

Frequency of *blaSHV* and *blaTEM* Genes in Clinical Isolates of *Enterobacter*, along with the Determination of Antibiotic and Probiotic Resistance Patterns

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Abstract

Background: Urinary tract infections are among the most prevalent human infections, primarily caused by *Enterobacteriaceae*. Currently, a significant number of *Enterobacteriaceae* produce extended-spectrum beta-lactamases (ESBLs), rendering them resistant to beta-lactam antibiotics and resulting in treatment failures.

Objectives: The aim of this study was to determine the frequency of *blaSHV* and *blaTEM* genes in *Enterobacter* isolates from clinical samples at Amir al-Momenin Hospital in Maragheh city in 2023, as well as to assess their antibiotic and probiotic resistance patterns.

Methods: One hundred urine samples from patients with urinary tract infections hospitalized at Amir Al-Momenin Hospital were included in the study. ESBL-producing bacteria were identified using the agar disk diffusion method according to CLSI criteria, employing 30 µg ceftazidime and cefotaxime antibiotic disks, both with and without clavulanic acid. PCR was utilized to amplify the genes for examining the frequency of *blaTEM* and *blaSHV* genes. Electrophoresis of the samples was conducted on a 1% agarose gel.

Results: Of the 21 samples, the ESBL index was negative in 7 samples, resulting in a frequency of 33.33%, while it was positive in 14 samples, with a frequency of 66.67%. The frequency of the *blaTEM* gene in positive bacterial samples was 95.23%, and the frequency of the *blaSHV* gene was 92.52%.

Conclusion: The disk diffusion test on antibiotic-sensitive samples found that the highest average growth inhibition zone was associated with the CTC antibiotic, while the lowest average growth inhibition zone was related to the NA antibiotic.

Keywords: Disk Diffusion, *Enterobacteriaceae*, ESBL, *blaTEM*, *blaSHV*

1. Background

Enterobacteriaceae are common gram-negative, rod-shaped bacteria. Some species are motile with flagella, while others are not. Notable genera include *Escherichia*, *Klebsiella*, and *Salmonella*. These bacteria do not produce spores, typically measure 1-6 microns in length, are oxidase negative, catalase positive, ferment sugars, and reduce nitrate to nitrite.¹ *Enterobacter aerogenes* is a bacterium commonly found in the gut or as a free-living organism, associated with urinary tract infections and sepsis. It differs from *Klebsiella* in being motile, ornithine decarboxylase positive, and urease negative. *Enterobacter* species are generally resistant to certain antibiotics, with common pathogens including *E. aerogenes*, *E. cloacae*, and *E. sakazakii* can be identified by their unique fermentation and pigmentation characteristics.² *Enterobacter cloacae* is an aerobic gram-negative bacillus and an opportunistic pathogen commonly found in hospital settings. It is responsible for various acquired diseases

and poses significant infection risks due to its resistance to multiple antibiotics.³ Infections caused by *Enterobacter cloacae* can affect the urinary tract, respiratory system, skin, soft tissue, and blood. They can also lead to non-hospital infections such as endocarditis and infections of the nervous system. The improper use of antibiotics significantly contributes to the spread of antibiotic resistance, making infections harder to treat.⁴ The overuse of beta-lactam drugs, aminoglycosides, and carbapenems, combined with prolonged hospital stays, has led to the emergence of multidrug-resistant bacteria. This complicates infection treatment and increases mortality rates. Studies indicate that drug resistance patterns can vary significantly by country and hospital, highlighting the need for tailored approaches to combat these resistant strains.⁵ The rise of multidrug-resistant *Enterobacter* species makes treating *enterobacterial* bacteremia quite challenging. Key factors in choosing the right treatment include understanding the resistance patterns of *Enterobacter* in specific hospitals

