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College of Veterinary Medicine
Department of Microbiology**



**Molecular and Biochemical Identification of Gram-Negative
Bacteria in Infants Suffering from Diarrhea in Thi-Qar
Province**

A Thesis

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Shatrah, in Partial Fulfillment of the Requirements for a **Master's
Degree** in Veterinary Science (Microbiology)

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بِسْمِ اللَّهِ الرَّحْمَنِ الرَّحِيمِ

﴿وَقُلْ رَبِّ زِدْنِي عِلْمًا﴾

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Dedication

-To the one whose name I carry with pride, whom God honored me with and chose to be my father; the source of my strength, and honor ,my beloved father.

-To my joy, my light, and the happiness of my life; to my beloved inspiration and my first teacher; to the one who granted me life, hope, and endless support, and eased my hardships through her prayers, my mother, Suad.

-To the steadfast pillars of my life, my solid ground and strongest wall; to the source of my motivation and success , my brothers and sisters: Hajar, Talib, Yousif, Mohammed Sadiq, Zainab, and Enas.

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Hawraa Abbas Khudhur

Summary

Diarrhea is a leading cause of illness and death among children under five years ,particularly in developing countries. It is defined as passing three or more loose stools per day, which can lead to dehydration and electrolyte imbalance. Key contributors include infections,malnutrition,poor sanitation, and unsafe water, despite being preventable and treatable,diarrhea remains a significant public health issue, breastfeeding, oral rehydration therapy,proper nutrition,and improved sanitation can mitigate its effect. However, there are still challenges with access and implementation. Improving these measures is crucial for reducing child illness and death rates.

This study aims to identify the bacterial causes of diarrhea,with a particular focus on Gram-negative bacteria. It utilizes biochemical tests,such as the Vitek system, and also aim to validate the findings with molecular analysis methods and determine the most effective antibiotics against the identified Gram-negative bacterial isolates.

A total of (132) stool samples were collected from children presenting with diarrhea to investigate possible bacterial causes. Following the initial assessment,(32)samples were excluded due to contamination or confirmed parasitic infections, leaving (100) samples eligible for bacterial analysis. These samples were first enriched using peptone broth before being cultured on MacConkey agar for the isolation of Gram-negative bacteria. Bacterial identification was done using the VITEK II system. Among the isolates, seven Gram-negative bacteria were found. *Escherichia coli* was the most common,making up (59%)of the isolates. It was followed by *Klebsiella* spp. at (32%) (including 17% *K.pneumoniae* and 7% *k.oxytoca*, *Klebsiella aerogenes* at 8%)and lower rates of *Citrobacter freundii* and *Routella orinthinoytica*. The gender distribution was almost equal, with infections

present in (51%) of females and (49%) of males. Molecular confirmation was conducted using PCR amplification of the 16S ribosomal RNA (rRNA) gene, which is specific for Gram-negative bacteria, producing a clear band of approximately (353) bp on agarose gel electrophoresis. The identity of the isolates was confirmed through DNA sequencing, and the sequences obtained were compared with entries in the NCBI database. Antibiotic susceptibility testing showed that meropenem was the most effective, with (100%) sensitivity against *E. coli*, *Klebsiella* spp., *Citrobacter freundii*, and *Enterobacter* spp. Imipenem and piperacillin/ tazobactam also proved to be highly effective. However, trimethoprim showed (100%) resistance in *C. freundii* and (81%) in *E. coli*. High resistance rates of (88% to 100%) were observed for cefazolin, cefuroxime, and cefuroxime axetil, indicating a rise in multidrug-resistant Gram-negative pathogens. In conclusion, Gram-negative bacteria *E. coli* and *Klebsiella* species, play a significant role in causing diarrhea in children. The findings emphasize the importance of careful antibiotic use, ongoing monitoring of resistance patterns, and implementing preventive measures such as breastfeeding, improved nutrition, sanitation, and access to safe drinking water. These strategies are crucial for lowering the rate of diarrheal diseases and the risk from multidrug-resistant pathogens.

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List of Abbreviations

Abbreviations	Expansion
aEPEC	Atypical Enteropathogenic E. coli
AMC	Amoxicillin/clavulanic acid
BHIA	Brain heart infusion agar
CFU	Colony-forming unit
CIP	Ciprofloxacin
CN	Gentamicin
DNA	Deoxyribonucleic Acid
GN	Gram negative
GNEBPs	Gram-negative enteric bacterial pathogens
GP	Gram positive
IBD	inflammatory bowel disease
IBD	Inflammatory bowel disease
IgE	Immunoglobulin E
IMP	Imipenemase
KPC	Klebsiella pneumoniae carbapenemases
MDR	Multi-drug resistance
MEM	Meropenem
NCBI	National center for Biotechnology information
pb	Base pair

PCR	Polymerase chain reaction
RNA	Ribonucleic Acid
rpm	Revolution per minute
rRNA	ribosomal ribonucleic acid
SPSS	Statistical package for the social Sciences
T _m	Melting temperature
UV	Ultraviolet
VF _s	virulence factors
WHO	World Health Organization

Chapter One

Introduction

1.1. Introduction

One of the leading causes of morbidity and mortality is diarrhea , which is widespread throughout the world and affect all ages , Its causes death annuly to approximately two million people , the most significiant proportion of children under the age of five (Shen *et al.*, 2016). Diarrhea was defiened as the recurrence of loose stool and increased frequency. Diarrhea means that for most patients, passage of loose stool is accompanied by increased bowel movement frequency. Passage of loose stool more than twice a day is considered abnormal (Adam *et al.*, 2024).

The etiology of diarrheal disease , caused primarily by a wide variety of bacterial , viral , and parasitic pathogens , varies depending on several conditions , including geographic and climatic conditions , socioeconomic situation and host factors (Hodges and Gill, 2010). Diarrhea is one of the most common disease , in the world in children , which truly threatens global health , as it is considered the second leading cause of death in children after respiratory disease .

The primary causes of bacterial diarrhea are *Salmonella* spp., *Campylobacter jejuni* and *Enterohemorrhagic E.coli* (Gómez-Duarte *et al.*, 2009). Diarrheal disease can be classified into three primary clinical syndrome : acute watery diarrhea ,bloody diarrhea and persistent diarrhea .The incidence of this disesase accounts for 63%of global health problems (Walker *et al.*, 2013; Zhang *et al.*, 2016). Accurate and quick diagnosis of the causes of diarrhea is critical ,standard methods in diagnosis take along time to determine the bacterial causes of diarrhea , and previous studies have had difficulty diagnosing the causes of approximately 40% of diarrhea cases (Ugboko *et al.*, 2020).

For children who are under the age of five , diarrhea is dangerous to them because it cause a severe lack of fluid in their bodies , as it negatively affects electrolytes and causes dehydration that requires emergency therapeutic intervention , especially if it is accompanied by fever and presence for more than twenty four hours (WHO).The infection rate in adults is considered relatively low compared to the infection rate in children under five . Studies have confirmed that the incidence of diarrhea in children is a higher in groups of children under 24 months old and the percentage decrease with increasing age .

The immune condition and loss of production of antibodies to endotoxins , such as those from E.coli , play crucial role in the pathogenesis of diarrhea in children . Consequently , the percentage of antibodies to these bacteria increases as children age. Therefore , developing countries , due to the development of antibiotics resistance in their populations , are the regions most vulnerable to the disease (Eckburg *et al.*, 2005). The WHO estimates that 88%of diarrhea cases are due to unsafe water supplies and inadequate sanitation .

1.2. Rationale and Aim of the Study

Diarrheal diseases remain a significant global health problem, especially in developing countries . poor sanitation , limited access to clean water , and rising antimicrobial resistance increase the risk .Even though diarrhea can be prevented and treated ,it continues to be a leading cause of death among children under five ,emphasizing the need for better diagnostic and management strategies .Tradionals methods for diagnosing the bacterial causes of diarrhea often require a lengthy process and may miss pathogens in many cases .Quickly and accurately identifying the causes is crucial ,It

helps ensure proper treatment , reduces the disease burden ,and prevents complications like severe dehydration .

Additionally ,the misuse and overuse of antibiotics have led to the development of resistant strains of Gram- negative bacteria ,making treatment outcomes more challenging . Understanding the antibiotic resistance patterns of diarrheal pathogens can provide valuable insights into the management of these infections .It can help shape effective treatment practices and developed local antibiotic management programs , This study is therefore justified as it aims to :

1. Isolate and identify Gram-negative bacteria causing diarrheal infections using morphological and biochemical tests .
2. Confirm the bacterial isolates by molecular techniques(PCR)
3. Evaluate the antibiotics sensitivity test patterns of the identified Gram-negative bacteria .

Chapter Two

Literature Review

2. Literature Review**2.1. Diarrhea: Definition and Impact**

The WHO defines diarrhea as the passage of three or more watery or loose stool within a (24) hour period . while researchers often focus on frequency , change in stool consistency might to be more important in a clinical setting. Diarrhea has many causes , including infections, allergies , immune disorders , and tumors , with infections being the most common worldwide (Thapar *et al.*, 2004).

Historically , diarrhea led to about (4.6) million child deaths each year during 1980s , mainly due to dehydration . These death were largely preventable with proper treatment . From (2000- 2003) , global childhood deaths from diarrhea had dropped to nearly 2 million per year , but diarrhea still ranks as the second leading cause of death for children after the neonatal period . The rate of illness however , has remained relatively unchanged (Bryce *et al.*, 2005). The pathogens that cause diarrhea in children include viruses , bacteria , and parasitic .Rotaviruses are leading viral causes , affecting nearly all children by the age of three . It account for 40%of diarrhea cases in developing countries and 60%in developed nations (Thapar *et al.*, 2004).

