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Original Article

Prevalence of Erythromycin Resistance Genes in Gram-Positive and Gram-Negative Bacteria From Secondary Infections

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Abstract

Background: Bacterial secondary infections in patients can exacerbate disease symptoms and significantly increase mortality rates. This study aimed to evaluate the prevalence of bacterial agents responsible for secondary infections in hospitalized patients and to identify the presence of erythromycin resistance genes (*ermB*, *ermA*, and *mefA*) in bacterial isolates.

Methods: Samples from secondary infections were collected from hospitalized patients to determine the most prevalent bacterial pathogens and assess their resistance profiles using the polymerase chain reaction method.

Results: The bacterial species most frequently associated with secondary infections were *Escherichia coli* (32.2%), *Klebsiella pneumoniae* (31.2%), *Enterococcus faecalis* (24.3%), and *Acinetobacter baumannii* (11.3%). Testing for antibiotic susceptibility indicated that most bacterial isolates were resistant to erythromycin and ciprofloxacin. Notably, the *ermB* gene was detected in 89.6% of the isolates, while *ermA* and *mefA* genes were absent in all bacterial isolates.

Conclusion: These findings underscore the significant level of erythromycin resistance among pathogens responsible for secondary infections in hospitalized patients, with a predominant association with the *ermB* gene. This highlights the pressing need for improved antibiotic stewardship and the development of alternative therapeutic strategies to combat the growing threat of secondary bacterial infections in clinical settings.

Keywords: Secondary infections, Erythromycin, Resistance genes, ermB protein, Multidrug-resistant

Introduction

Erythromycin, a macrolide antibiotic, has long been a cornerstone in the treatment of bacterial infections, particularly those caused by gram-positive organisms, such as *Streptococcus* and *Staphylococcus* species. However, resistance to erythromycin has also emerged among gram-negative bacilli, raising significant concerns for infection control and public health (1).

Erythromycin is a macrolide antibiotic that inhibits bacterial protein synthesis by binding to the 50S ribosomal subunit. This action is more effective against gram-positive bacteria because their ribosomes are more accessible. However, gram-negative bacteria have an outer membrane that can restrict erythromycin's entry, thus making them less susceptible. Gram-negative bacilli exhibit resistance to erythromycin primarily through efflux systems that actively pump the antibiotic out of the cell, thereby preventing its accumulation. These systems, often encoded by genes such as *mef(A)*, significantly lower the intracellular concentration of erythromycin, rendering it ineffective. Although this mechanism is more common in gram-positive bacteria, certain gram-negative bacilli can also acquire resistance through the methylation of ribosomal RNA by *erm* (erythromycin ribosome methylase) genes. This methylation reduces erythromycin's binding affinity to the 23S ribosomal RNA in bacterial ribosomes, further diminishing its efficacy (2).

The spread of erythromycin resistance among gram-negative bacilli presents a significant challenge in the treatment of bacterial infections. This resistance not only limits the effectiveness of erythromycin and related macrolides but often necessitates the use of alternative antibiotics. The problem is particularly acute in hospital settings, where resistant bacteria are more prevalent (3).

Despite the introduction of newer antibiotics that have reduced mortality rates associated with secondary

infections, the overall incidence of such infections remains high. Moreover, the overuse of antibiotics has contributed to the emergence and spread of resistant bacterial strains, further complicating treatment efforts (4,5). Secondary infections have led to substantial human and financial losses worldwide in recent years. Secondary infection occurs when a new infection develops following a primary infection, often due to a weakened immune system, tissue damage, or disrupted microbiota. Preventive measures include vaccination, proper antibiotic use, and good hygiene maintenance to reduce the risk of complications. The availability of clinical data on bacterial infections is essential for guiding effective treatment strategies and mitigating the impact of these infections (6). This study aims to investigate the bacterial agents associated with secondary infections and evaluate the prevalence of erythromycin resistance genes among these pathogens.