and considering the patient's antibiotic history. *Enterobacter*, commonly found in the human digestive tract, can lead to infections such as urinary tract infections, pneumonia, and sepsis. Treatment typically involves antibiotics like imipenem or a combination of antipseudomonal penicillin with an aminoglycoside.⁶ *E. agglomerans* is a diverse group of yellow-pigmented organisms capable of growing at 4 °C and typically testing negative for decarboxylase/dihydrolase. *Enterobacter*, part of the ESKAPE group, comprises 22 species, including seven within the *Enterobacter cloacae* complex. This gram-negative bacterium presents significant challenges in hospital infections, particularly due to the increasing rates of antibiotic resistance. Understanding the resistance patterns and the antibiotic history of patients is essential for selecting effective treatments.⁶ *E. sakazakii* is distinct from *E. cloacae* as it does not ferment D-sorbitol and does not produce a yellow pigment. On the other hand, *E. agglomerans* is a diverse group of organisms, often yellow-pigmented, capable of growing at 4 °C, and typically tests negative in decarboxylase/dihydrolase tests. This differentiation is important for accurate identification and treatment in clinical settings.⁷ Effective antibiotics, such as gentamicin, are recommended for treating *Enterobacter* infections. Broad-spectrum beta-lactamase types TEM and SHV are common enzymes associated with resistance, with 174 TEM and 119 SHV subtypes reported. The amino acid sequences for 1,2-TEM and 1-SHV can be generated for further analysis, which is crucial for understanding resistance mechanisms and developing targeted treatments.⁹ The production of beta-lactamase enzymes by *Enterobacteriaceae* poses significant health challenges, as it leads to antibiotic resistance. The prevalence of these enzymes varies by location and over time, making it essential to monitor resistance patterns in different regions. This variability can impact treatment options and necessitates ongoing surveillance to inform effective therapeutic strategies.¹⁰ Antibiotic resistance is indeed a critical global health issue. In 2014, the World Health Organization highlighted that it jeopardizes the advancements made in modern medicine. Persistent infections can exacerbate this problem, as they may require more aggressive treatments and can lead to increased morbidity and mortality. Addressing antibiotic resistance through better stewardship, research, and public awareness is essential to safeguard the effectiveness of existing antibiotics and ensure better health outcomes.¹¹ Probiotics and probiotic products have gained immense popularity worldwide, transcending cultural, geographic, and social boundaries. For these beneficial microbes to exert their positive effects, they need to reside in the intestinal tract for varying durations. This presence can enhance gut health, support immune function, and improve overall well-being. The growing interest in probiotics reflects a

broader trend towards preventive health and natural remedies, making them a staple in many diets across the globe.¹² Probiotic bacteria play a vital role in maintaining gut health by preventing harmful bacteria from colonizing the intestinal tract. They achieve this by attaching to enterocytes (the cells lining the intestine) and producing various substances, including bacteriocins, lactic acid, and toxic oxygen metabolites. These compounds inhibit the growth of pathogenic bacteria, support a balanced microbiota, and contribute to the overall health of the digestive system.¹³ Our aim in this study was to determine the frequency of *blaSHV* and *blaTEM* genes in *Enterobacter* isolated from clinical samples at Amir al-Momenin Hospital in Maragheh city in 2023, as well as to assess their antibiotic and probiotic resistance patterns.

2. Objectives

The aim of this study was to determine the frequency of *blaSHV* and *blaTEM* genes in *Enterobacter* isolates obtained from clinical samples at Amir Al Momenin Hospital in Maragheh city in 2023. These genes are significant indicators of antibiotic resistance, particularly in *Enterobacter* species, which are known to cause various infections in hospitalized patients. By identifying the prevalence of these resistance genes, we can gain valuable insights into the current state of antimicrobial resistance within the local healthcare setting. In addition to assessing the frequency of these genes, the study also aimed to evaluate the antibiotic resistance patterns of the *Enterobacter* isolates. This involves testing the isolates against a range of commonly used antibiotics to determine their susceptibility or resistance. Understanding these patterns is crucial for guiding effective treatment options for infections caused by *Enterobacter* species. Furthermore, the study will explore the probiotic resistance patterns of these isolates. Probiotics are beneficial bacteria that can help restore the balance of the gut microbiota, and understanding their interaction with resistant *Enterobacter* strains is essential for developing strategies to combat infections while preserving beneficial microbial populations. Overall, this research will contribute to the growing body of knowledge on antimicrobial resistance and inform clinical practices in the region.