2.2. Gut Microbiota and Dysbiosis

The human gastrointestinal tract contains a complex microbial community that is vital for metabolism and immune function . Over (1,000) different microbial species have been identified , although many have not been cultured (Rajilić-Stojanović *et al.*, 2014).Gut colonization is significant for newborns , as it supports metabolic and immune development .

Disrupting this process can lead to dysbiosis , characterized by the loss of beneficial bacteria , reduced microbial diversity , or an overgrowth of harmful species .Factors that contribute to dysbiosis include genetics , antibiotics use , diet, stress, and chronic illness. Dysbiotic microbes can change how the host metabolizes nutrients and may lead to disease , but it is often unclear whether dysbiosis causes illness or is the result of it .

In cases of diarrhea, harmful bacteria often take over the gut microbiome , inhibiting beneficial bacteria and producing toxins .These toxins disrupt gut balance and trigger immune responses , ultimately resulting in diarrhea (Li *et al.*, 2021).

2.3. The Primary Etiologic Agents of Diarrhea

While some cases of diarrhea are attributed to metabolic faults, chemical imbalances, or organic disturbances, the vast majority are caused by infectious pathogens.

1. Bacterial Infections

Particularly, Gram-negative enteric bacterial pathogens (GNEBPs) are the common causes of the syndrome. The group includes bacteria that reside mainly in the intestine. Genera such as *Escherichia*, *Shigella*, *Campylobacter*, *Salmonella*, *Enterobacter*, *Yersinia*, *Klebsiella*, *Serratia*, *Proteus*, and others are included in the group. However, the most common and significant pathogens are *S. enterica*, *E. coli*, *Campylobacter*, and *Shigella spp* (Beyene *et al.*, 2022).

2. Viral Infections

Viruses have been major pathogens causing acute gastroenteritis for a long time. *Rotavirus group A* and *norovirus* are the most common causes of acute diarrhea in children. Human *adenovirus* and classic human *astrovirus* are also significant causes of sporadic and outbreak diarrhea in children (Lu *et al.*, 2021). Rotavirus is estimated to account for around (600,000-875,000) child fatalities each year. In temperate regions, rotavirus epidemics typically occur during the cooler months, while in tropical areas, they can happen year-round. The virus is mainly passed through the fecal-oral route. Additionally, other viral families are responsible for causing (Dennehy, 2005).

3. Parasites

The host's immune defenses predominantly influence the severity of parasitic diarrhea; when these defenses are compromised, the incidence and severity of parasitic diarrhea increase significantly. In developing nations, protein-energy malnutrition is the leading cause of immune deficiency. Diarrhea resulting from *Strongyloides* or *Giardia* is frequently observed in malnourished children, whereas well-nourished children often serve as asymptomatic carriers. Additionally, diarrhea caused by *cryptosporidium* spp. can be particularly severe in malnourished or immune compromised children, with recovery generally contingent upon nutritional rehabilitation or addressing the underlying immunodeficiency (Gendrel *et al.*, 2003).

4. Food Intolerances

The manifestations of classic IgE-mediated food allergy can vary in intensity and may encompass a range of symptoms, including laryngeal

edema , nausea , vomiting , diarrhea , urticaria ,and hypotension .Allergic reactions can be elicited by a variety of food, with the most frequently implicated being cow's milk , eggs , peanuts , soy, tree nuts,fish, wheat ,and shellfish. Allergies to eggs and milk typically arise during childhood .They are often outgrowth, whereas allergies to tree nuts and peanuts can develop in both childhood and adulthood and are less likely to resolve being associated with a higher risk of severe reactions.Given the potential for triggering life threatening responses , it is essential to identify the possibility of cross reactivity across food groups. The diagnosis of food allergy is established through skin prick testing specific serum IgE testing , and oral food challenges. Management strategies primarily involve the prevention of allergenic and cross reacting foods ,as well as the prompt identification and treatment of allergic reactions. Current research is exploring treatment protocols aimed at desensitizing patients to specific food allergens (Patel *et al.*,2015).

5. Reaction to Medicines

The identification of a potential cause of chronic diarrhea presents a significant challenge in patients who are on multiple medications. This condition may be associated with intestinal mucosal alterations that resemble diseases such as celiac disease, or it may be purely functional, showing no histopathological changes. Drug-induced diarrhea can be linked to malabsorption of nutrients, although this association is not always present. Clinical improvement may be observed within days following the cessation of the offending drugs, or it may take longer if mucosal injury is involved in diabetic patients. Diarrhea is often mistakenly attributed to poor management and lack of control, but it might stem from the use of oral

hypoglycemic agents Chemotherapy can lead to either spread or segmental colitis, while olmesartan and few other medications can infrequently induce a condition that mimics celiac disease, albeit without gluten intolerance. Therefore, heightened awareness of drug-induced diarrhea and a comprehensive understanding of its clinical manifestation are essential for clinicians in addressing this complex issue (Philip *et al.*, 2017).

6. Intestinal Diseases

The pathogenesis of diarrhea associated with inflammatory bowel disease (IBD) is complex and arises from multiple factors primarily due to mucosal injury induced by chronic inflammation. This condition results in dysregulation of intestinal ion transport, compromised epithelial barrier integrity, and an increased susceptibility of the intestinal mucosa to pathogenic organisms. The primary mechanism underlying diarrhea in IBD is the altered expression and function of epithelial ion transport and channels, resulting in electrolytes retention and excess fluid accumulation within the intestinal lumen. Additionally, impaired barrier function contributes to diarrhea through leak flux mechanisms. The invasion of enteric pathogens into mucosa further disrupts microbial balance (dysbiosis) and intensifies immune responses, thereby exacerbating tissue damage and diarrhea associated with IBD (Anbazhagan *et al.*, 2018).

2.4. Transmission Routes

Diarrhea infection in infants have been linked with pathogens such as *Campylobacter jejuni*, *enterotoxigenic and enteropathogenic E.coli*, *shigella*, *rotaviruses* and *cryptosporidium*. These microorganisms are primarily transmitted within the home environment through the feces of

animals , contaminated food and water , and direct person to person contact . A significant transmission route appears to be the weaning food , which is frequently contaminated due to improper preparation and inadequate sanitation of utensils . Therefore enhancing feeding practices , minimizing exposure to animals feces, and improving personal and domestics hygiene are essential strategies for reducing the high prevalence of diarrhea in developing countries among infants (Black *et al.*, 1989).

2.5. Types of Diarrhea

1. Acute Diarrhea

Acute diarrhea, defined as diarrhea lasting less than four weeks (Meisenheimer *et al.*, 2022), is a nonspecific response of the intestines to various conditions. These can include infections, adverse drug reactions, inflammatory bowel disease, and ischemia. Certain drugs and poorly absorbed sugars can cause acute diarrhea. Although most cases of diarrhea are a result of infections (Table2-1)

Table (2-1). Etiology of acute Diarrhea infectious

Bacteria	Viruses	Parasites
<i>Salmonella</i> sp.	<i>Rotavirus</i>	<i>Giardia lamblia</i>
<i>Shigella</i> sp.	<i>Norwalk virus</i>	<i>Entamoeba histolytica</i>
<i>Enterobacter</i> sp.	<i>Norwalk-like agents</i>	<i>Cryptosporidium</i> sp.
<i>campylobacter</i> sp.	<i>Enteric adenovirus</i>	<i>Cyclospora</i> sp.
<i>Enterotoxigenic E. coli</i>	<i>Small round viruses</i>	
<i>Enterohemorrhagic E.coli</i>	<i>Calcivirus</i> sp.	
<i>Enteroinvasive E.coli</i>	<i>Coronavirus</i> sp.	

<i>Enteropathogenic E.coli</i>	<i>Herpes simplex virus</i>	
<i>Yersinia</i>	<i>Cytomegalovirus</i>	
<i>Clostridium difficile</i>	<i>Astrovirus</i>	
<i>Clostridium perfringens</i>		
<i>Vibrio</i>		
<i>Staphylococcus aureus</i>		
<i>Chlamydia</i>		
<i>Treponema pallidum</i>		

2. Chronic Diarrhea

Chronic diarrhea , characterized by a change in stool consistency lasting longer than four weeks, is a prevalent yet complex clinical condition (Juckett and Trivedi, 2011). Chronic diarrhea in pediatric patients presents significant challenges in both etiological diagnosis and mangements. The underlying causes and pathological mechanisms differ considerably across age groups , with congenital or genetic etiologies being more prevalent in neonates . At the same time , infections, allergies, and immune mediated processes are more common in older children .

A comprehensive patient history and through physical examination are essential for determining the necessity of further diagnostics investigations. The management approach should be age specific and primarily informed by the underlying pathophysiological mechanisms. The characteristics of the stool, such as its watery , bloody , or fatty nature (steatorrhea), can provide critical insights into likely etiology and the organ system involved . Following intial routine assessments , additional evaluations may include

specific serological tests , imaging studies , endoscopy (such as gastroscopy/colonoscopy), histopathological examination of intestinal mucosa , breath tests , or radionuclide imaging to establish a definitive diagnosis .

Genetic testing is significant in cases of congenital diarrhea , monogenic inflammatory bowel disease (IBD), and immunodeficiency disorders .The management strategy aims to stabilize the patient, provide nutritional support, and implement etiology specific treatments . Therapeutic interventions can range from simple dietary exclusions to complex procedures such as small bowel transplantation .Given the complexity of these cases , timely referral to specialized care is crucial for minimizing morbidity , including nutritional deficiencies , and enhancing overall patient outcomes (Tripathi and Srivastava, 2024).