Materials and Methods

Bacterial Strain

This study was conducted on 100 samples collected from patients with secondary infections hospitalized in medical centers in Isfahan in 2023. The specimens were cultured on Eosin-Methylene Blue agar and blood agar. Bacterial colonies were assessed for phenotypic characteristics, followed by Gram staining and biochemical tests to identify bacterial isolates.

The Kirby-Bauer disk diffusion method was employed to evaluate the antimicrobial susceptibility profiles of the isolates to commonly used antibiotics. Antibiotic discs containing erythromycin (15 μg), azithromycin (5 μg), penicillin G (10 μg), vancomycin (30 μg), ciprofloxacin (5 μg), gentamicin (10 μg), tetracycline (30 μg), and linezolid (10 μg) were placed on Mueller-Hinton agar plates inoculated with the isolates. The diameters of the inhibition zones around the discs were compared against the Clinical and Laboratory Standards Institute (CLSI) guidelines. Based on these standards, the isolates were categorized as resistant, intermediate, or susceptible (7).

Minimum Inhibitory Concentration and Minimum Bactericidal Concentration Determination

Erythromycin MIC was determined using a microdilution approach in 96-well microtiter plates. The amount of antibiotic required to prepare the stock solution was calculated based on CLSI guidelines using the following formula:

Weight of powder (mg) = [volume of solution (mL) × concentration (μg/mL)] / [potency of powder (μg/mg)]

The MIC was defined as the lowest concentration of the antibiotic at which no turbidity was observed, indicating the inhibition of visible bacterial growth. To determine the MBC, aliquots from wells without visible growth were subcultured onto fresh agar plates. The

MBC was identified as the lowest antibiotic concentration that achieved a 99.9% reduction in the initial bacterial count. The results were then compared with the standard reference values provided by the CLSI (8).

Molecular Identification of Selected Isolates

Target genes (*ermA*, *ermB*, and *mefA*) were identified via polymerase chain reaction (PCR) amplification using gene-specific primers listed in Table 1 (9,10). Genomic DNA was obtained through a heat-based extraction protocol.

The PCR mixture consisted of 25 μL, including 0.25 μL (1.25 U/μL) of Taq DNA polymerase, 0.5 μL (0.25 mM) of deoxynucleotide triphosphates (dNTPs), 0.75 μL (1.5 mM) of $MgCl_2$, 1 μL (5 pmol/μL) of forward and reverse primers, 17 μL of double-distilled water, and 2.0 μL of template DNA. PCR was initiated with a denaturation step at 94 °C for 3 minutes, followed by 30 cycles comprising denaturation (94 °C for 30 seconds), annealing (55 °C for 30 seconds), and extension (72 °C for 45 seconds). A final elongation was performed at 72 °C for 5 minutes. Amplification products were resolved by electrophoresis on a 1.5% agarose gel and visualized using SYBR Green fluorescence (11).

Data Analysis

Data processing and statistical evaluation were conducted using SPSS (version 21), and a *P*-value less than 0.05 was considered indicative of a significant difference.

Results

The study included 100 samples from patients with secondary infections, of which 66.0% were female and 34.0% were male. The frequency of secondary infections was significantly higher in females than in males, as determined by the chi-square test (*P* = 0.001). The age of the patients with secondary infections ranged from 1 year to 96 years, with an average age of 59.38 ± 23.94 years (Table 2). The highest frequency of secondary infections was observed in the age group of 60–79 years (34.0%). Based on the chi-square test, a significant difference in infection frequency was found across different age groups (*P* < 0.001). However, Fisher's exact test did not reveal a significant difference in infection frequency between males and females.

A total of 115 bacterial isolates were recovered from 100 samples of secondary infections. One bacterial isolate was

Table 1. The Specifications of Primers Used for Erythromycin Resistance Genes

Product Length (bp)	Nucleotide Sequence	Gene Name
189	5'-AAGCGGTAAACCCCTCTGA-3' 5'-TTCGCAAATCCCTTCTCAAC-3'	*ermA*
424	5'-GGAACATCTGTGGTATGGCG-3' 5'-CAT TTAACGACGAAACTGGC-3'	*ermB*
348	5'-AGTATCATTAATCACTAGTGC-3' 5'-TTCTTCTGGTACTAAAAGTGG-3'	*mefA*

obtained from each of the 85 samples, while two isolates were obtained from each of the remaining 15 samples. The identified isolates included 37, 36, 14, and 28 *Escherichia coli*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, and *Enterococcus faecalis*, respectively (Figure 1).