3. Methods

3.1. Study Sample

Urine samples from 100 patients with urinary tract infections at Amir al-Momenin Maragheh Hospital and outpatients were tested. After 24 hours, the culture medium was examined for bacterial colony growth. EMB agar served as a selective medium for Gram-negative bacteria. Various tests were conducted to identify and confirm the bacteria.

3.2. Antibiotic Sensitivity Testing

Initially, the lids of the plates were left open for 3-5 min to allow excess moisture to evaporate. A sterile swab was then dipped into a 0.5 McFarland suspension, pressed against the wall of the tube, and rotated to remove any excess solution. The swab was uniformly spread across the Mueller Hinton agar plate, rotated approximately 60 °C, and the swabbing process was repeated three times. Finally, the swab was drawn in a complete circle around the edge of the agar. Antibiotic disks, including AMP, CP, NA, SXT, IMP, CAZ, and CTC, were used in the design and stored at the appropriate temperature to preserve their effectiveness. The disks were placed using dispenser or sterile forceps, and the plate was incubated at 35 °C for 16 to 18 hours. After incubation, bacterial growth was assessed, and the diameter of the zones of inhibition was measured in millimeters.

3.3. Treatment *Enterobacter* with Probiotics

First, *Lactobacillus* bacteria from dairy products were isolated and cultured on specialized MRS agar and broth. The GasPak anaerobic system provided an oxygen-free environment for these bacteria. Gram staining was utilized for identification. After 48 hours, the culture's supernatant was centrifuged to settle the bacteria, followed by filtration. A microbial suspension equivalent to a 0.5 McFarland standard of *Enterobacter* was prepared and inoculated into a test tube using a sterilized swab. Wells were created in the agar, with one serving as a control. Different volumes of physiological saline and filtered probiotic liquid were added to the wells. After incubation at 37 °C for 24 hours, the diameter of the zones of inhibition was measured.

3.4. DNA Extraction

For DNA extraction, the Vira Gene Company's DNA extraction kit was utilized. Initially, a 0.5 McFarland suspension was prepared from the sample. Next, 200 µl of cell/blood lysis solution was added to the tube, followed by the addition of 20 µl of proteinase K, which was incubated for 10 min at 37 °C. After mixing in 100 µl of isopropanol, the tube was centrifuged at 12,000 rpm for one minute. The supernatant was discarded, and 500 µl of wash solution 1 was added, followed by another centrifugation at 12,000 rpm for one minute, with the supernatant discarded afterward. This process was repeated using 500 µl of wash solution 2. A small column was placed in a collection tube, and the mixture was poured into it, followed by the addition of 100 µl of elution buffer. After one final minute of centrifugation at 12,000 rpm, the quantity and quality of the extracted DNA were assessed using a Nanodrop, and its integrity was verified through 1% agarose gel electrophoresis.

3.5. Polymerase Chain Reaction (PCR)

To determine the frequency of the TEM and SHV genes, the primers were validated for specificity using the NCBI database. The sequences of the primers are presented in Table 1. The PCR method was employed to identify the *blaTEM* and *blaSHV* genes. The PCR reaction mixture consisted of 10 µl of PCR master mix (Ampliqon, Denmark), 1 µl of forward primer, 1 µl of reverse primer, 3 µl of template DNA, and 5 µl of water, totaling 20 µl. Various temperature programs and cycles were designed, with the optimal annealing temperature determined using a gradient thermocycler. The thermal profile for the genes was executed over 35 cycles. A 2% agarose gel electrophoresis was performed to analyze the PCR products.