3. Dysentery

Bloody diarrhea , also known as dysentery , is characterized by inflammation of the intestines particularly the colon resulting in abdominal pain and severe diarrhea containing blood or mucus. It represents a significant public health concern , especially in developing countries , where it contributes to high morbidity rates among children under five years of age. several bacterial and parasitic pathogens are responsible for dysentery , including *shigella* , *campylobacter* , *salmonella* , *schistosoma mansoni* and , *entamoeba histolytica*. In tropical regions , *shigella* is the predominant causative agent, with epidemic outbreaks of *shigella* dysentery type I associated with mortality rates as high as (6.2%). In contrast , *salmonella* is primary pathogen causing dysentery in developed countries (Traa *et al.*, 2010).

2.6. Risk Factors of Diarrhea**1. Age of the Child**

Young age (under 23 months) has been identified as a key risk factor positively associated with all forms of diarrhea due to the natural physiology of their bodies to developing and growing (Gladstone *et al.*,2010), whereas age under 60 months has been determined to be significantly positively associated to diarrhea by two studies (Gladstone *et al.*,2008).

2. Malnutrition

In their seminal (1968) review , Scrimshaw , Taylor and Gordon highlited the bidirectional relationship between infection and malnutrition. They explained that infections can adversely affect nutritional statutes by decreasing food intakes, impairing nutrients absorption , increasing tissue catabolisim and depleting essential nutrients needed for growth and tissue repair. Conversely , malnutrition compromises the body's defense mechanisms , including the integrity of the skin and mucous memembranes,and impairs immune funiction, thereby increasing susceptibility to infection. This interaction has been further supported by subsequent research , including (Brown.,2003), who emphasized the cyclical nature of this relationship and its implications for children health , particularly in low resource setting.

3. Anemia

Previous studies have demonstrated a significant association between diarrhea, fever, and anemia in children under five years of age living in urban slum areas. Micronutrient deficiencies particularly of vitamin A and zinc have also been implicated in exacerbating anemia linked to chronic inflammation, due to their essential roles in maintaining immune competence and protecting against infectious diseases (Fraker *et al.*, 2000). Children suffering from deficiencies in these nutrients are more susceptible to diarrheal illnesses, which may in turn contribute to the development or worsening of anemia through prolonged inflammatory responses (Untoro *et al.*, 2005).

4. Water-Related Factor

Contaminated water and food have been widely recognized as major contributors to diarrheal diseases. Several water-related factors have been shown to significantly influence the incidence of diarrhea, including the distance to the water source (Gascon *et al.*, 2000) and unsafe water storage practices (Jensen *et al.*, 2002). For example, drawing water by dipping into storage containers, rather than using hygienic dispensing methods, increases the risk of contamination. In addition, the use of untreated or unclear water sources—such as rivers, dams, streams, lakes, wells, and other surface water bodies—has been associated with higher rates of diarrheal illness (Tumwine, 2002; Brooks *et al.*, 2003). Storing water in wide-mouthed containers has also been identified as a risk factor (Jensen *et al.*, 2002). Furthermore, low per capita water consumption may contribute to poor hygiene practices, thereby indirectly increasing the risk of diarrheal diseases.

5. Sanitation and Rainy Factors

The latest report from the Intergovernmental panel on climate change(IPCC) emphasized the escalating impacts of climate changes , which is crucial for understanding its effects on human health and well being (Geremew *et al.*, 2024). Tow contrasting hypotheses have emerged regarding the relationship between rainfall increase the concentration and persistence of pathogens due to poor enviromental cleansing (Mukabutera *et al.*, 2016). In contrast , the second hypothesis argues that heavey rainfall can increase the incidence of diarrhea by washing pathogens into water sources, particulariy in setting lacking piped water and improved sanitation.This view is supported by findings indicating that higher mean temperatures and increased rainfall are associated with more diarrhel cases in households relying on unimproved water source or sanitation facilities (Kemajou, 2022).

6. Breastfeeding

The absence of exclusive breastfeeding for infants aged (0-5) months , as well as lack of continued breastfeeding for children aged (6-23) months , has been associated with increased rate of diarrhea related morbidity and mortality in developing countries . Among infants aged (0-5) months , exclusive breastfeeding provides significantly greater protection against diarrheal diseases compared to predominant or partial breastfeeding (Lamberti *et al.*, 2011). This protective effect appears to opearate through two primary mechanisms : reducing both the incidence and duration of diarrheal episodes. Notably, the effect size is greater for the reduction in diarrhea prevalence than for incidence, suggestion that the primary way in

which breastfeeding reduces diarrhea related mortality is by shortening the length of diarrheal episodes .

7. Hygiene Practices

Ensuring access to basic hygienic services , including continuous access to water at the household level (24 hours per day) (Soe *et al.*, 2024) , has been identified as a key factor in reducing diarrheal disease. Additional associated factors include promoting hand washing with soap (Lakew *et al.*, 2024), proper disposal of children's waste items , the duration of drinking water storage , water treatment methods , and the cleanliness of water storage containers . Collectively , these findings suggest that improvements in water , sanitation , and hygiene infrastructure and behaviors represent effective interventions for reducing the burden of diarrheal diseases , particularly in low resource settings (Abera *et al.*, 2024).

8. Immunodeficiency

Patients with primary immunodeficiency now referred to as inborn errors of immunity (IEI) demonstrate an increased susceptibility to autoimmunity and neoplasia, however , the predominant clinical manifestations are recurrent infections , including those affecting the gastrointestinal tract (Notarangelo *et al.*, 2020). Several studies have reported differences in the composition of intestinal microflora between patients with common variable immunodeficiency (CVID) and healthy individuals , which may contribute to gastrointestinal symptoms such as diarrhea (Bosák *et al.*, 2021). Diarrhea is also a common clinical manifestation in HIV infected children , with an incidence ranging from (9.9% to 24.5%) (Zash *et al.*, 2016). Furthermore ,

children living with HIV are (5.6) times more likely to experience diarrhea compared to their HIV negative counterparts (Acácio *et al.*, 2018).

2.7. Gram-Negative Bacteria Etiology and Pathogenesis

The main bacterial species that are considered responsible for causing diarrhea in children include *E.coli* ,*campylobacter* ,*salmonella* , *shigella* , *Yersinia enterocolitica* , and *vibrio cholera* .These bacteria are significant pathogens that impact children's health , leading to acute gastrointestinal symptoms (Dennehy, 2005).

Research has provided new insights into the mechanisms of diarrhea caused by various pathogens that are classified as noninflammatory, inflammatory or invasive. These three groups of organisms cause two diarrheal syndromes—noninflammatory diarrhea and inflammatory diarrhea. The noninflammatory diarrheas are caused by enterotoxin-producing organisms such as *Vibrio cholerae* and enterotoxigenic *Escherichia coli*, or by viruses that adhere to the mucosa and disrupt the absorptive and/or secretory processes of the enterocyte without causing acute inflammation or mucosal destruction. Inflammatory diarrhea is caused by two groups of organisms—cytotoxin-producing, noninvasive bacteria (e.g. *enteroaggregative Escherichia coli*, *enterohemorrhagic Escherichia coli* and *Clostridium difficile*), or by invasive organisms (e.g. *Salmonella* spp., *Shigella* spp., *Campylobacter* spp., *Entamoeba histolytica*). The cytotoxin-producing organisms adhere to the mucosa, activate cytokines and stimulate the intestinal mucosa to release inflammatory mediators. Invasive organisms, which can also produce cytotoxins, invade the intestinal mucosa to induce an acute inflammatory reaction, involving the activation of

cytokines and inflammatory mediators. Regardless of the underlying mechanism they use, these various types of pathogen have all successfully evolved to evade and modulate the host defense systems (Navaneethan and Giannella., 2008).

2.7.1. *E.coli*

Escherichia coli was first described in (1885) by Theodor Escherich as bacterium coli commune, linked to diarrhea in children, and was later renamed in his honor. Genome analysis of the original isolate revealed that it was nonpathogenic and lacked virulence factors, such as shiga toxin (Meric *et al.*, 2016). Now *E.coli* one of the most studied model organisms, and plays a key role in understanding the genetic code, DNA transcription, and restriction enzymes (Lehman *et al.*, 1958; Crick *et al.*, 1961; Linn and Arber, 1968). *E.coli* is a facultative anaerobic Gram negative rod (0.4×2-3µm), motile via peritrichous flagella, and grows optimally at 37°C. It exhibits both respiratory and fermentative metabolisms, forms smooth, convex colonies on standard media. Serotyping based on O, H, K, and F antigens classifies strains into serogroups and serotypes (Sora *et al.*, 2021). Phylogenetic analysis categorizes *E.coli* into groups A, B1, B2 and D, with extraintestinal virulent strains predominantly found in group B2 (Clermont *et al.*, 2000).

2.7.1.1. Classification of *E.Coli* that Causes Diarrhea

Diarrheagenic *E.coli* (DEC) is leading causes of diarrhea in children, spread through contaminated food and water. The six main types are enterotoxigenic *E. coli* (ETEC), enteropathogenic *E. coli* (EPEC), enterohemorrhagic *E. coli* (EHEC), enteroaggregative *E. coli* (EAEC),

enteroinvasive E. coli (EIEC), and *diffusely adhering E. coli* (DAEC). Identifying these strains requires the use of biochemical , cultured –based , and molecular methods ,such as PCR .To prevent infection ,strategies include sanitation ,access to safe water , exclusive breastfeeding ,rehydration therapy ,zinc supplementation ,and the careful use of antibiotics (Suleiman *et al.*, 2022). Strain O157:H7 can lead to serious complication (Khan *et al.*, 2022). Factors influencing adhesion , cytotoxicity , proteolysis , iron acquisition , and cell cycle modulation contribute to the virulence of DEC. virulence genes (SVGs) include :

A. Colonization : *ehaD,cah,nleB*

B. Cytotoxicity : *sat , espC , pet , hylA , stx1*

C. Proteolysis : *pic , eatA , espP*

D. Cyclomodulins : *cnf , pks , cif*

E. Iron acquisition : siderophore –related genes (Mare *et al.*, 2021)

2.7.2. Campylobacter

The name campylobacter came from the Greek words *kampylos* (“crooked”) and *bakteria* (“rod”), which reflects the bacterium’s unique S-shaped form (Robert, 2018). Initially , two types of vibrios were linked to enteric disease : *Vibrio fetus* and thermophilic strains.In (1963), scientists proposed the genus *campylobacter* due to differences in sugar utilization and G+C content compared to *vibrio* spp. Later ,isolation methods for thermophilic *campylobacter* were first developed from human feces and linked to diarrhea by Dekeyesr and Butzler in (1972) .Subsequently , advanced diagnostic methods were developed by Moore and Matsuda in (2002). *Campylobacter* is now known as leading cause of bacterial diarrhea

worldwide and most common source of foodborne illness in the UK, affecting about 280,000 people each year (Fitzgerald, 2015; Higham *et al.*, 2018).