The highest prevalence of secondary infections was observed in urinary tract infections (UTIs) (81.7%), followed by blood infections (8.7%). On the other hand, the lowest prevalence was associated with joint fluid, peritoneal fluid, cerebrospinal fluid, wound cultures, and tracheal tube samples (Table 1). Fisher's exact test revealed no significant relationship between the type of infection and the type of bacterial isolate ($P = 0.096$).

Based on the results of the antibiotic susceptibility testing, all bacterial isolates were resistant to at least one antibiotic. The highest resistance was found against erythromycin (89.6%), followed by ciprofloxacin (62.1%) and gentamycin (75.0%). Interestingly, resistance to azithromycin was observed in 70.3% of the isolates. Significant differences in resistance were noted for erythromycin, ciprofloxacin, and linezolid among the bacterial isolates. In terms of antibiotic resistance profiles, *E. coli* isolates were more sensitive to antibiotics compared to other isolates, whereas *A. baumannii* isolates exhibited the lowest sensitivity to antibiotics (Table 2).

The MIC values of erythromycin for isolates in the susceptibility test were determined using the broth microdilution method (Table 3). The frequency of erythromycin-resistant bacteria ranged from 16.4% in *E. coli* to 90.9% in *A. baumannii*, as determined by the microdilution plate method.

An investigation into the presence of erythromycin resistance genes in the isolates revealed that *ermB* was the most detected gene in *E. coli*, *K. pneumoniae*, *E. faecalis*, and *A. baumannii* (Figure 2).

The *ermA* and *mefA* genes were not observed in any

Table 2. Antibiotic Resistance Patterns Among Isolates From Secondary Infections

Antibiotic	*E. coli*		*K. pneumoniae*		*E. faecalis*		*A. baumannii*		*P* Value
	No.	%	No.	%	No.	%	No.	%	
Erythromycin	34	91.9 [a]	34	94.4 [a]	25	89.3 [a]	8	57.1 [b]	0.021
Azithromycin	26	70.3 [a]	27	75.0 [a]	25	89.3 [b]	6	42.8 [c]	0.064
Vancomycin	-	-	-	-	16	57.1 [b]	-	-	0.086
Ampicillin	35	94.6 [a]	33	91.7 [a]	17	60.7 [b]	14	100.0 [a]	0.063
Ciprofloxacin	31	83.8 [a]	31	86.1 [a]	18	64.3 [b]	14	100.0 [c]	0.032
Tetracycline	35	94.6 [a]	33	91.7 [a]	22	78.6 [b]	14	100.0 [a]	0.508
Gentamycin	30	81.1 [a]	33	91.7 [a]	20	71.4 [b]	13	92.9 [a]	0.891

Note. E. coli: Escherichia coli; K. pneumoniae: Klebsiella pneumoniae; E. faecalis: Enterococcus faecalis; A. baumannii: Acinetobacter baumannii. *Numbers marked in a row with the same letters had no significant difference in terms of antibiotic resistance.

Table 3. MIC Values of Erythromycin for 115 Isolates

Bacteria	MIC (µg/mL)								
	> 0.2	> 0.4	> 0.8	> 0.16	> 0.32	> 0.64	> 0.128	> 0.256	> 0.512
E. faecalis	-	-	-	2	-	1	2	13	10
E. coli	1	-	3	1	3	3	4	15	7
K. pneumoniae	1	-	1	1	3	1	4	18	7
A. baumannii	2	1	-	1	1	3	-	2	3