Table 1. Sequence of Primers of TEM and SHV Genes

Gene	Forward primer	Reverse primer	Pair bases
SHV	AGCCGCTTGAGCAAATTAAC	ATCCCGCAGATAAATCACCAC	713
TEM	GAGTATTC AACATTTCGTGTC	GGGCGAAA ACTCTCAAGGATC	177

4. Results

4.1. Biochemical and Microscopic Tests

Results of biochemical tests, including citrate utilization, motility, indole production, Methyl Red (MR), Voges-Proskauer (VP), Triple Sugar Iron Agar (TSI), Lysine Iron Agar (LIA), and Urea culture medium are presented in Table 2.

4.2. AntibioGram Results

In this experiment, the following antibiogram discs were used: AMP (ampicillin), CP (ciprofloxacin), NA (nalidixic acid), SXT (sulfamethoxazole), IMP (imipenem), CAZ (ceftazidime), and CTC (cefotaxime clavulanic acid). Table 3 presents the sensitivity and resistance protocols to the antibiotics used in this study, based on the size of

the inhibition halo formed. Out of 21 samples, the ESBL index was negative in 7 samples with a frequency of 33.33% and positive in 14 samples with a frequency of 66.67%. Figure 2 shows the halos created by *Enterobacter* bacteria, and Table 4 details the antibiotic resistance patterns observed. In this study, all samples were found to be resistant to the ampicillin. Additionally, 61.90% of the samples exhibited resistance to ciprofloxacin, while 33.33% were sensitive and 4.65% were semi-sensitive to this antibiotic. Resistance rates for other antibiotics included 61.90% for nalidixic acid, with 33.33% sensitive and 4.76% semi-sensitive. For sulfamethoxazole, 52.38% of samples were resistant, while 47.61% were sensitive. Imipenem resistance was observed in 52.38% of samples, with 33.33% sensitive and 14.28% semi-sensitive.

Table 2. Biochemical and Differential Tests to Detect Isolates of *Enterobacter*

	Simmons citrate	TSI	Urea	MR	VP	LIA	SIM
<i>Enterobacter</i>	+	+	+	-	+	-	M+
	Color change from green to blue	Orange top yellow bottom No gas				purple top yellow bottom	I-

Table 3. The Average Growth Inhibition of Antibiotics

Antibiotics	Sensitive	Semi-sensitive	Resistance
Ampicilin 10 mg	X≥17	14-16	X≤ 13
Ciprofloxacin 5 mg	X≥23	20-22	X≤ 19
Nalidixi acid 30 mg	X≥19	14-18	X≤ 13
Sulfamethoxazole	X≥16	11-15	X≤ 10
Imipenem 10 mg	X≥23	20-22	X≤ 19
Ceftazidim	X≥21	-	X≤ 20
CTC	X≥26	23-25	X≤ 22



Figure 1. Antibiogram Test Results.

Disk diffusion test

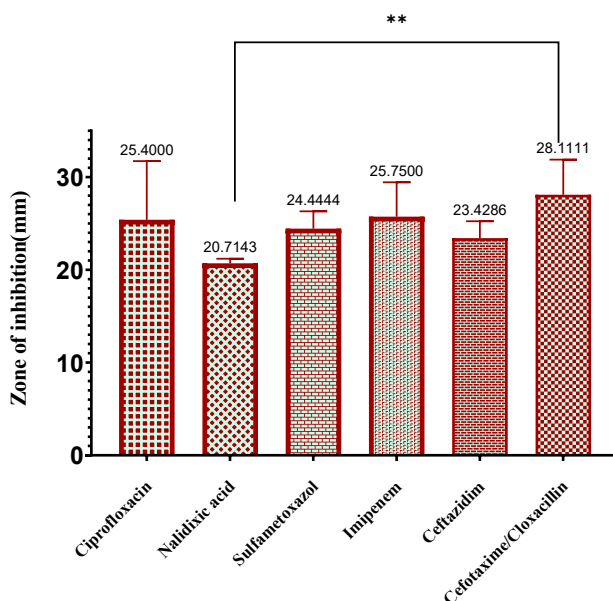


Figure 2. Antibiogram Test on Isolates of *Enterobacter* (** indicate $P < 0.01$).