Campylobacter spp. Are microaerophilic , Gram –negative , non-spore-forming , helical rods (0.2-0.8×0.5-5µm);some species have a straight rod shape (Vandamme *et al.*,2005)Thermophilic species thrive at 42°C and cannot grow below 30°C because they lack cold-shock protein genes(Silva *et al.*, 2011; Bolton, 2015). Particular species , including *C.gracilis* , *C.hominis* , *C.ureolyticus* , and *C. blaseri* , do not move (Shinha, 2015; Vandamme *et al.*, 2010).

These bacteria live in the gastrointestinal tracts of birds and mammals and belong to the class Epsilonproteobacteria (Rossler *et al.*, 2019; Griekspoor *et al.*, 2013). Unlike most Gram negative bacteria , campylobacter doesn't have lipopolysaccharide but has lipooligosaccharide (LOS) and capsular polysaccharides, which are the basis of penner serotyping (Pike *et al.*, 2013; Hameed *et al.*, 2020). The incidence of campylobacteriosis is increasing , especially among children under five in tropical developing regions (Terefe *et al.*, 2020; Acheson *et al.*, 2001). This bacterium is primarily transmitted through contact with animals, contaminated water , or person to person contact (Huang *et al.*, 2015; Kaakoush *et al.*, 2015; Lopes *et al.*, 2021).

2.7.2.1. Classification of *Campylobacter*

The genus *campylobacter*, which belongs to the family *campylobacteraceae* order campylobacterales, class Epsilonproteobacteria, and phylum proteobacteria, currently includes (33) species and subspecies.

2.7.2.2. Virulence Factor of *Campylobacter* and Mechanism of Diarrhea

Campylobacter's ability to cause disease relies on several factors, including flagella, secreted proteins, adhesins, cytotoxic distending toxin (CDT), LOS, and serine protease HtrA (Tegtmeyer *et al.*, 2021). These factors facilitate movement toward, attachment to, and invasion of cells, as well as disrupt the epithelial barriers, allowing the spread to deeper tissue. Its infection usually starts by invading the mucosal layer, which leads to damage of the epithelial barriers through the disruption of tight junctions and the subsequent induction of cell death. This results in the loss of water and solutes, leading to leaky-flux diarrhea, and promote the absorption of antigens, a condition often referred to as “leaky gut syndrome.” Damage to the barrier also triggers the release of pro-inflammatory cytokines, which affects sodium absorption due to dysregulation of ENaC. Future research aims to elucidate the molecular mechanisms underlying these processes and to develop preventive or therapeutic strategies (Murugesan *et al.*, 2022).

2.7.3. *Salmonella* spp.

In (1879), Karl Joseph Eberth discovered a bacillus in the lymph nodes and spleen of a typhoid patient. This bacillus was later named “Eberth Bacillus” (Popa and Papa, 2021). Robert Koch confirmed its link to typhoid fever in 1884 (Gryglewski and Chlipala, 2020). The genus *salmonella* was named after Daniel Elmer Salmon, who, with Theobald

Smith, isolated the bacterium from pigs with hog cholera in (1884). This work helped improve the understanding of bacterial infections in both animals and humans (Salmon, 1884; Popa and Papa, 2021). Historical analysis suggests that typhoid fever may have led to devastating outbreaks, including the plague in Athens in 430 B.C. (Littman, 2009; Marineli *et al.*, 2013).

Salmonella spp., member of the Enterobacteriaceae family, are Gram negative, flagellated, and facultative anaerobic bacteria measuring $2-3 \times 0.4-0.6 \mu\text{m}$. They do not form spore and are negative for oxidase, indole, urease, lactose, and sucrose. They cause typhoidal (TS) and non-typhoidal salmonellosis (NTS), leading to enteritis, septicemia, abortion, and meningitis in human and animals (Oludairo *et al.*, 2022). *Salmonella* is closely related to *shigella* and *E. coli*, based on 16S and 23S rDNA sequences, which are transmitted through contaminated food, contact with animals, or exposure to the environment, making it a significant global health issue. Ongoing research works to improve understanding, prevention, and treatment (Oludairo *et al.*, 2022).

2.7.3.1 Classification of *Salmonella* spp.

Salmonella taxonomy has shifted from biochemical-based systems to the Kauffman – White scheme, which classifies strains based on O(somatic) and H(flagellar) antigens. This method identified over (2,500) serological variants (D'Aoust and Maurer, 2007). Currently, the genus includes two species: *S. enterica* and *S. bongori*. The *S. enterica* is divided into six subspecies: *enterica*, *salamae*, *arizonae*, *diarizonae*, *houtenae* and *indica*, which are further classified into serogroups and serovars (Brenner *et al.*,

2000). Molecular serotyping methods now offer higher reproducibility and better distinguishing power , although limited access to equipment and reagents restricts their use in developing countries (Seif *et al.*, 2019; Mohammed and Thapa, 2020).

2.7.3.2. Virulence Factor of *Salmonella*

Both bacterial and host factors influence *Salmonella*'s ability to cause disease. Host susceptibility varies by age, genetics, and environmental conditions. Bacterial virulence is associated with various factors, including effector proteins, fimbriae, flagella, toxins, and plasmids. Effector proteins help *Salmonella* survive and replicate within its own vacuoles. Classic virulence factors support adhesion, invasion, and evasion of the immune system. There is ongoing debate about the definition of “virulence factors,” particularly concerning plasmids, which play an indirect role in host-pathogen interactions (Dos Santos *et al.*, 2019).

2.7.4. Other Bacteria Causing Diarrhea

2.7.4.1. *Shigella*

In areas with limited resources , *Shigella* spp are a significant cause of diarrhea in children , with death rate hitting (28%) in severe cases. Symptoms can vary widely, ranging from watery diarrhea to dysentery and complications like encephalopathy. The WHO recommends rehydration , zinc supplements and specific antibiotics for dysenteric infections, with ciprofloxacin suggested for children who have blood in their stool. This advice is based on the link between *Shigella dysenteriae* type 1 and dysentery, as well as the proven effectiveness of the antibiotic, despite the

challenges of obtaining stool cultures in low-resource areas. Non-dysenteric infections still lead to significant illness and death (Khan *et al.*, 2013).

The *Shigella* genus comprises four species: *S. dysenteriae*, *S. flexneri*, *S. sonnei*, and *S. boydii*, each of which contains multiple serotypes. *S. dysenteriae* type 1 and, to a lesser degree, *S. flexneri*, are most closely linked to bloody stool. However, global cases of *S. dysenteriae* type 1 have decreased significantly. For instance, the Global Enteric Multicenter study (2007–2011) looked at (9,439) children with moderate-to-severe diarrhea across seven countries and found no cases of *S. dysenteriae* type 1. Likewise, another multi-country study reported no instances in (56,958) episodes of diarrhea. In Bangladesh, surveillance has not detected *S. dysenteriae* type 1 infections since (2005), while other *shigella* serotype remain stable (Tickell *et al.*, 2017). This change in epidemiology may lower the number of children with bloody diarrhea, and as a result, reduce the use of antibiotics in accordance with current guidelines.

2.7.4.2. *Vibrio cholerae*

Cholera is a severe diarrheal disease and remains a significant public health issue because it can lead to high mortality rates. The bacteria responsible for cholera are *Vibrio cholera*, primarily serogroups O1 and O139, which can be detected in stool, vomit or food samples. The standard method for diagnosis toxigenic *V. cholerae*, especially during outbreaks, is through traditional phenotypic fingerprinting. However, this method is labor-intensive and time-consuming, which can be challenging in remote or resource-limited areas (Tarh, 2020).

2.8. Traditional Biochemical Methods for Identification of Pathogens**2.8.1. Basic Biochemical Tests**

Bacterial functionality necessitates the production of a unique suite of enzymes, rendering these enzymes potential targets for biochemical assays designed to identify specific bacterial genera. Tests such as catalase, oxidase, and urease, among others, provide reliable means for preliminary bacterial identification by assessing strain-specific enzymatic activities. Distinct strains exhibit varied reactions in these tests, which are dictated by their enzymatic profiles, resulting in alterations in the pH and color of the culture medium. The catalase test, for example, exploits the activity of the catalase enzyme, which catalyzes the conversion of hydrogen peroxide into water and oxygen. Each biochemical test requires adherence to specific protocols and accurate interpretation of results (Hafezi and Khamar, 2024).

2.8.2. Selective Media

Bacterial growth media provide essential nutrients for microbial proliferation. Due to varying nutritional needs, a single medium cannot support the growth of all microorganisms, and in many cases, these requirements remain undefined. Selective media facilitate the growth of specific bacterial classes while inhibiting the growth of other bacterial classes. For instance, MacConkey agar selectively supports the growth of Gram-negative bacteria, particularly those of the Enterobacteriaceae family while inhibiting the growth of many Gram-positive species. The use of selective media is critical for isolating pathogens from infection sites, enabling accurate identification, diagnosis, and treatment. Furthermore, it serves as a crucial initial step in the microbiological analysis of

environmental samples. Despite the availability of commercial selective media, research continues to develop improved media for more efficient bacterial isolation and identification (Blooshi et al., 2021).

