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Biochemical Identification and Antibiotics Resistance of *Citrobacter freundii* Isolated from Diarrheal Cases in Thi-Qar Province, Iraq

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Abstract

Citrobacter freundii is an opportunistic pathogen associated with gastrointestinal infections in children, particularly in areas with limited healthcare and inadequate antimicrobial management. This study aimed to determine the prevalence, resistance patterns, and colony traits of *C. freundii* in stool samples from children under two years old admitted to Al-Husain Teaching Hospital in Thi-Qar Province, Iraq. A total of 100 stool samples were collected and cultured. Among the Gram-negative isolates, *C. freundii* made up 3%. Antimicrobial susceptibility testing revealed complete resistance (100%) to first- and second-generation cephalosporins (cefazolin and cefuroxime), as well as the third-generation cephalosporin ceftriaxone. There was moderate resistance (60%) to amoxicillin-clavulanic acid. In contrast, all isolates were fully susceptible to carbapenems, aminoglycosides, and fluoroquinolones. These results confirm that the VITEK 2 Compact system is a quick and accurate method for identifying *C. freundii* and determining its susceptibility to antibiotics. Additionally, the high resistance to cephalosporins underscores the urgent need for regular monitoring of antimicrobial resistance and for informed choices about antibiotic use in managing pediatric diarrhea caused by multidrug-resistant *C. freundii*.

Keywords: Antimicrobial Resistance, *Citrobacter freundii*, Pediatric diarrhea, VITEK 2

الخلاصة

تعد بكتيريا سيتروباكتر فريوندي من الممراضات الانتهازية المرتبطة بالإصابات المعوية لدى الأطفال، ولا سيما في البيئات التي تعاني من نقص الخدمات الصحية وضعف إدارة المضادات الميكروبية. هدفت هذه الدراسة إلى تحديد معدل الانتشار، وأنماط المقاومة، والصفات الشكلية لبكتيريا سيتروباكتر فريوندي في عينات البراز المأخوذة من الأطفال دون سن السنتين الراقدين في مستشفى الحسين التعليمي بمحافظة ذي قار، العراق. تم جمع مائة (100) عينة براز وزرعها، وقد شكّلت سيتروباكتر فريوندي نسبة 3% من مجموع العزلات سالبة الغرام. أظهر اختبار الحساسية للمضادات الحيوية مقاومة كاملة (100%) تجاه السيفالوسبورينات من الجيلين الأول والثاني (سيفازولين وسيفوروكزيم)، وكذلك السيفالوسبورين من الجيل الثالث (سيفترياكسون). كما سُجّلت مقاومة متوسطة (60%) تجاه الأموكسيسيلين-كلافولانيك أسيد. وعلى النقيض من ذلك، أبدت جميع العزلات حساسية تامة تجاه الكاربابينيمات، والأمينوغليكوزيدات، والفلوروكينولونات، والفوسفومايسين. تؤكد هذه النتائج أن نظام VITEK 2 Compact يُعد وسيلة سريعة وموثوقة لتشخيص سيتروباكتر فريوندي وتحديد أنماط حساسيتها للمضادات الحيوية. علاوةً على ذلك، يُبرز المستوى المرتفع لمقاومة السيفالوسبورينات الحاجة الماسة إلى تعزيز برامج المراقبة الدورية لمقاومة المضادات الميكروبية، واتخاذ قرارات علاجية مدروسة فيما يتعلق باستخدام المضادات الحيوية في التعامل مع حالات الإسهال الطفولي الناجم عن عزلات سيتروباكتر فريوندي المقاومة للمضادات الحيوية.

الكلمات المفتاحية: مقاومة مضادات الميكروبات، سيتروباكتر فريوندي، إسهال الأطفال، VITEK 2 Compact

Introduction

Diarrheal disease is a major global health issue that mainly impacts infants and young children, causing about 1.3 million deaths each year, mostly in low-income countries where sanitation, nutrition, and healthcare are lacking [1,2]. Diarrhea occurs when intestinal ion transport is disrupted, resulting in mucosal damage and the loss of fluids and electrolytes [3]. Common causes including viral and bacterial infections, malabsorption of bile acids, deficiencies in disaccharides ,carbohydrate malabsorption,and chronic inflammation. While

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mortality rates have decreased in recent decades, diarrhea remains a leading cause of pediatric hospital visits in parts of Asia and Africa [4,5,6,7,8].

