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Phenotypic Characterization and Virulence Profiling of *Enterobacter* Species Isolated from Clinical Specimens in Thi-Qar Province, Iraq

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ABSTRACT

Background: *Enterobacter species are nosocomial pathogens associated with urinary tract infections (UTIs), wound infections, and burns. Their virulence mechanisms, including biofilm formation, capsule production, and colonization factors, contribute to their pathogenicity and antimicrobial resistance.* ***Objective****: This study aimed to isolate and identify Enterobacter species from clinical samples (urine, wounds, and burns) in Thi-Qar province, Iraq, and to phenotypically evaluate their virulence factors.* ***Methods****: A total of 120 clinical samples (67 urine samples, 32 wound swabs, and 21 burn swabs) were collected. The isolates were cultured on MacConkey agar and identified using biochemical tests and VITEK-2. Virulence factors were assessed using the following phenotypic assays: biofilm formation (tube method), CFA expression (slide agglutination), capsule detection (India ink), and hemolysis (blood agar).* ***Results****: Among 42 Enterobacter isolates, E. cloacae (54.8%) predominated over E. sakazakii (40.4%) and E. aerogenes (4.8%). Urine samples yielded the highest isolates (71.4%), with E. cloacae predominant (47.6%), while E. sakazakii was more frequent in wounds (16.7%). Phenotypic analysis revealed non-hemolytic isolates (87–82.4% for E. cloacae and E. sakazakii), high capsule prevalence (87% and 82.4%), and biofilm formation (73.9% and 76.5%). E. sakazakii exhibited significantly higher CFA I (76.5% vs. 30.4%) and CFA III (88.2% vs. 52.2%) than E. cloacae ($p < 0.05$).* ***Conclusion****: Enterobacter species, particularly E. cloacae and E. sakazakii, are significant pathogens in clinical settings, with distinct virulence profiles that influence their niche preferences. The high prevalence of capsules and biofilms underscores their roles in persistence and antibiotic resistance.*

Keywords: Enterobacter cloacae, Enterobacter sakazakii, Virulence factors, Biofilm formation, Capsule, Nosocomial infections.

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INTRODUCTION

Gram-negative environmental bacteria, water, sewage, soil, and plants all contain *the Enterobacter species, which are members of the Enterobacteriaceae* family. Humans and animals typically carry these microorganisms, with E. cloacae being the most commonly isolated species. Members of this genus are opportunistic pathogens that are becoming more significant in nosocomial diseases, especially in neonates, immunocompromised patients in critical care units, patients in emergency rooms, urology wards, and patients in wards with skin and soft tissue infections (1), and have been found to be accountable for approximately 13.20 percent of UTI among patients in Iraq, as indicated by a prior investigation (2). There are at least 12 species in the genus *Enterobacter,* but the bacteria *E. cloacae, E. aerogenes, E. gergoviae, and E. hormaechei* are most commonly isolated from clinical samples. The two most common isolates of this

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group, *E. cloacae and E. aerogenes*, were recovered from the blood, urine, wounds, and CSF samples(3). *Enterobacter sakazakii* causes respiratory infections, wounds, meningitis, and bacteremia(4). However, not all species infect humans. *Enterobacter* species are responsible for most nosocomial infections and less frequent community-acquired diseases such as lung infections, soft tissue infections, osteomyelitis, endocarditis, and urinary tract infections (3). The microbiota of the gastrointestinal tract of mammals contains some species of this bacterium(5). The frequency and severity of nosocomial *Enterobacter* infections has increased, particularly in immunocompromised individuals. Notably, the most frequently affected groups included neonates and premature infants, patients in intensive care units, and postoperative patients.(6).

Some studies have shown that most strains of *Enterobacter* form biofilms (7, 8), which increases the risk of nosocomial infections by elevating the survival of bacterial populations in patients and on hospital surfaces. Several studies have established a link between biofilm formation and horizontal gene transfer(9). The ability of bacteria to thrive in various settings, both natural and man-made, like food processing facilities, primarily hinges on their capacity to adhere to surfaces(10).