Note. MIC: Minimum inhibitory concentration; E. coli: Escherichia coli; K. pneumoniae: Klebsiella pneumoniae; E. faecalis: Enterococcus faecalis; A. baumannii: Acinetobacter baumannii.
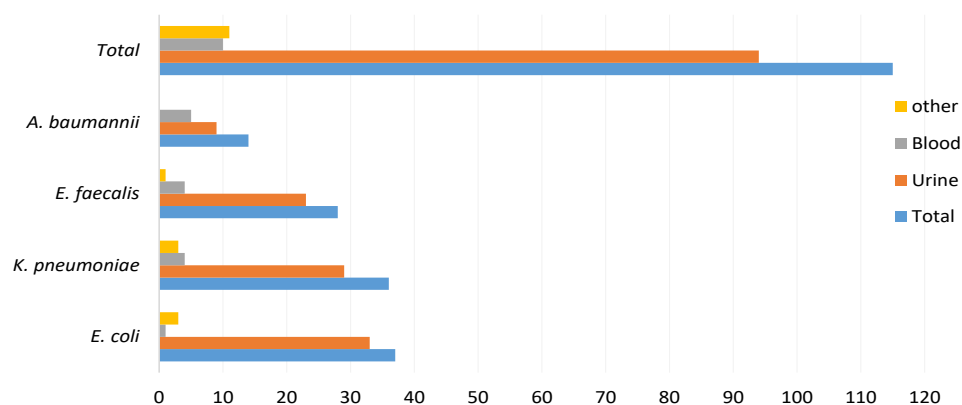

Figure 1. Distribution of Bacteria Isolated From Secondary Infections. *Note. E. coli: Escherichia coli; K. pneumoniae: Klebsiella pneumoniae; E. faecalis: Enterococcus faecalis; A. baumannii: Acinetobacter baumannii*

of the isolates. Additionally, the relationship between the presence of the *ermB* gene and resistance to erythromycin and azithromycin was examined, and no significant association was found in *E. coli* and *E. faecalis* (Figure 3).

Table 3 presents the relationship between the presence of the *ermB* gene and resistance to erythromycin and azithromycin in various isolates from secondary infections. According to the results of Fisher's exact test, there was no significant association between erythromycin resistance and the presence of the *ermB* gene in *E. coli* ($P=0.343$) and *E. faecalis* ($P=0.530$).

The *ermB* gene was detected in all isolates of *E. faecalis* and *A. baumannii*. Based on the results of Fisher's exact test (Table 4), no significant association was found between azithromycin resistance and the presence of the *ermB* gene in *E. coli* ($P=0.672$) and *E. faecalis* ($P=0.408$).

Discussion

The present study identified *E. coli*, *K. pneumoniae*, and *E. faecalis* as the most common causes of secondary infections, with UTIs being the most frequent (81.7%). In

addition, *E. coli* was the predominant pathogen in urinary infections, while *A. baumannii* was the most common cause of blood infections. Gram-negative bacilli, including *K. pneumoniae* and *A. baumannii*, were prevalent in secondary infections. In the study performed by Sharma et al on bacterial co-infections and secondary infections, *A. baumannii* and *K. pneumoniae* were the most common isolates (35.6% and 18.1%), respectively (12). Likewise, Saeed et al concluded that gram-negative (including *K. pneumoniae*, *P. aeruginosa*, multidrug-resistant *A. baumannii*, and *E. coli*) and Gram-positive (coagulase-negative *Staphylococci*, *E. faecalis*, *E. faecium*, and *S. aureus*) bacteria were the most common species (13).