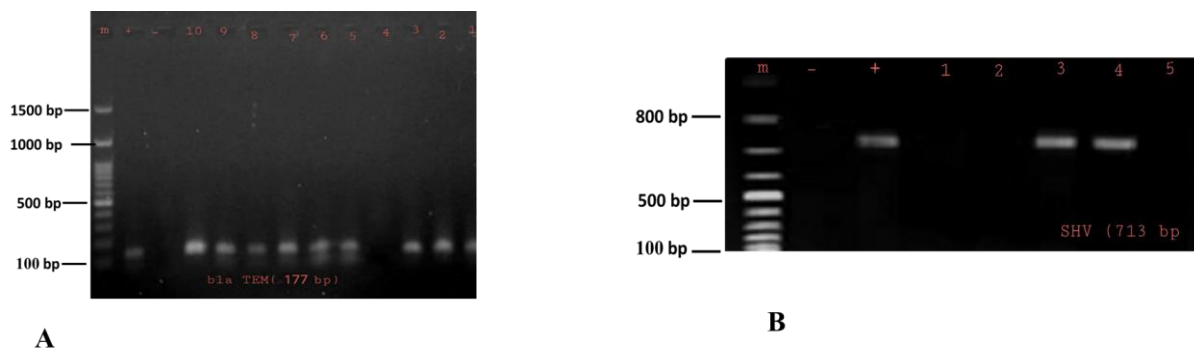


Figure 3. *blaTEM*(A) and *blaSHV*(B) electrophoresis the, (ladder 100 bp).

Ceftazidime showed a resistance rate of 66.67%, with 33.33% of samples being sensitive. Lastly, for cefotaxime clavulanic acid, 38.095% of samples were resistant, 38.095% were sensitive, and 23.809% were semi-sensitive (Figure 3).

4.3. Disk Diffusion Result

By conducting the disk diffusion test on the antibiotic-sensitive samples, it was observed that the highest average growth inhibition area was associated with the CTC antibiotic, measuring an average of 28.11 mm. Conversely, the lowest average growth inhibition area was linked to the NA antibiotic, with an average of 20.714 mm. Figure 4 illustrates that there is no significant difference between the average growth inhibition of the NA antibiotic and the CTC antibiotic, nor among the other groups.

4.4. blaTEM and blaSHV Genes Expression

To examine the presence of TEM and SHV genes, the PCR method was employed using gene-specific primers. The lane marked with "M" indicates a length of 100 bp, with the positive control well containing *Enterobacter* bacteria and the negative control well containing deionized water. The *Enterobacter* positive control was confirmed using the standard strain number ATCC 13048, obtained from Bahar Afshan Company. The results

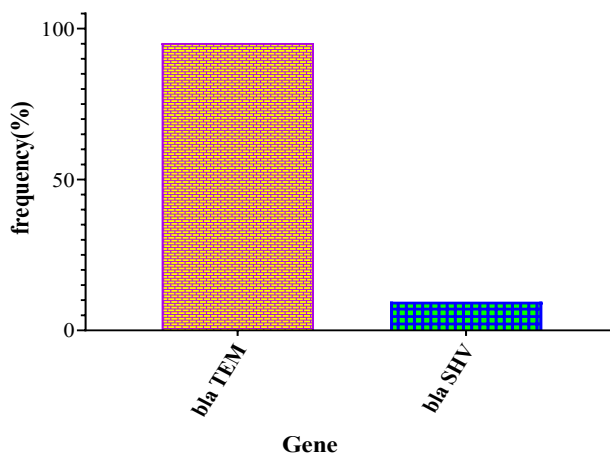


Figure 4. Frequency of *blaSHV* and *blaTEM* genes. The frequency of *blaTEM* gene in positive bacteria samples is 95.23% and the frequency of *blaSHV* gene is 9.52%.