The beginning of an infection are determined by a variety of complex and dynamic interactions between the microorganism and the host, which can vary based on the type of infecting microbe.These components can be found in the structure of cells or in substances that the body secretes, such as enzymes and poisons, Bacteriocin, siderophores, proteases, colonization factor antigen (11), haemolysin, and capsule are some of these virulence factors(12). The majority of *Enterobacter* strains that can create a variety of adhesive factors, including colonization factor antigen (11), which has been previously documented in the majority of commensals, and are pathogenic, may also produce pilli or fimbriae(13). CFA/Is are among the most prevalent *Enterobacteriaceae* species in humans.(14) . Capsules improve bacterial evasion of phagocytosis, which encourages tissue invasion and protects cells from biocidal chemicals (15). The objective of this study was to isolate and identify *Enterobacter species* that cause different clinical illnesses in humans, and to assess their virulence phenotypically.

METHODS

Collection of samples

During the study period, 120 samples were obtained from Al Hussein Teaching Hospital and Alshatra General Hospital in Thi-Qar Province from September 1, 2024, to February 1, 2025. The samples were drawn from different clinical patients like (67 urine samples, 32 wound smears, and 21 burn smears). After diagnosing the samples, 42 isolates of *Enterobacter.spp* spp. were distributed as follows: (30 isolates from urine samples, 9 from wound smears, and 3 from burn smears).

Isolation and identification of bacteria

Midstream urine samples (10–20 ml) were collected from non-catheterized patients. From the catheterized patients,

5–10 ml samples were drawn after discarding the first few drops of urine, and the wound and burns were debrided lightly with sterile saline solution to remove exudates or debris.

The surface material of the wound or exudate was swabbed with a sterile swab. Twist the swab To ensure good contact with the wound or burn surface, the swab was placed in sterile transport medium (e.g., Amies transport medium). If more than one site was sampled, a different swab was used for each wound site.

Amies transport medium was used to inoculate the swabs. After that, all samples were brought to the laboratory, where they were cultivated on MacConky agar and incubated for twenty-four hours at 37°C. Following the incubation period, the identification of bacterial isolates is based on biochemical and cultural characterization tests, and the Vitek-2 system is used to achieve definite identification in accordance with the manufacturer's instructions; Negative ID-Gram cards (ID-GN cards; bioMérieux,France)(16, 17)

Detection of virulence factors

Formation of Biofilms

This study was conducted to determine whether bacteria can build biofilms using the tube method. A successful outcome is achieved when biofilms develop as a purple coating on the inner walls and bottom of the tubes. After being transferred to glass test tubes filled with tryptic broth medium and 1% glucose at 37°C, the growing bacterial colonies were cultivated for a whole day (24 hour). The samples were poured out, and the tubes were cleaned with saline phosphate buffer during the incubation period. After drying, the were stained for three minutes with 1% crystal violet. The excess dye was drained and cleaned with deionized distilled water. The tubes were then turned upside down to dry. A purple layer of biofilm developed on the inner and lower walls of the tubes. (18).

Colonization Factor Antigen :

The isolates were cultured for a whole night at 37°C under static conditions on CFA agar, which was made of nutrient agar that had been enhanced with 0.5% D(+)-mannose and 0.15% yeast extract. To achieve a turbidity of 0.5, the McFarland standard, colonies were suspended in phosphate-buffered saline (PBS).

A sterile glass slide was coated with a drop of the bacterial suspension, and an equivalent volume of CFA-specific antiserum—obtained from reference sources or made using inoculated animals—was added. At room temperature, the slide was rocked gently for one–two minutes.A noticeable agglutination within two minutes was regarded as a positive indicator of colonization factor antigen expression, and the absence of obvious clumping suggested a poor outcome. (19).

Capsule formation

Capsule production was detected using India ink preparation. On a sterile glass slide, a tiny drop of regular saline solution was combined with a tiny amount of bacterial growth extracted from a 24-hour-old colony. Subsequently, using a transfer loop, a drop of India ink was added to the

slide and blended. A glass coverslip was then placed on the slide. The liquid was spread as a thin layer under a coverslip, and the slide was examined under an oil-based lens. The presence of a transparent halo around the bacterial cells, unstained by India ink, was evidence of the presence of a capsule. (20) .