On MHA-C15, the growth of Gram-negative bacterial pathogens, including *E. coli*, *K. pneumoniae*, *S. typhimurium*, and *Pseudomonas aeruginosa*, was observed. Conversely, none of the major Gram-positive bacterial pathogens, such as *Staphylococcus aureus*, *Streptococcus pneumoniae*, *Streptococcus pyogenes*, *Streptococcus mutans*, *Bacillus* spp., and *Enterococcus* spp., exhibited growth under the same conditions (Blooshi et al., 2021). Moreover, their media is selective for species such as CHROMagar salmonella (CAS), a recently developed selective chromogenic medium designed to identify *salmonella*, which manifests as mauve colonies after (18-24) hours of incubation.

Other Enterobacteriaceae family members typically appear as blue or colorless colonies (as per CHROMagar Salmonella Product Information, CHROMagar Microbiology, Paris, France). The initial CAS medium formulation demonstrated sensitivity comparable to that of HEA in detecting *salmonella* spp., both on primary plating and following enrichment in broth culture (Maddocks and Chen, 2002).

2.8.3. Limitations

Several factors limit the effectiveness of the Catalase/Biochemical (CB) test. It struggles to culture particular bacterial species, including strict anaerobes that die in the presence of oxygen unless handled meticulously in airtight containers. Additionally, viable but dormant, non-culturable bacteria and fastidious pathogens, which require enriched media, pose challenges.

Rare fastidious pathogens may evade detection due to the need for specific biochemical test , which maybe unavailable in some lower and middle income countries (LMICs). Mixed culture can also obscure results, as different bacterial species produce varying metabolites .Furthermore ,the CB test may be unreliable when bacterial phenotybic and biochemical profiles are easily alerted by stress .Crucially ,the CB test can be time-consuming for identifying fastidious or slow-growing pathogens , potentially leading to patient morbidity, mortality ,unnecessary broad-spectrum antibiotics use ,and the risk of pathogens transmission, particularly in LMICs where infection prevention and control (IPC) strategies may be under resourced .

2.9. Molecular Approaches to the Identification of Gram-Negative Bacteria

The challenge of identifying bacterial pathogens and their associated antimicrobial resistance (AMR) genes, stemming from the absence of rapid diagnostic techniques has historically been addressed in clinical settenigs through the use of board-spectrum antibiotics. Early identification of bacterial pathogenes is crucial for prompt and aggressive antibiotics treatment , as this can prevent chronic infections, such as wound infections , pneumonia , and catheter related bloodstream infections, which can lead to sepsis. Consequently, development of rapied and sensitive moleculare methods for detecting highly virulent, drug-resestant bacteria in clinical samples is essential for guiding antibiotic therapy and optimizing infection control measure.

Currently , the gold standard for microbial identification relies on phenotypic approachaches, including the use of enriched and selective culture

media for isolating pathogenic bacteria, automated biochemical testing for identification and antimicrobial susceptibility testing to guide therapeutic decisions (Tsalik *et al.*, 2018; Sinha *et al.*, 2018). Cultivation methods are primarily limited by the need for microbe-specific growth media, extended incubation periods for microbial proliferation and the time required to obtain results. Conversely, molecular diagnostics offer the advantage of directly detecting microbes from clinical specimens, thereby reducing occupational exposure of laboratory personnel to infectious agents (Mulani *et al.*, 2019). Advancements in molecular techniques, combined with progress in bioinformatics tools, have enabled the development of novel technologies that have revolutionized the diagnosis of bacterial infections.

2.9.1. PCR

Nucleic acid amplification technologies (NAATs) represent advanced methodologies for the swift and specific detection of pathogen-associated nucleic acids. Polymerase Chain Reaction (PCR), pioneered by Mullis in 1985, was the initial DNA amplification technique, enabling the generation of numerous copies from a minimal number of DNA molecules. PCR employs thermal cycling, involving repeated heating and cooling cycles to synthesize DNA. Short DNA fragments, known as primers, which are complementary to the target sequence, facilitate the exponential amplification of the target sequence, utilizing a thermostable DNA polymerase. Given the critical need for the prompt identification of pathogens and their resistance mechanisms to curb the spread of antibiotic resistance, numerous endpoint PCR variants have been developed (Zhu *et al.*, 2020).

2.9.1.1. Real-Time PCR

Real-time PCR, A technique derived from PCR methodologies is widely used in clinical setting for diagnosis infectious disease due to its superior sensitivity ,specificity ,and rapid turnaround time .This technology enables real-time monitoring of target amplification ,employing either non-specific intercalating fluorescent dyes or specific fluorescently labeled probes.The fluorescent signal is produced only upon probe hybridization with its complementary target , directly correlating with the quantity of PCR amplicons. Desspite the availability of commercial kits for detecting pathogens and resistance genes in biological samples , previous studies have documented instances of both false-positive and false-negative results across various genes(Chen *et al.*, 2018).These discrepancies may arise from factors such as genomic gene rearrangements and the acquisition of novel genes through horizontal transfer .furthermore ,while the detection of the mec gene serves as an indicator for methicillin-resistant staphylococcus aureus (MRSA), identifying resistance to β -lactam antibiotics in Gram-negative bacteria present greater complexity given the presence of numerous β -lactamase-encoding genes.Consequently ,diverse PCR platforms have been developed to target the most prevalent carbapenemases, including *blaKPC*,*blaOXA-48*,*blaOXA-23*,*blaOXA-24/40*,*blaNDM*,*blaVIM*,and *blaIMP*.

The limitation of RT-PCR include a restricted number of targets due to the limited availability of distinct fluorescent dyse. Additionally , its sensitivity and specificity may diminish with increased PCR multiplexing, due to the production of non specific amplification products and primer dimer formation (Elnifro *et al.*, 2000). Moreover, RT-PCR instruments often

incur significant maintenance expenses .Recent advancements in RT-PCR have facilitated the development of various isothermal amplification techniques ,such as loop-mediated amplification (LAMP), helicase dependent amplification (HDA), nucleic acid sequence-based amplification (NASBA),and transcription-mediated amplification (TMA).

2.9.1.2 Multiplex PCR

Multiplex polymerase chain reaction (PCR) can be designed to amplify either a limited set of DNA regions (e.g., two amplicons) or a large number of distinct DNA segments (e.g., thirteen or more). This technique may serve as a final analytical step or be used as a preliminary method for subsequent analyses, such as sequencing or hybridization. Regardless of the number of amplified fragments , the fundamental principles of multiplex PCR development and the advantages of simultaneous amplification remain consistent across different systems (Edwards and Gibbs, 1994).

2.9.1.3 Other Molecular Techniques

2.9.1.3.1.Whole-Genome Sequencing

Whole –genome sequencing (WGS) involves the comprehensive determination of the complete nucleotide sequence within a microorganism’s genome ,achieved through a single assay. Over the past twenty years ,WGS has demonstrated considerable utility across various domains , including (1) the identification of virulence factors in specific pathogens (2) the elucidation of disease transmission pathways during outbreak investigation (3) the profiling of antimicrobial resistance(AMR) and (4) the identification of sources contributing to recurrent infections and patients- to –patients transmission event.Despite its significant potential,the

widespread adoption of WGS in clinical settings has been a gradual process. However, the recent emergence of more affordable benchtop sequencing platforms may facilitate the broader integration of bacterial WGS within clinical practices (Gilchrist *et al.*, 2015).

2.10. Antibiotic Resistance in Gram-Negative Bacteria

Beta-lactam antibiotics comprise a significant group of antimicrobial agents characterized by a β -lactam ring within their molecular structure. This class encompasses penicillins, cephalosporins, monobactams, and carbapenems. Carbapenems often serve as the final therapeutic option for infections caused by multidrug-resistant (MDR) Gram-negative bacteria. These agents exhibit bactericidal properties, leading to rapid bacterial eradication and a broader spectrum of antimicrobial activity compared to other antibiotic classes. Except for Ertapenem, these antibiotics demonstrate efficacy against clinically relevant pathogens, including *P. aeruginosa*, *Burkholderia cepacia*, and *Acinetobacter spp.* (Kattan *et al.*, 2008).

In Gram negative bacteria, presence of AmpC, VIM, and TEM beta-lactamase genes is frequently observed, whereas PER, VEB, and SHV beta-lactamase genes are less commonly detected. Given their capacity for dissemination via mobile genetic elements (MGE), these genes pose a significant concern, as they facilitate the transfer of resistance traits across diverse bacterial species and geographical regions. Consequently, routine monitoring of antibiotic resistance patterns is essential for effective control and mitigation of emerging antimicrobial resistance (Satir *et al.*, 2016).

2.11. Antibiotic Stewardship

The advent of antibiotics represents a pivotal advancement in the history of medicine. However, the inappropriate utilization of these agents has fueled the emergence of antibiotics resistance and associated adverse outcome, including *Clostridioides difficile* infection (CDI), extended hospital stays, and increased readmission rates, collectively contributing to heightened morbidity and mortality. Antibiotics stewardship programs (ASPs) have been recognized as a crucial global strategy to address antibiotics resistance by various professional, governmental, and regulatory bodies. Consequently, ASPs are experiencing increased implementation across diverse healthcare settings. Effective ASPs leverage a multidisciplinary team of healthcare professionals and administrators, employing a range of strategies to optimize antibiotics use and monitor clinical outcomes. Furthermore, the scope of antibiotics stewardship practices is expanding to encompass outpatient settings, nursing facilities, and other healthcare environments (HA *et al.*, 2017).