of the PCR test revealed that the frequency of the TEM gene was 95.23%, while the frequency of the SHV gene was 9.52% among the 21 samples studied. Figure 3A displays the electrophoresis results for the *blaTEM* gene, where the first well is 100 bp, the second well is the positive control of *Enterobacter* bacteria, and the third well is the negative control (deionized water). Figure 3B shows the electrophoresis results for the *blaSHV* gene, with the first well at 100 bp, the second well as the negative control (deionized water), and the third well as the positive control of *Enterobacter* bacteria. The frequency of the *blaTEM* gene in positive bacteria samples is 95.23% and the frequency of the *blaSHV* gene is 9.52% (Figure 4).

5. Discussion

Gamboratto et al. conducted a study on intestinal vancomycin-resistant *enterococci* (VRE) colonization in France, reporting a prevalence of 37% among inpatients and 12% among outpatients.¹³ Gikas et al. reported a 20% intestinal carriage of vancomycin-resistant *enterococci* (VRE) in Greece in 2005. Additionally, a European study conducted in 2003 found a 2.9% resistance to vancomycin in rectal swabs and clinical specimens, with vanA strains exhibiting 89% resistance to ampicillin and 80% resistance to gentamicin.¹⁴ Broad-spectrum β-lactamases of TEM and SHV types are the enzymes responsible for this resistance. Currently, 174 different subtypes of TEM and 119 subtypes of SHV have been reported.¹⁵ The TEM enzyme is the first identified extended-spectrum β-lactamase (ESBL) and is widely spread within the *Enterobacteriaceae* family, making it the most common β-lactamases. Notably, it has even been transferred to *Haemophilus influenzae* and *Neisseria gonorrhoeae*. Most broad-spectrum β-lactamases, including TEM and SHV, arise from changes in the amino acid sequence of TEM-1, TEM-2, and SHV-1. In many strains, the gene encoding SHV beta-lactamases is originally located on the chromosome; however, over time, this gene has transferred to plasmids, facilitating its spread among bacterial strains.¹⁶ In this study, we tested 100 patient urine samples and 21 samples were diagnosed with *Enterobacter*. Out of the 21 samples, the ESBL

index was negative in 7 samples with a frequency of 33.33% and positive in 14 samples with a frequency of 66.67%. Among the studied isolates, resistance to ampicillin was very high at 100%. Following inappropriate experimental treatments, even sensitive organisms can develop resistance. This occurs through the induction of enzymes that inactivate antibiotics or through mutations in coding genes, cell wall channels (outer membrane porins), secretion systems, or via plasmid transfer. One of the most problematic mechanisms of antibiotic resistance is related to beta-lactamases.¹⁷ Beta-lactamase-producing bacteria are on the rise, with over 400 different types of β -lactamase defined in clinical samples. The organisms that produce broad-spectrum beta-lactamases are particularly significant in terms of therapy. In recent years, these bacteria have become widespread globally. Through disk diffusion tests on antibiotic-sensitive samples, it was found that the highest average growth inhibition zone was associated with the CTC antibiotic, averaging 28.11 mm, while the lowest average growth inhibition zone was related to the NA antibiotic, averaging 20.714 mm.¹⁸ As illustrated in the chart, there is no significant difference between the average growth inhibition of the NA antibiotic and that of the CTC antibiotics, as well as among other groups. The high level of healthcare in developed countries, along with effective planning, has contributed to a decrease in antibiotic resistance compared to third-world countries.¹⁹ This difference may indicate an increase in resistance to broad-spectrum cephalosporins in recent years. This trend serves as a warning for the treatment of infections caused by this bacterium, which is one of the most commonly isolated bacteria from urinary patients and a frequent cause of urinary tract infections in both hospital and community settings. Treatment with cephalosporins has often been unsuccessful, leading to treatment failures. Consequently, there has been a shift towards using broad-spectrum drugs such as fluoroquinolones and carbapenems, but strains resistant to these medications are also on the rise.²⁰ The results of the antimicrobial sensitivity test in this study indicate that the highest level of resistance among third and fourth generation cephalosporins is observed with ceftriaxone and cefotaxime, while cefepime shows the lowest resistance. This finding aligns with the general principle that activity against Gram-negative bacteria increases from the first to the fourth generation of cephalosporins. However, a notable number of strains exhibited moderate sensitivity, raising concerns about multidrug resistance in TEM-producing strains compared to aminoglycosides. In our study, 52.6% of the isolates showed resistance to ciprofloxacin, while the lowest resistance was observed with imipenem at 5.3%, which also demonstrated the highest sensitivity. This is consistent with findings from other studies in Iran and globally that have reported high sensitivity levels.