Hemolysin production

The bacterial isolates were streaked onto blood agar plates using a sterile loop and incubated aerobically for 24 h at 37°C. Plates were inspected for hemolysis zones surrounding the colonies following incubation.Alpha (α) hemolysis: A greenish tint surrounds colonies due to partial lysis of red blood cells.The total disintegration of red blood cells, known as beta (β) hemolysis, causes a clear, colorless zone to encircle colonies.Gamma (γ) hemolysis: The medium around colonies stays the same and there is no hemolysis. (21)**.**

STATISTICAL ANALYSIS

SPSS version 24.2 and Microsoft Excel were used in the statistical analysis of the study to identify significant differences. This was accomplished using the chi-square test, and significant differences were identified at various probability levels.

RESULTS

After collecting the samples, they were distributed among the urine samples, which had the highest percentage of 67 (56%), followed by wound smear at 32(27%), and the lowest percentage of burn swabs at 21(17%) **Fig 1**

Gram staining and cultural, morphological, and biochemical tests were among the parameters used to initially identify the bacterial specimens; the results were consistent with the categorization schemes used previously (16, 17). The automated vitek-2 compact system, which is distinguished by its convenience, speed, and accuracy of biochemical diagnosis confirmation, was used for final identification. GN-ID cards containing 64 biochemical tests and one negative control were used. **(table1) .**

Shows the percentage of *Enterobacter* species isolated from clinical samples. The percentage of *Enterobacter cloacae* was higher at (54.8%) that of *Enterobacter sakazakii* at (40.4 %) and *Enterobacteraerogenes* aerogenes (4.8 %) out of 42 *Enterobacter* isolates **(Table 2)**.to ascertain whether these frequencies substantially depart from an equal distribution among species, a chi-square goodness-of-fit test was used. The outcome was statistically significant (χ^2 = 19.71, df = 2, p = 0.00005), suggesting that *E. cloacae* was substantially more common and that the distribution of *Enterobacter* species was not uniform.

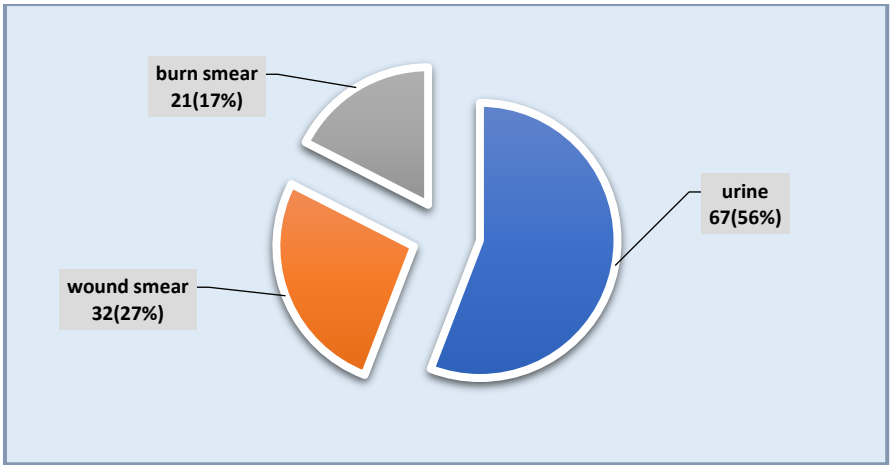

Figure 1: Distribution (percentage) of study samples.

Table 1: Phenotypic tests (Morphological and microscopic) and biochemical tests for the diagnosis of *Enterobacter* species.

Phenotypic tests	***E. cloacae***	***E.sakazakii***	***E.aerogenes***
Gram Stain	**(-) Rods**	**(-) Rods**	**(-) Rod**
Oxidase	-	-	-
Catalase	+	+	+
Motility	+	+	**Motile**
Indole	-	**V (often +)**	-
Methyl Red (MR)	+	**V**	-
Voges-Proskauer (VP)	+	+	+
Citrate Utilization	+	+	+
Urease	**V**	+	-
Lysine Decarboxylase	**V**	-	+
Ornithine Decarboxylase	+	+	+
Arginine Dihydrolase	+	**V**	+
H2S Production	-	**V (rarely +)**	-
Gelatin Hydrolysis	-	+	+
DNase Test	-	+	-
ONPG Test (β-galactosidase)	+	+	+
Glucose Fermentation	**+ (acid/gas)**	**+ (acid/gas)**	**+, with gas production**
Lactose Fermentation	+	**Slow fermenter**	+
Sucrose Fermentation	+	+	+
Melibiose Fermentation	+	-	+
Inositol Fermentation	+	-	+

+ / Positive result/Negative result V/variable

Table 2: The Number and Percentage of *Enterobacter* species isolated from study samples.