Chapter Three

Materials and

Methods

3. Materials And Methods**3.1. Materials**

3.1.1. Equipment and Instruments: The main equipment and instruments used in this study are summarized in Table 3.1. They include devices for sterilization, microbiology, and molecular biology.

Table (3-1): List of equipment and instruments.

NO.	Type Of Instrument	Company	Country
1.	Autoclave	Hirayama	Japan
2.	Cooling centrifuge	Hettich	Germany
3.	Oven	ESCO	Singapore
4.	Micropipettes	DARWELL	China
5.	Hotplate magnetic stirrer	Heidolph	Germany
6.	Incubator		
7.	Laminar-Flow cabinet	BIOBAN36	Italy
8.	Applicator wooden stake		China
9.	Disposable syringe		China
10.	Disposable loop		China
11.	Cotton swaps	Afco	Jordan
12.	Slides		China
13.	Plastic petri dishes	Afco	Jordan
14.	Glass wear	Pyrex	England
15.	Benzene burner	Hirayama	Japan
16.	Eppendorf tubes	Himedia	India
17.	Refrigerator	Gongord	Lebanon
18.	Micropipette tips	Gelson	France
19.	Microfuge IB Centrifuge	Beckman Coulter	Germany
20.	Dry microtubes incubator	Ae	UK
21.	Optimus 96G thermal Cycler	QLS	UK
22.	Electrophoresis system	Cleaver	UK
23.	Kern PFB balance	Kern & Sohn	Germany
24.	UVP	Analytik Jena	UK
25.	Microscope	Olympus	Japan
26.	Slide and Coverslide	AmScope	-

3.1.2. Chemical and biological materials**Table (3.2):**Chemicals, reagents, and kits used in this study.

NO.	Type Of Instrument	Company	Country
1	G-spin Genomic DNA Extraction Kit for Bacteria	17121	Intron/Korea
2	Agarose LE	32034-50	Intron/Korea
3	RedSafe nucleic acid staining solution	21141	Intron/Korea
4	GoTaq® G2 Green Master Mix	M7822	Promega/USA
5	Safe-Green 100bp Opti-DNA Marker	G473	ABM/Canada
6	10X TAE (Tris Acetate EDTA) buffer	V4271	Promega/USA
7	Primers	---	Macrogen/Korea
8	Glycerol	Fluka	England
9	TE buffer	Bio Basic	Canada
10	Turbidity Buffer	-----	-----

3.1.3. Culture Media

- Brain heart infusion broth
- MaCconky agar
- Peptone broth water

3.1.4. Susceptibility Test of Bacteria

All antibiotics used are listed in Table 3.3 with their corresponding symbols.

Table (3-3): List of antibiotics used in the susceptibility tests

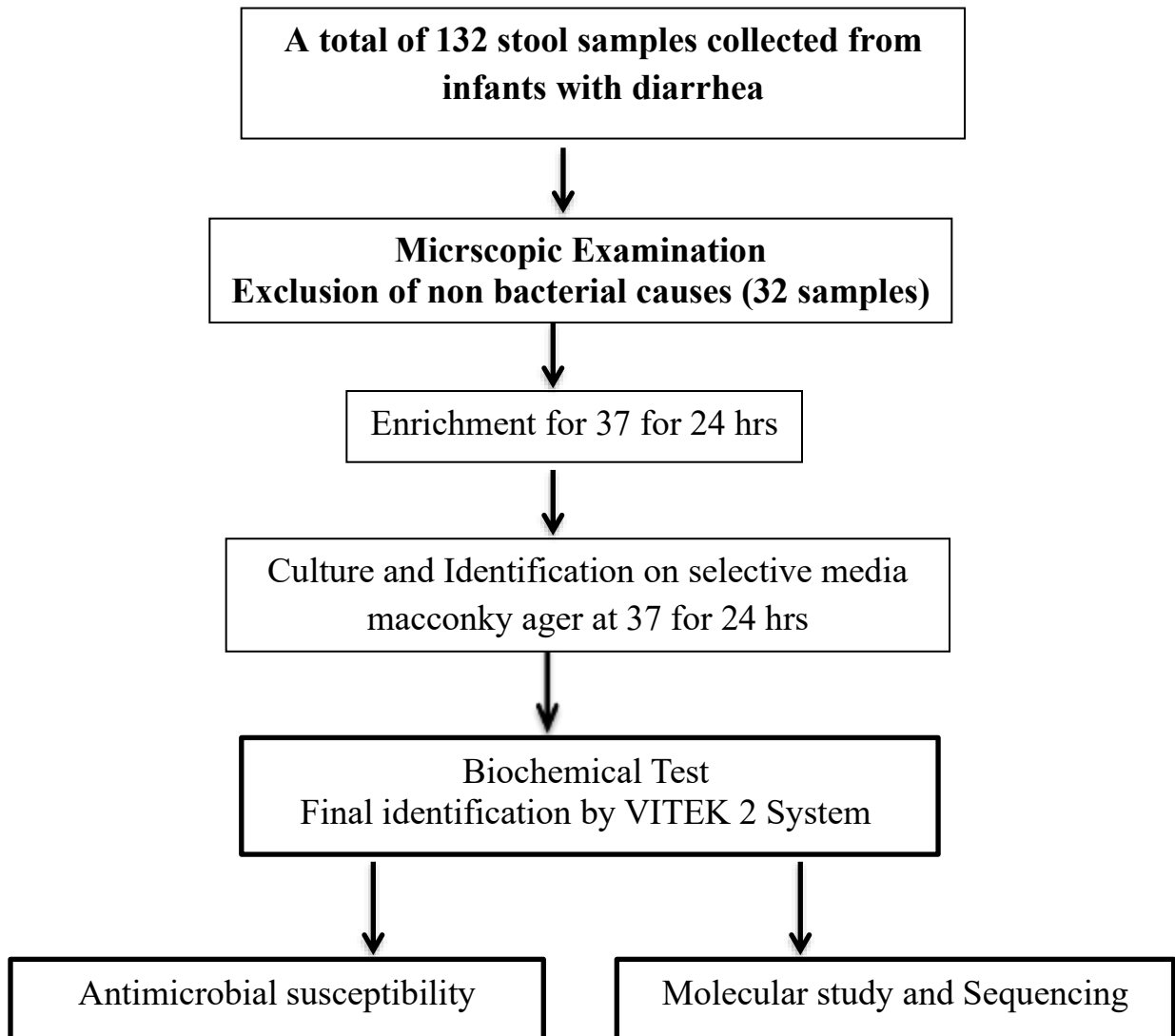
NO.	Antibiotic	Symbol
1.	Amoxicillin/Clavulanic Acid	AMC
2.	Piperacillin/Tazobactam	TZP
3.	Cefazoline	CFZ
4.	Cefuroxime	CXM
5.	Cefuroxime Axetil	CXT
6.	Ceftazidime	CAZ
7.	Ceftriaxone	CRO
8.	Cefepime	FEP
9.	Ertapenem	ETP
10.	Imipenem	IPM
11.	Meropenem	MEM
12.	Amikacin	AMK
13.	Gentamicin	GEN

14.	Ciprofloxacin	CIP
15.	Trimethoprim/Sulfamethoxazole	SXT
16.	Cefotaxime	CTX
17.	Ceftolozane/Tazobactam	C/T
18.	Tigecycline	TGC
19.	Colistin	COL
20.	Aztreonam	ATM
21.	Minocycline	MIN
22.	Ticarcillin	TIC
23.	Tobramycin	TOB
24.	Ampicillin/Sulbactam	SAM

3.1.5. Genomic DNA Extraction Kit: The Genomic Extraction Kit solutions are illustrated in Table 3-4.

Table (3-4): Solution of Genomic DNA extraction

NO.	Solution	Volume
1	G-Buffer	300µl
2	Washing buffer	500µl
3	Bidding buffer	250µl
4	Elution buffer	50-250µl
5	G-spin™ column	
6	1 X TBE buffer	

3.1.1. Study Design

3.2. Methods**3.2.1. Sterilization Method**

1. Autoclaving (culture media): Culture media were sterilized with an autoclave at 121°C and 15 psi for 15 minutes. This method uses steam under pressure to kill all microorganisms, including spores. It works well for heat-stable liquids and media.
2. Dry Heat (Glassware): Glassware was sterilized in a dry heat oven at 180°C for 2 hours. Dry heat kills microorganisms by oxidation and protein denaturation. It is best for glassware and metal instruments that cannot be sterilized with moisture.

3.2.2. Preparation of Culture Media

The majority of the media was prepared according to the instructions provided on the package.

3.2.2.1. MacConkey Agar

50.03 gm of the compound are dissolved in 1 liter of distilled water. Subsequently, the solution is heated to boiling to ensure complete dissolution of all components. Following this, the solution is autoclaved at 121°C and 15 pounds per square inch for 15 minutes. Finally, the prepared medium is incubated at 37°C for 24 hours to verify the absence of contamination.

3.2.2.2. Brain heart infusion broth (BHIB)

Dissolve 37 grams of the medium in 1000 mL of distilled water. Then, add 15% glycerol to the solution. The mixture is then poured into sterile test tubes. Finally, the test tubes are autoclaved to sterilize the medium.

3.3. Sample collection and Macroscopic/ Microscopic Examination

A total of (132) stool samples were collected from patients presenting with diarrhea. Each sample was assessed for microscopic characteristics including consistency, color, odor, volume, and the presence of mucus, pus, or blood. samples were transported to the laboratory under appropriate condition for further analysis.

