may focus on assessment of whole-genome sequencing, longitudinal design, and evaluation of efflux pump inhibitors. Such studies may enable to elucidate the antibiotic susceptibility pathways and facilitate targeted strategies for treatment.

CONCLUSION

This study illustrated a high prevalence of MDR in samples retrieved from AOM patients, particularly among those admitted to ICU and surgical wards. The efflux pump activity and associated genes were predominantly involved in mediating antibiotic resistance. The significantly higher AMR in hospital-derived isolates indicate the enhanced antimicrobial stewardship, routine resistance surveillance, and the development of novel therapeutic approaches. One possible approach to overcome the MDR may be the treatments targeting the efflux pump activity. However, large cohort longitudinal studies are warranted to establish these findings.

Declarations

Competing Interests

The author deny any conflicts of interest.

Data Availability

All relevant data have been presented in the manuscript.

Ethical Approval

Ethical approval was granted by the Al-Nahrain Specialist Laboratory, Dhi Qar Governorate, Iraq.

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Author Contributions

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Visualization: ASH, AAJA, OMM

Writing – original draft: RHJ, TAH, FHM, ASH, AAJA, OMM, IA

Writing – review & editing: RHJ, TAH, FHM, ASH, AAJA, OMM, IA

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