Enterobacter species	**No. of isolates**	**%**
Enterobacter cloacae	23	54.8 %
Enterobacter sakazakii	17	40.4 %
Enterobacter aerogenes	2	4.8 %
Total	42	100 %
χ^2 = 19.71	**df = 2**	**p = 0.00005**

Three different sample types—urine (71.4%), wound (21.5%), and burn (7.1%)—had a combined 42 *Enterobacter* isolates. Of them, The majority of urine samples (47.6%) included *Enterobacter cloacae*, whereas wound samples (16.7%) contained *E. sakazakii*. A chi-squared test of independence was used to assess the association between *Enterobacter* species and sample type. The test results showed that the distribution of *Enterobacter* species by sample type differed significantly (χ^2 = 18.92, df = 4, p = 0.004). As p = 0.004 < 0.05, there was a significant difference between the sample type and bacterial species from the genus *Enterobacter* **(table 3)**.

The results of the phenotypic detection of virulence factors in **Table 4** showed that most of the *Enterobacter* isolates were non-hemolytic, capsule-forming, and biofilm-forming. *E. sakazakii* produced the colonization factor CFA I, and CFA III was higher than *E.cloacae*. Colonization and adherence to host cells, which is regarded as the initial stage of infection, are influenced by the majority of virulence factors that have been investigated. The relationship between the presence of particular virulence factors and bacterial species (*E. cloacae and E. sakazakii*) was investigated using a series of chi-squared tests. Hemolysis (p = 0.493), capsule formation (p = 1.000), CFA III (p = 0.108), and biofilm production (p = 1.000) were not significantly different between the two species. However, CFA I (χ^2 = 6.38, p = 0.012) and CFA III (χ^2 = 2.58, p = 0.108) showed statistically significant associations, indicating that *E. sakazakii* is more likely than *E. cloacae* to express CFA I and CFA III than E. cloacae.

Table 3: The Number and Percentage of *Enterobacter* species according to type of sample.

Sample	**No and % of samples**	**Number and % of *Enterobacter spp.***		
		E. cloacae	***E.sakazakii***	***E.aerogenes***
Urine	30 (71.4%)	20 (47.6 %)	8 (19 %)	2 (4.8 %)
Wound	9 (21.5 %)	2 (4.8 %)	7 (16.7 %)	0
Burn	3 (7.1 %)	1 (2.4 %)	2 (4.8 %)	0
Total	42 (100%)	23 (54.8 %)	17 (40.4 %)	2 (4.8 %)
χ^2 = 18.92		**df = 4**		**p = 0.004**

Table 4: Phenotypic identification of several *Enterobacter* species virulence factors.

Virulence factors		***Enterobacter cloacae***		***Enterobacter sakasakii***	
		Positive results	**Negative results**	**Positive results**	**Negative results**
Hemolysis		3 (13%)	20	4 (23.5%)	13
χ^2 = 0.47 df = 1 p = 0.493					
Capsule		20 (87%)	3	14 (82.4%)	3
χ^2 = 0.00 df = 1 p = 1.000					
CFA	CFA I	7 (30.4%)	16	13 (76.5%)	4
	χ^2 = 6.38 df = 1 p = 0.012				

	CFA II	4(17.4%)	19	1 (5.9%)	16
	$\chi^2 = 4.54$ df = 1 p = 0.033				
	CFA III	12(52.2%)	11	15 (88.2%)	2
	$\chi^2 = 2.58$ df = 1 p = 0.108				
Biofilm formation		17 (73.9%)	6	13 (76.5%)	4
$\chi^2 = 0.00$ df = 1 p = 1.000					

DISCUSSION

Enterobacter species cause skin, soft tissue, and urinary tract infections (caused by *Enterobacter* species. The objective of this study was to identify and isolate *Enterobacter* spp. and assess their pathogenicity based on their phenotypic characteristics. Primary isolate identification was based on a few parameters using the categorization schemes used previously (16, 17). GN-ID cards, which are distinguished by their simplicity, speed, and accuracy in confirming biochemical diagnosis, were used for the final identification process using the automated VITEK-2 system. Numerous studies have demonstrated that VITEK-2 technology and VITEK-2 ID cards may yield accurate and dependable findings for gram-positive cocci and gram-negative bacilli(22).