3.4. Isolation of Gram-Negative Bacteria

Stool samples first underwent an enrichment step to encourage the growth of Gram-negative bacteria. Each sample was added to Brain Heart Infusion (BHI) broth and incubated at 37°C for 24 hours in aerobic conditions. After enrichment, the cultures were streaked onto MacConkey agar, a medium that selects for and differentiates Gram-negative bacteria, and incubated at 37°C for an additional 24 hours. Colonies that exhibited typical characteristics on MacConkey agar were selected and subcultured onto new MacConkey agar plates to obtain pure, isolated colonies.

3.5. Biochemical Study**3.5.1. VITEK2 Automated Microbiology System**

The VITEK® 2 Compact System (bioMerieux, France) is an automated platform used in clinical and industrial microbiology laboratories

for the rapid identification of microorganisms and testing for antimicrobial resistance. The system operates on a growth-based principle, where microbial metabolism causes color change in specially designed reagent cards. These changes are continuously monitored, and the results are interpreted automatically. This process minimizes manual work and lowers the chances of human error (El-Amier *et al.*, 2024).

3.5.1.1. System Format

VITEK 2 Compact. This version is mainly used in industrial microbiology and low- to mid-volume clinical labs. It meets 21 CFR Part 11 standards, which ensure secure electronic records and signatures. The compact system features a BCL card to aid in identifying spore-forming Gram-negative bacilli, including species of the genus *Bacillus*.

3.5.1.2. Operational Principle

The workflow includes inoculating microbial isolates into the reagent cards. These cards contain several substrates meant to detect specific metabolic activities. As microorganisms break down these substrates, color changes happen in the card well. The system monitors these changes continuously. The software examines these changes to produce automated identification and susceptibility reports, improving both accuracy and efficiency.

3.5.1.3. Reagent Cards

Each reagent card has (64) wells, each with a different test substrate to measure metabolic activities. The cards have an optically clear film for

oxygen transmission and pre-inserted transfer tubes for inoculation. Barcodes provide product information and a unique identifier. Four cards are available for identifying different organism classes: GN (Gram-negative bacilli), GP (Gram-positive cocci and bacilli), YST (yeasts), and BCL (Gram-positive spore-forming bacilli).

3.5.1.4. Suspension Preparation

For transferring a sufficient number of colonies of a pure culture, a sterile swab or applicator stick is used to suspend the microorganism in 3.0 mL of sterile saline (aqueous 0.45% to 0.50% NaCl, pH 4.5 to 7.0) in a 12 x 75 mm clear plastic (polystyrene) test tube. The turbidity is then adjusted accordingly and measured using a turbidity meter called the DensiChek™.

Table (3-5): McFarland turbidity range

Product	McFarland Turbidity Range
GN	0.50-0.63

Optical System

A transmittance optical system enables the interpretation of test reactions by utilizing various wavelengths within the visible spectrum. During incubation, each test reaction is read every 15 minutes to measure either turbidity or the colored products of substrate metabolism. Furthermore, a special algorithm is employed to eliminate false readings caused by the presence of small bubbles(El-Amier *et al.*,2024).

3.5.1.5. Test Reactions

Raw data is analyzed and compared to established thresholds to determine the results of each test. On the VITEK 2 Compact, test reaction results are displayed as “+”, “-”, “(-)” or “(+)”. Reactions shown in parentheses indicate weak reactions that are close to the threshold for a positive result.

3.5.1.6. Identification Levels

Table (3-6): The percentage of the vitkes system

ID message confidence level	Choice	Probability	Comments
Excellent	1	96 to 99	N/A
Very good	1	93 to 95	N/A
Good	1	89 to 92	N/A
Acceptable	1	85 to 88	N/A
Low discrimination	2 to 3	Sum of choice =100; after resolution to one choice, the percent probability reflects the number associated with the selected choice.	2 to 3 taxa exhibit the same biopattern. Separate by supplemental testing.
Unidentified Organism	< 3 or 0	N/A	Either > 3 taxa exhibit the same biopattern, or a very typical biopattern. Does not correspond to any taxon in the database. Check Gram-negative stain and purity.

There are (47) biochemical tests and one negative control well. Final identification results are available in approximately (10) hours or less. The list of test substrates is shown in Appendix I.

In a recent multi-site study, the performance of the VITEK 2 GN was evaluated using 562 isolates of both commonly and rarely observed species of Gram-negative bacilli, including (153) non-fermentative strains. The reference identification was determined using API 20E and API 20NE identification kits. Overall, the VITEK 2 GN correctly identified 96.8% of the isolates, including 6.4% with low discrimination, for which the correct species was still listed. Misidentifications occurred at (3.0%), and no identifications occurred at (0.2%) (El-Amier *et al.*,2024).

3.6. Molecular Study

3.6.1. Genomic DNA Extraction

Genomic DNA was extracted from bacterial cells using the G-spin Genomic DNA Extraction Kit, following the manufacturer's instructions with some minor modification. The steps were as follows

Bacterial cells were cultured to an OD₆₀₀ of 0.8-1.0. A 1-2 ml culture volume was harvested and centrifuged at 13,000 rpm for 1 minute, and the supernatant discarded. The cell pellet was resuspended in 300 µl of G-Buffer, mixed, and incubated at 65°C for 15 minutes with intermittent mixing to ensure lysis. Then, 250 µl of binding buffer was added to the lysate and mixed. The lysate was transferred to a G-spin™ column and centrifuged at 13,000 rpm for 1 minute, with the flow-through discarded.

The column was washed with 500 µl of Washing Buffer A and centrifuged at 13,000 rpm for 1 minute, followed by a wash with 500 µl of Washing Buffer B under the same conditions. Residual liquid was removed by centrifuging the column for 1 minute at 13,000 rpm. Finally, the column was placed in a clean tube, and 50-200 µl of Elution Buffer was added directly to the membrane. After incubation at room temperature for 1 minute, DNA was eluted by centrifugation at 13,000 rpm for 1 minute. The resulting solution contained the purified genomic DNA.

3.6.2. Preparation of Primers

Following the manufacturer's instructions, the lyophilized primers were dissolved in nuclease-free water (ddH₂O) to reach a final concentration of (100 µM/µl). This stock solution was stored at (-20°C). A working solution of (10 µM/µl) was then made from the stock for use in the experiment.

Table (3-7): Primers used in this study

Gene	Primer Sequence 5'- 3'	PCR size	Accession number	Reference
16S rRNA	F: ATGACGTCAAGTCATCATGG	353 bp	CP147165. 1	(Klauegger <i>et al.</i> , 1999)
	R: AGGAGGTGATCCAGCCGCA			

3.6.3. PCR Amplification Using GoTaq® G2 Green Master Mix

PCR reactions utilized GoTaq G2 Green Master Mix (Promega, Madison, WI, USA), a pre-made 2× solution containing Taq DNA Polymerase, dNTPs, and reaction buffer. The master mix also has an inert green dye and stabilizers. This feature enable PCR products to be loaded

directly onto agarose gels without the need for extra loading dye. PCR conditions were optimized based on the needs of the primers and templates.

Table (3-8): Preparation of PCR solutions

Components	Concentration	Volume (50 μ l)
GoTaq® G2 Green Master Mix	1X	25 μ l
Forward primer	10 μ M/ μ l	4 μ l
Reverse primer	10 μ M/ μ l	4 μ l
ddH ₂ O	-	13 μ l
DNA	40 ng/ μ l	4 μ l

Table(3-9): PCR conditions

Phase	T _m (°C)	Time	Cycles
Initial denaturation	94°C	5 min	1X
Denaturation	94°C	30 sec.	35X
Annealing	55°C	30 sec.	
Extension	72°C	1 min	
Final extension	72°C	5 min	1X

3.6.4. Agarose Gel Electrophoresis

Electrophoresis was performed to assess the quality og the DNA extraction and visualize the size of the PCR products after the PCR program was completed .The concentration of the gel varied based on the type of product .Usually , a (0.7%) agarose gel was used for DNA quality checks , while a (1-2%) agarose gel was used to visualize regular PCR products.

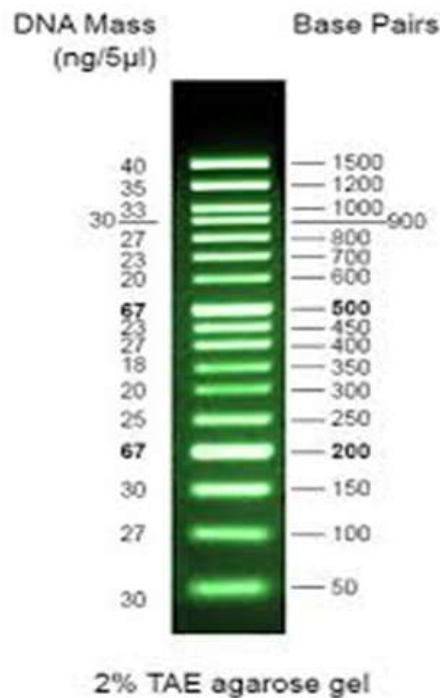
3.6.4.1. Preparing the Agarose Gel

Agarose gel preparation followed the method from Sambrook *et al.* (1989). First, (1) gram of agarose was dissolved in about (100 ml) of (1X) TBE buffer and heated to boiling in a microwave. After cooling to (45-50°C) ,(5 µl) of redSafe nucleic acid staining solution was added to the gel mixture .

This mixture was then poured into a gel cast .The gel solidified for about (30) minutes .The gel plate was transferred to a gel tank and submerged in (1X) TBE buffer until the gel was entirely covered .

3.6.4.2. Loading the PCR Products

Approximately five microliters of each PCR products were loaded into the corresponding well in the 1% agarose gel . (5) microliters of Safe-Green (100pb) opti-DNA Marker were added to the first well in each lane of the gel, serving as a size marker for the PCR products.