Additionally, 67% of the isolates were confirmed as ESBL producers, a significant statistic. The elevated level of ESBL in the samples studied may be attributed to the indiscriminate use of broad-spectrum antibiotics and the larger sample size in this study compared to others. Zandi et al. reported an ESBL frequency of 30% in their isolates, which is lower than what was found in the present study. The frequency of ESBL varies across different communities; in some, it is lower than the results of this study, while in others, it is similar. Additionally, there are studies indicating an increase in ESBL-producing isolates, highlighting the dynamic nature of antibiotic resistance patterns in different regions. This variability underscores the importance of ongoing surveillance and tailored treatment strategies in addressing antibiotic resistance effectively.²¹ The results of Vachvanichsanong's studies indicate a prevalence of 30-40% of ESBL in bacteria among individuals with urinary tract infections. In a study by colleagues, TEM beta-lactamase was identified as the most common ESBL, with a prevalence of 55.5%. Additionally, findings from Bajpai et al. revealed that 45% of *Enterobacteriaceae* isolates carried the TEM gene. When comparing our study to previous research conducted in Iran on the detection of ESBL-producing bacteria, we found that our results varied—showing higher prevalence in some cases and lower in others. This inconsistency highlights the need for continuous monitoring and research to understand the evolving landscape of antibiotic resistance in different populations.²³

6. Conclusion

According to the findings of the present study, the *blaTEM* gene, unlike the *blaSHV* gene, can be considered a significant indicator for diagnosing *Enterobacteria*. However, further studies with larger sample sizes are needed to assess its sensitivity and specificity. Our results also indicated an increase in antibiotic resistance among *Enterobacter*, with at least one-third of the isolates demonstrating resistance to all tested antibiotics. Interestingly, we found that probiotics play a crucial role in reducing antibiotic resistance. The frequency of resistance to antibiotics decreased with increased probiotic usage. In the group that consumed 200 ml of probiotics, the resistance rate dropped to 28%, highlighting the potential

Research Highlights

What Is Already Known?

Many *Enterobacteriaceae* produce extended-spectrum beta-lactamases (ESBLs), making them resistant to beta-lactam antibiotics and leading to treatment failures.

What Does This Study Add?

The study found that the *blaTEM* gene is a more reliable indicator for diagnosing *enterobacteria* compared to the *blaSHV* gene.

importance of probiotics in combating antibiotic resistance. This suggests that incorporating probiotics into treatment regimens could be a valuable strategy in managing antibiotic resistance in *Enterobacter* infections.

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Author Contributions

FGH, ZHB, and ST developed the theoretical formalism, conducted analytic calculations, and performed numerical simulations. All authors contributed to the final version of the manuscript. ZHB supervised the project.

Conflict of Interest Disclosures

All authors declared that they have no conflict of interest.

Ethical Approval

Not applicable.

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