The most frequent study samples were urine samples, and bacterial isolation from the study samples was *E. cloacae* which had the highest frequency in urine compared to *E. sakazakii* and some isolates of gram-negative bacteria that were neglected. This could be the consequence of bacteria being present in the natural flora of the human stomach and spreading to the urine tract(23). *E. cloacae* has also been found to be highly prevalent in skin and soft tissue infections; these infections may be caused by bacteria colonizing the skin as part of the normal flora, causing an endogenous infection(24). The frequency of *E. cloacae* in clinical specimens is in line with previous research, which indicates that bacteria are commonly associated with hospital-acquired diseases, particularly tract infections. Both species were presumably distributed rather evenly among the isolates, as the test revealed that the distribution difference was not statistically significant ($p > 0.05$). This finding was similar to that of a previous study (25). The highest frequency and percentage of *E. cloacae* isolated from UTIs in medical diagnostic laboratories in Shahrekord, Iran. It also agreed with the study(26) Examine the frequency of multidrug-resistant *E. cloacae* as a major cause of UTIs and identify any patterns of antibiotic resistance in patients who are treated at Vadodara's tertiary care hospital. In addition, a comparison with(27) demonstrated the possibility of isolating *Pantoea.sp* from patients with UTIs. The genus *Pantoea* has recently descended from *Enterobacter*, as it shares most of its diagnostic features.

E. cloacae and *E. aerogenes*, which have been seen in hospital wards , and are a members of the ESKAPE group, are the main culprit behind human infections, according to recent reports(28) according to testing, the most frequent bacterial infections that cause UTIs (20.0%) *E. cloacae* (29). According to the results of sensitivity testing, *Enterobacter spp.* exhibit a high degree of resistance to Gram-negative bacteria (30).

The majority of isolates (71.4%) were found in urine samples, with *E. cloacae* making up 47.6 % and *E. sakazakii* 19 %. Although *E. sakazakii* was more commonly recovered from wounds (16.7%) than *E. cloacae* (4.8%), wound and burn samples showed lower isolation rates .this raises the possibility of niche preferences or different pathogenic capacities among species, which may be impacted by the profiles of virulence factors.Although urine samples represented the highest proportion of isolates, the association between sample type and species was not statistically significant ($p > 0.05$).

Investigations revealed that *E. sakazakii* was the primary cause of most illnesses, even though all *Enterobacter* species were identified from clinical material(31). Clinical symptoms of *Enterobacter* infections generally include necrotizing enterocolitis, septicemia, meningitis, and wound and urinary tract infection(32). Therefore, it was isolated and confirmed to be present in the sample.

Our study showed that the lowest isolation rate of burns was 3 (7.1%) ,It is consistent with (33) study when he isolated gram-negative bacteria from burn wounds, and found *Enterobacter* at a rate of (3.22%). Gram-negative pathogenic infections caused by *Enterobacter* species, such as *E. cloacae* and *E. sakazakii*, are increasingly isolated from human clinical specimens. Premature birth, low birth weight in neonates, presence of a foreign device, and immunocompromised patients from any cause are some of the major risk factors for contracting *Enterobacter* infections(1).

The two species differed significantly according to the virulence factor analysis. In both species, hemolysin production was comparatively uncommon; it was found in only 13% of isolates of *E. cloacae* and 23.5% of isolates of *E. sakazakii*. Although its presence may increase host cell damage in vulnerable patients, this low frequency suggests that hemolysin may not play a significant role in the virulence of these isolates. Less closely related to the study by(34) Found (87.5% and 16.7%) *Enterobacter.spp* strains were capable of β-hemolysis on blood agar base . Similar to a previous study (35), 7 of the 50 *E. cloacae* strains from clinical isolates produced small turbid zones of hemolysis in blood agar plates. Not compatible with (12), the study showed that none of the *Enterobacter* didn't produced any type of hemolysin.