The prevalence of secondary bacterial infections and the types of bacteria involved vary across studies and regions. While the current study and that of Saeed et al (13) found a high prevalence of gram-negative bacilli, studies conducted by Sharma et al (12) and Moreno-García et al reported different results in terms of the most common bacterial isolates (14). These variations may be due to regional bacterial strains, hospital settings, and patient

Table 4. Association Between the Presence of the *ermB* Gene and Resistance to Azithromycin and Erythromycin in Secondary Infection Samples

Bacteria	Azithromycin						Erythromycin					
	Sensitive		Resistant		Carrying *ermB*	P Value	Sensitive		Resistant		Carrying *ermB*	P Value
	%	No.	%	No.			%	No.	%	No.		
E. coli	72.7	8	80.8	21	+		100.0	3	76.5	26	+	
	27.3	3	19.2	5	-	0.672	0.0	0	23.5	8	-	0.672
	100.0	3	100.0	34	Total		100.0	3	100.0	34	Total	
K. pneumoniae	100.0	9	100.0	27	+		100.0	2	100.0	34	+	
	0.0	0	0.0	0	-	-	0.0	0	0.0	0	-	-
	100.0	9	100.0	27	Total		100.0	2	100.0	34	Total	
E. faecalis	66.7	2	87.0	20	+		100.0	2	83.3	20	+	
	33.3	1	13.0	3	-	0.408	0.0	0	16.7	4	-	0.408
	100.0	3	100.0	23	Total		100.0	2	100.0	24	Total	
A. baumannii	100.0	7	100.0	6	+		100.0	5	100.0	8	+	
	0.0	0	0.0	0	-	-	0.0	0	0.0	0	-	-
	100.0	7	100.0	6	Total						Total	

Note. *E. coli*: *Escherichia coli*; *K. pneumoniae*: *Klebsiella pneumoniae*; *E. faecalis*: *Enterococcus faecalis*; *A. baumannii*: *Acinetobacter baumannii*.
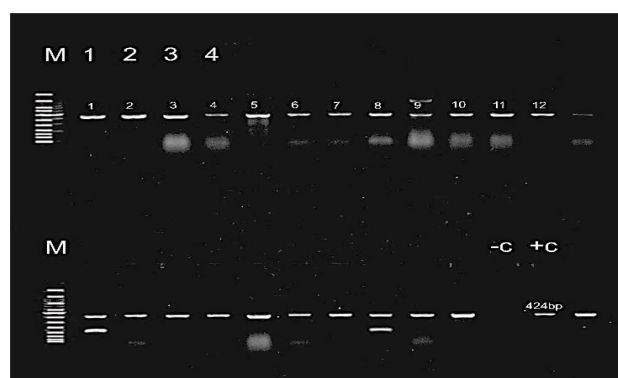

Figure 2. Agarose Gel Electrophoresis of the *ermB* Gene. *Note*. *S. aureus*: *Staphylococcus aureus*. A *S. aureus* strain with *ermB* and distilled water were the positive control (C+) and the negative control (C), respectively. Bands corresponding to the *ermB* gene are observed at 424 bp
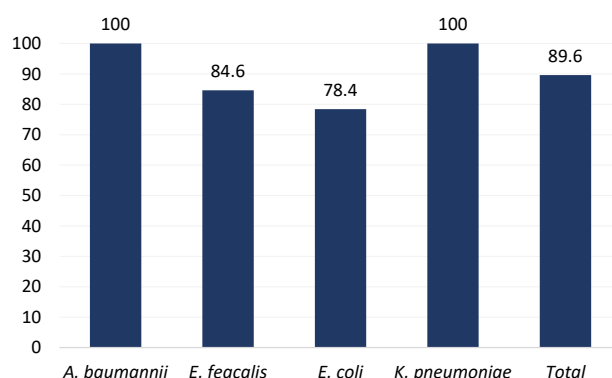

Figure 3. Prevalence of the *ermB* Resistance Gene in Isolates From Secondary Infections. *Note*. *E. coli*: *Escherichia coli*; *K. pneumoniae*: *Klebsiella pneumoniae*; *E. faecalis*: *Enterococcus faecalis*; *A. baumannii*: *Acinetobacter baumannii*

populations. However, all studies emphasized the presence of drug-resistant bacteria, highlighting the importance of continuous monitoring and targeted antibiotic treatment strategies for patients with secondary bacterial infections.

Studies on erythromycin resistance in gram-negative bacilli indicate significant concerns regarding antibiotic resistance, especially from infections acquired in hospitals and the community. Resistance mechanisms in gram-negative bacteria often involve the production of efflux pumps, antibiotic-modifying enzymes, and alterations to the bacterial cell envelope, preventing effective penetration of antibiotics like erythromycin (15).