C. freundii is a Gram-negative facultative anaerobe in the Enterobacteriaceae family. It is increasingly seen as a healthcare associated pathogen [9]. This bacterium is found in soil, water, sewage, and the intestinal microbiota. C. freundii can lead to opportunistic infections like urinary tract infections, pneumonia, septicemia, and wound infections, especially in neonates, young children, and immunocompromised individuals [10,11].Recent surveillance data show that most citrobacter infections happen in hospitals. More than 95% of cases reported between 2000 and 2022 are connected to healthcare related exposures [12,13,14,15].

C.freundii's increasing presence in clinical setting presents a serious treatment challenge due to widespread antibiotics resistance ,including the production of extended-spectrum β-lactamase (ESBLs) and carbapenemase like blaCTX-M ,and blaNDM. Resistance rates differ significantly by region .In Switzerland , show 44% of strains show resistance to third-generation cephalosporins. In China, more than 50% are resistant to ceftriaxone, while in France, more than 90% are resistant to ertapenem [16,17]. These high resistance levels lead to more treatment failures and poorer patient outcomes. Additionally, hospital wastewater has been identified as a potential source and transmission route, increasing the public health risk [18,19.20].

The pathogenicity of C.freundii involves adhesion ,biofilm formation ,LPS endotoxin ,shiga-like toxin, heat-stable enterotoxins ,and the cholera toxin B subunit .These factors disrupt intestinal absorption and lead to inflammation[21,22].Genomic studies have revealed that the vi polysaccharide capsule and Type VI secretion system enable the bacteria to evade the immune response ,from biofilms ,and adhere to host cells [23] .Increasing antibiotic resistance from ESBLs, AmpC β-lactamase ,and carbapenemase makes treatment more challenging [24,25,26,27,28,29].Studies conducted in high-income countries ,revealing gaps in understanding the epidemiology and resistance in at-risk children. .

This study aims to evaluate the prevalence, antibiotic resistance, and characteristics of C. freundii in stool samples from infants with diarrhea at Al-Husain Teaching Hospital in Thi-Qar Province, Iraq. It will also assess the VITEK 2 Compact system for quick identification and susceptibility testing. The findings are expected to enhance knowledge about C. freundii as a diarrheal pathogen and aid in the development of effective treatment strategies in under-resourced regions.

Materials and Methods

Collection of samples

A total 0f 100 stool samples were collected from male and female infants under the age of two with diarrhea .These samples were collected from hospitals and public health laboratory in Thi-Qar province from November 24 to February 25. All stool samples were collected in sterile tubes with peptone water as the transportation medium to maintain the viability of the bacteria.The stool samples were immediately transported to the bacteriology lab in a cool box, which maintains microbial viability throughout the transportation period .

Ethical Approval and Informed Consent

The study was reviewed and approved by the Ethical Committee of the College of Veterinary Medicine

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and Surgery/University of Shatrah, Iraq. All procedures were performed in accordance with the Declaration of Helsinki and local ethical standards. Prior to collecting any stool sample, the parents or legal guardians of each infant signed an informed consent form. National and international ethical principles and standards were also followed.

Isolation and Culture of Bacteria

The stool samples were incubated in a peptone water broth for twenty-four hours at 37°C upon receipt at the laboratory to enrich for potential enteric pathogens. Following this enrichment, the samples were streaked for isolation on a selective and differential medium for Gram-negative bacteria, MacConkey agar, and returned for an additional twenty-four-hour incubation at 37°C. Colonies were selected for further evaluation on the MacConkey agar based on distinct morphology after incubation, such as red-pink colonies that were lactose fermenters. The evaluation of the MacConkey agar plates was completed by using a biochemical assay (Vitek-2).