In both species, Capsule formation was significantly more common (87% in *E. cloacae* and 82.3% in *E. sakazakii*). The high frequency of capsules highlights their function in phagocytosis resistance and immune evasion, which is in line with their potential pathogenicity in immunocompromised individuals. Similar to the study (12), all *Enterobater* species isolates possessed a polysaccharide capsule around the bacterial cell, with the exception of one *E. cloacae* strain (98.8% versus 1.2%). In addition, with (28), when he explained Update on Taxonomy, Clinical Aspects to *Enterobacter.spp* Most of its species contain capsules. These findings concur with those of (36), who discovered that every *E. cloacae* isolate recovered from UTIs had a capsule. *E. hormaechei's* capsule is a crucial immune evasion factor, making it a possible target for treatment and preventive measures against MDR human infections(37).

There are species-specific patterns of colonization factor antigens (11). *E. sakazakii* exhibited a higher prevalence of CFA I (76.5%) and CFA III (88.2%) compared to *E. cloacae* (30.4% and 52.2%, respectively). These variations might be due to *E. sakazakii's* improved adherence mechanisms of E. sakazakii, which support its capacity to colonize epithelial surfaces, especially those of wounds. On the other hand, although still rather rare in both species, CFA II was more prevalent in *E. cloacae* (17.4%) than in *E. sakazakii* (5.9%). Consistent with (12) study , a high level of CFA production was seen in CFA / III produce, which was produced by 95% of *Enterobacter* isolates, according to (38), 95% of *Enterobacter* isolates were able to produce CFA/III, which is what causes the bacteria to colonize the surfaces of urinary catheters(39), 32% of isolates were able to produce CFA/I, and 10% were able to produce CFA/II, which causes chicken blood to agglutinate and acts to adhere bacteria with specific and complex carbohydrate receptors of small intestinal epithelial cells(40).

A significant percentage of both species showed biofilm formation: (76.5%) and *E. sakazakii* and (73.9%) in *E. cloacae*. The clinical challenge posed by these organisms in

persistent infections is highlighted by the fact that their capacity to form biofilms is a critical factor in chronic infections and resistance to antimicrobial agents. Consistent with the study (41) , *E. cloacae* was found to be 6.80% of UTIs. Among the isolated strains, those that produced biofilms were identified. agree with the study(42) Bacterial isolates that formed biofilms showed higher levels of antibiotic resistance, according to a phenotypic and genotypic investigation of biofilm formation in multi-drug resistant *Enterobacter* species in the province of Al-Najaf. Similar to the study by (43), the isolates of *E. cloacae* from clinical and environmental samples showed a positive biofilm in 100% of cases, moderate biofilm production in 40% of cases, and strong biofilm production in 60% of cases.Although both species showed notable virulence characteristics overall, *E.sakazakii* seemed to have a wider range of adherence related characteristics, which might help explain why it was able to colonize wounds more successfully. Our study was similar to (12), when he revealed the virulence factors of *Enterobacter* species and he explained that most of them are non-hemolytic, contain capsules, form biofilms, and produce CFA / III at a rate of 80(95%) isolated from various clinical samples.

Pathogens that cause infection in burn wounds are *Enterobacter spp.* because of their weakened immune systems and compromised skin barriers; patients with burns are especially susceptible to infection. In addition, prolonged hospital stays, invasive diagnostic and therapeutic procedures, and the immunocompromising effects of burns all contribute to nosocomial infections in burn patients. Infection is influenced by the patient's size, depth, and age as well as the type, number, and pathogenicity of the microorganisms present(44). Variations in environmental conditions and attitudes regarding burn wound care can cause variations in the frequency rates of the most common pathogen, *Enterobacter spp.* by (16.19%) (45).

CONCLUSION

This study highlights the predominance of *Enterobacter cloacae* in urinary tract infections and *Enterobacter sakazakii* in wound infections, driven by the differential expression of virulence factors such as colonization antigens (CFA I/III). The widespread presence of capsules and biofilms among isolates suggests mechanisms that facilitate persistence and resistance in hospital environments. The significant association of *E. sakazakii* with CFA I/III underscores its enhanced adhesive capacity, potentially contributing to its clinical success in wound colonization. These results advocate integrated approaches that combine phenotypic virulence profiling with antimicrobial stewardship to mitigate infections caused by *Enterobacter* species, particularly in immunocompromised populations.

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