This study revealed the significant prevalence of the *ermB* gene in the bacterial isolates. However, the detection of the *ermB* gene did not show a direct association with azithromycin resistance in *E. coli* and *E. faecalis*, implying that other factors might play a role in the development of antibiotic resistance. These findings underscore the importance of continuously monitoring both antibiotic resistance patterns and the presence of resistance genes in bacterial infections.

Considering that erythromycin is not the preferred antibiotic for eliminating infections caused by gram-negative bacilli according to CLSI guidelines, there are limited published studies in this area (16). Nonetheless, given that erythromycin and azithromycin were used to treat secondary infections, they were considered in this study. The resistance levels to these two antibiotics were assessed in all gram-positive and gram-negative isolates. Rostami et al found that the *ermTR* and *mefA* genes were present in 37% and 11.1% of isolates, respectively (17). In the study by Akrami et al, the prevalence of secondary infections in southern Iran was reported as 72.7%, with *S. aureus* and *K. pneumoniae* being the most frequently isolated bacteria. Their findings also revealed that 68.7% of these isolates demonstrated multidrug resistance (18).

Based on the results of Fallah et al, erythromycin resistance in *S. pneumoniae* isolates was 84.93%. The *ermB* and *mefA* genes were identified in 58.06% and 16.13% of erythromycin-resistant *S. pneumoniae*, respectively (19). This is particularly concerning given the role of these bacteria in critical infections, including UTIs, respiratory tract infections, and infections associated with surgical wounds.

Roohbakhsh Ghiasi et al investigated the frequency of erythromycin resistance genes in *Klebsiella*, *Enterobacter*, and *Proteus* isolates from UTIs. The frequency of *ermA* and *ermB* genes in the 120 studied isolates was 22.5% and 20.6%, respectively, while phenotypic resistance to erythromycin was 76.7% (20). The high levels of resistance suggest that these bacteria may harbor multiple resistance mechanisms, such as the production of extended-spectrum beta-lactamases, which complicates treatment strategies. The presence of the *ermB* gene, which is linked to resistance to macrolides like erythromycin, has been frequently observed in various isolates. However, it is important to note that while the *ermB* gene may contribute

to resistance, other genetic factors (e.g., modifications in antibiotic target sites or efflux pump activity) might also play a role in conferring resistance (21). Consequently, understanding these mechanisms is crucial for managing resistance in clinical settings, particularly as resistance continues to rise among gram-negative pathogens.

Conclusion

This study highlights the alarming prevalence of erythromycin resistance among bacterial pathogens associated with secondary infections in hospitalized patients, with the *ermB* gene emerging as the dominant resistance determinant. The detection of *ermB* in both gram-positive and gram-negative isolates, particularly in *E. coli*, *K. pneumoniae*, *E. faecalis*, and *A. baumannii*, underlines the gene's potential role in widespread macrolide resistance. The absence of *ermA* and *mefA* suggests a localized genetic pattern of resistance that warrants further investigation. Our findings emphasize the urgent need for comprehensive antibiotic stewardship programs, continuous surveillance of resistance genes, and the development of novel antimicrobial therapies to mitigate the growing threat of multidrug-resistant infections in healthcare settings.

Authors' Contribution
Conceptualization: Maryam Mohammadi-Sichani, Soodabeh Rostami.
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Project administration: Maryam Mohammadi-Sichani.
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Validation: Soodabeh Rostami.
Visualization: Mohammadsaid Golabi.
Writing-original draft: Mohammadsaid Golabi.
Writing–review and editing: Maryam Mohammadi-Sichani.

Competing Interests
The authors have no conflict of interests to declare.

Ethical Approval
This study was performed with the approval of the Medical Ethics Committee (ID: IR.IAU.PS.REC.1400.196). Informed consent for blood sampling and the bronchoalveolar lavage fluid procedure was obtained from each guardian.

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