Biochemical Identification and Antibiotic Susceptibility Testing

Clinical Gram-negative bacilli were identified using the VITEK-2 system with ID-GN cards, which contain 64 biochemical tests each. This method provides results in about 10 hours for Enterobacteriaceae and non-glucose-fermenting species. Antibiotics susceptibility was determined using AST-N417 cards ,following the manufacturer's instructions [15].A 0.5 McFarland bacterial suspension was diluted in 3 ml of sterile saline ,resulting in approximately 8×10^6 CFU/ML , and 200 µl was inoculated per card. The system automatically incubates ,reads and interprets MICs, adjusting results based on phenotypic profiles for various antibiotics ,including Caftazidime , Cefepime , Imipenem , Meropenem , Gentamicin , Ciprofloxacin , Trimethoprim , Ertapenem , Amoxicillin/Clavulanic acid , and Tigecycline [31,32,33,34].

Statistical Analysis

Statistical analysis was conducted to evaluate antimicrobial susceptibility patterns. Following the guidelines of Clinical and Laboratory Standards Institute (CLSI), the results were classified as sensitive (S), intermediate (I), or resistant (R). Microsoft Excel was used to calculate descriptive statistics, including the quantity and proportion of isolates in each antibiotic susceptibility category. IBM SPSS Statistics version 26 was used to conduct a chi-square (χ^2) test of independence to ascertain whether variations in the antibiotics' susceptibility distributions were statistically significant. The statistical significance was set at $p < 0.05$. A 95% confidence interval was used for all tests.

Results

Identification and Colony Morphology of C. freundii

C.freundii isolates were grown on MacConkey agar and incubated at 37° C for 24 hours .The colonies appeared smooth,moist ,medium to large and slightly raised with smooth edges.They exhibited a distinctive color range,from pink to dark pink,indicating lactose fermentation (Figur1 A)There was no swarming or hemolytic activity. These physical characteristics helped identify C. freundii as a lactose-fermenting member of the Enterobacteriaceae family.

For definitive identification, the VITEK 2 Compact automated system (bioMérieux, France), based on biochemical profiles, was used. As shown in Figure

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1B, C. freundii made up about 3 % of all Gram-negative isolates, while other genera, including Escherichia, Klebsiella, and Enterobacter, accounted for the remaining 97 %. Although C. freundii was isolated less frequently, it remains important in clinical settings due to its growing role in diarrheal disease and potential antimicrobial resistance.

Antimicrobial Susceptibility Pattern of C. freundii

The antimicrobial susceptibility profile of C. freundii isolates is shown in Figure 2 and Table 1. All isolates were 100% sensitive to piperacillin/tazobactam, ceftazidime, cefepime, imipenem, meropenem, amikacin, gentamicin, ciprofloxacin, trimethoprim, and ertapenem. There was complete resistance (100%) to cefazoline, cefuroxime, cefuroxime axetil, and ceftriaxone. Amoxicillin/ clavulanic acid exhibited moderate resistance, with approximately 60% of isolates displaying resistance.

Discussion

The identification and antimicrobial suscepitibility results of C.freundii offer important insights into its clinical relevance and resistance profile in pediatric cases of diarrhea .Its ability to produce smooth, moist, pink colonies on MacConkey agar (Figure 1A) supports its initial classification as a lactose fermenter in the Enterobacteriaceae family. Confirmation using the VITEK 2 compact system supplies a reliable method for definitive identification .Although C. freundii accounted for approximately 3% of Gram-negative isolates in this study (Figure 1B), it remains a relatively uncommon isolate compared to other enteric pathogens.

The antimicrobial susceptibility pattern (Figure 2) shows a concerning level of resistance to several commonly used drugs. Complete resistance to first-generation cephalosporins (cefazoline, cefuroxime), third-generation ceftriaxone, and trimethoprim aligns with the production of chromosomal β-lactamases. These enzymes grant broad resistance to many β-lactam antibiotics [35]. Additionally, the moderate resistance (~60%) to amoxicillin/clavulanic acid suggests that some common oral treatments used in community settings may be less effective [36,26,27].

On the other hand, the isolates showed 100% susceptibility to a wide range of antibiotics, including carbapenems, aminoglycosides, and fluoroquinolones. This maintained susceptibility is promising, especially for managing severe or invasive infections. In pediatric populations ,the full effectiveness of carbapenems may be due to limited exposure and reduce selective pressure; however ,ongoing monitoring is essential to identify emerging resistance [37].

Employing automated systems ,such as VITEK 2,for routine diagnostics is necessary to enhance the accuracy and efficiency of bacterial identification and antimicrobial susceptibility testing. The employing conventional methods can result in misidentification due to overlapping biochemical characteristics within the Enterobacteriaceae family [38]. Automated systems represent a quality assurance process that decreases errors while increasing data quality. This is crucial for targeting therapies, especially in low-resource environments or high-risk settings [39]. While C. freundii was detected at a low frequency, its impact on clinical samples should not be disregarded. Importance needs to be placed on the bacteria's intrinsic resistance mechanisms, their ability to acquire resistance genes, as well as their potential to spread in hospitals. These observation underscore the

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importance of ongoing monitoring and robust antimicrobial steawrdship initiatives [22,9].

This highlights the need to consider C. freundii as an emerging diarrheal pathogen and confirm the implementation of evidence-based strategies to control infections, particularly in at-risk children [39, 40].

Conclusion

This study elucidates regarding the emerging function of C.freundii in causing diarrhea in children. Although uncommon, this organism's profound resistance to commonly used first-line antibiotics, including cephalosporins and trimethoprim, poses significant challenges. Careful consideration must be given to the selection of antibiotics for this organism. The aforementioned treatments remain highly effective, as the bacteria remain susceptible to carbapenems, aminoglycosides, and fluoroquinolones. Timely and accurate identification, as well as susceptibility testing facilitated by automated systems such as VITEK 2, are crucial. It is essential to actively track and manage antimicrobial resistance and provide tailored intervention strategies ,which are crucial for controlling the spread of multidrug-resistance C.freundii in healthcare setting ,particularly in resource-limited areas.

Study Limitations

The small sample size and focus on a single geographic area may limit the generalizability of the findings to other populations. Moreover, the molecular analysis of virulence genes was not performed in this study. This would have been a good opportunity for understanding the pathogenicity of C. freundii. Further genomic analysis and multicenter monitoring are necessary to validate these results.

Authors' contributions

H.A.K. collected samples and conducted laboratory experiments. H.J.K. and S.H.A. designed and supervised the research and data interpretation. H.A.K. and H.J.K. jointly analyzed the data and wrote the manuscript. All authors reviewed and approved the final version of the manuscript.

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Declaration of competing interests

The authors declare no conflicts of interest related to this work.

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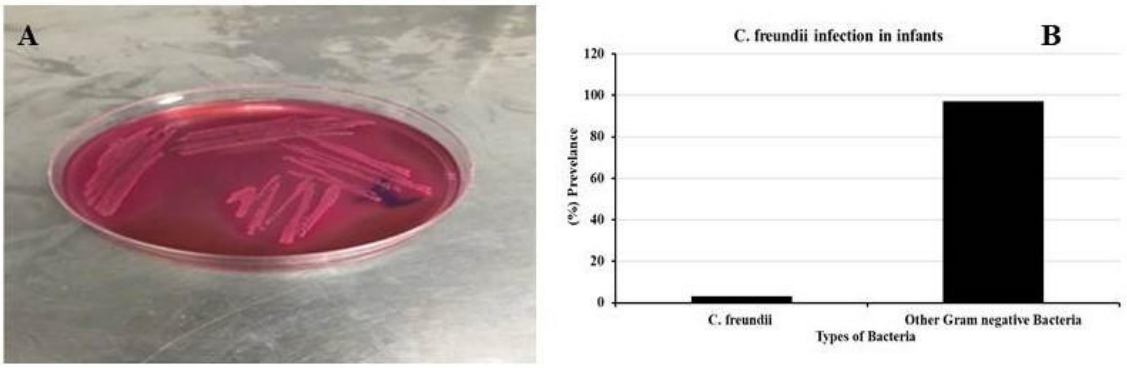

Figure 1. (A) *C. freundii* colonies on MacConkey agar after 24 h at 37 °C, showing pink coloration due to lactose fermentation. (B) Proportion of *C. freundii* (3%) among Gram-negative isolates identified by the VITEK 2 system.

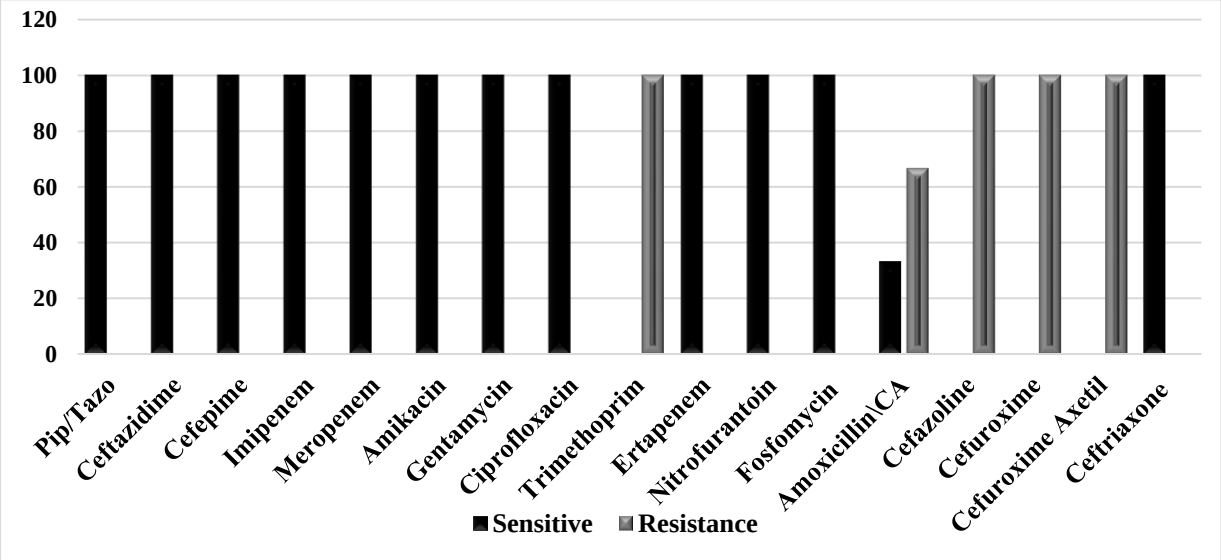

Figure 2. Antimicrobial susceptibility profile of *C. freundii* isolates. Black bars represent the percentage of isolates sensitive to each antibiotic, while gray bars indicate resistance. High sensitivity was observed to most antibiotics, with complete resistance to several cephalosporins and moderate resistance to amoxicillin/clavulanic acid.

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Table 1: Distribution of sensitivity, intermediate, and resistance rates of *C. freundii* to various antimicrobial agents and their statistical significance ($p < 0.01$).

C. freundii	Sensitive		Intermediate		Resistance	
	No.	%	No.	%	No.	%
Pip/Tazo	3	100	0	0.0	0	0.0
Ceftazidime	3	100	0	0.0	0	0.0
Cefepime	3	100	0	0.0	0	0.0
Imipenem	3	100	0	0.0	0	0.0
Meropenem	3	100	0	0.0	0	0.0
Amikacin	3	100	0	0.0	0	0.0
Gentamycin	3	100	0	0.0	0	0.0
Ciprofloxacin	3	100	0	0.0	0	0.0
Trimethoprim	0	0.0	0	0.0	3	100
Ertapenem	3	100	0	0.0	0	0.0
Amoxicillin\CA	1	33.33	0	0.0	2	66.67
Cefazoline	0	0.0	0	0.0	3	100
Cefuroxime	0	0.0	0	0.0	3	100
Cefuroxime Axetil	0	0.0	0	0.0	3	100
Ceftriaxone	3	100	0	0.0	0	0.0
Susceptibility %	72.55		0.0		27.45	
$CalX^2 = 47.6$	$TabX^2 = 26.3$		DF= 28		p. value $<0.01^{**}$	