Safe-Green 100bp Opti-DNA Marker**3.6.4.3. Electrophoresis Condition**

After loading the Safe-green (100 pb) opti-DNA marker and the samples , the electrophoresis system was set to (90) volts with a constant current for (45) minutes .The gel was then moved to a UVP system to visualize the PCR products under (320nm) UV light source.

3.6.5. DNA Sequencing

The PCR-amplified products underwent DNA sequencing with the Snger dideoxy methods. Macrogen in Seoul ,Korea ,carried out the sequencing reaction according to standard protocols. The obtained sequences were used for further analysis ,including confirmation of the target gene identity and comparsion with refrence sequences in public databases.

3.7. Statistical Analysis

The data of the current study were statistically analysed by using the Statistical Package of Social Science (SPSS) version(26), based on using Chi-Square at p-value for independent $0 < 0.05$. Each p-value has two stars, indicating a high significance at a p-value of (0.01) .A – p value has one star ,indicating a high significe at (0.05) ,while p-value without a star indicates a non-significant difference.

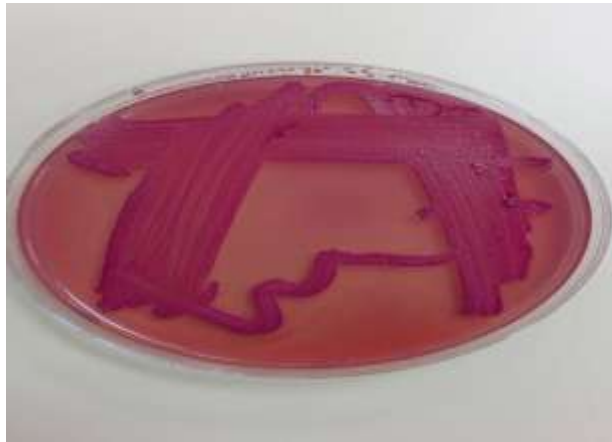
Chapter Four

Results

4. Results**4.1. Isolation and Identification of Gram-Negative Bacteria**

Preliminary microscopic evaluation was performed to exclude non-bacterial causes of diarrhea. Microscopy focused on detecting motile bacteria, leukocytes, red blood cells, epithelial cells, parasitic forms, and signs of bacterial overgrowth. Based on these findings, (32) samples showing non-bacterial infection were excluded, resulting in a final study sample of (100) specimens.

Microbial culture of the sample on MacConkey agar enhanced the growth of different types of bacteria, including *Escherichia coli*, *Klebsiella* spp., *Enterobacter* spp., *Citrobacter freundii*, *Salmonella* spp., and *Raoultella ornithinolytica*. *E. coli* colonies appeared smooth, non-mucoid, and pink to reddish, with a round shape. However, the *Klebsiella* spp. colonies appeared large, mucoid, pink in color, and had a mild, unpleasant odor. Furthermore, *Enterobacter* spp. are facultative anaerobic rods on MacConkey agar, typically appearing as large, round, moist, and pink colonies. Additionally, when cultured on MacConkey agar, *Citrobacter freundii* typically forms smooth, moist, round, non-mucoid colonies with a pink to pale color. Additionally, *Raoultella ornithinolytica* was observed as round, moist, large, and mucoid pink colonies. Its colony morphology was nearly identical to that of *Klebsiella pneumoniae*, with a fruity odor.



(A)



(B)



(C)



(D)



(E)

Figure 4-1. Bacterial isolation culture (A) *E.coli*. (B) *Klebsiella pneumoniae* (C), *Klebsiella aeruginosa* (D), *Raoultella ornithinolytica*. (E), *Citrobacter freundii*

4.2. VITEK II System for Confirming Gram-Negative Species Isolated

The Vitek II system is used to detect bacteria using biochemistry tests to confirm the detection of Gram-negative bacterial species isolates. There are 64 wells on the Gram-negative bacteria Reagent card, each containing a

different test substrate. The system monitors changes in color, turbidity, carbohydrate fermentation, enzymatic activity, H₂S production, acidification, and alkalization.

4.2.1. Prevalence of Pathogenic Bacteria in Pediatric Patients with Diarrhea

The current study showed a significant difference ($0 < 0.05$). It was recorded among (100) pediatric patients, with (59.0%) infected with *E. coli*, (32.0%) infected with Klebsiella Spp, (17.0%) with *K. pneumoniae*, (7.0%) with *K. oxytoca*, and (8.0%) infected with *k. aeruginosa*. The lowest isolated bacteria were *C. freundii*, and *E. cloacae*, as shown in Figure 4.2.

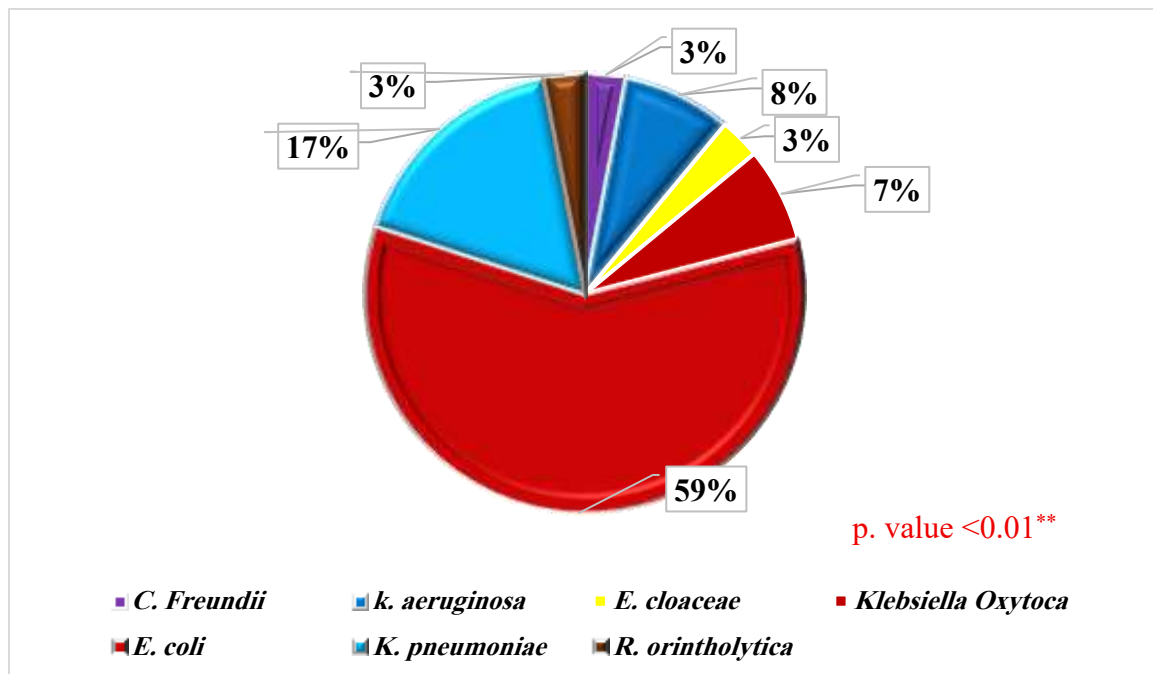


Figure 4-2: Prevalence of pathogenic bacteria in pediatric patients with diarrhea

4.2.2. Prevalence of Pathogenic Bacteria in Pediatric Patients with Diarrhea According to Sex

The current study found out a significant difference ($p < 0.05$) in the prevalence of gram-negative bacteria among pediatric patients with diarrhea, according to gender. Based on the gender of the patient, females were more frequently infected with diarrhea (51.0%) compared with males (49.0%). Regarding the species of bacteria, the study showed that *E. coli* was the most isolated bacterium in both males and females (60.78% and 57.14%, respectively). Additionally, the lowest isolated bacteria were *R. orinolithyca* in female group 1 (1.96%) and *C. freundii* in male group 2 (2.05%) (Table 4-1).

Table 4-1: Prevalence of pathogenic bacteria in pediatric patients with diarrhea according to sex

Sex	Male		Female		Total	
	No.	%	No.	%	No.	%
<i>C. freundii</i>	1	2.04	2	3.92	3	3.00
<i>k. aeruginosa</i>	4	8.16	4	7.84	8	8.00
<i>E. cloacae</i>	3	6.12	0	0.00	3	3.00
<i>E. coli</i>	28	57.14	31	60.78	59	59.0
<i>K. pneumoniae</i>	4	8.16	13	25.49	17	17.0
<i>K. oxytoca</i>	7	14.29	0	0.00	7	7.00
<i>R. orinolithyca</i>	2	4.08	1	1.96	3	3.00
Total	49	49.0	51	51.0	100	100
CalX ² = 15.5 TabX ² = 12.59 DF= 6 p. value 0.016						

4.2.3. Prevalence of Pathogenic Bacteria in Pediatric Patients with Diarrhea According to Age Group

The current study revealed significant differences ($p < 0.05$) based on patient age. The data showed that the most infected age group was the (7-12) months group (41.0%), followed by the (19-24) months group (27.0%). The lowest infected age group was the (13-18) months group (12.0%). In terms of bacterial species, the results revealed that the most isolated bacteria were *E. coli* in the (19-24) months age group (74.07%), followed by the (7-12) months age group (63.41%), and then *K. pneumoniae* in the less than (6) months age group (30.0%). Additionally, *C. freundii* was detected in ≤ 6 months, (13-18) months, and (19-24) months age groups at a rate of (0.0%). *R. orinolithyca* in (7-12) months, (13-18) months, and (19-24) months age groups was also (0.0%), but *k. aeruginosa* was (0.0%) in the less than (6) months age group (Table 4.2).

Table 4-2: Prevalence of pathogenic bacteria in pediatric patients with diarrhea according to age group

Age groups	≤ 6 Months		7-12 months		13-18 months		19-24 months	
	No.	%	No.	%	No.	%	No.	%
<i>C. freundii</i>	0	0.00	3	7.32	0	0.00	0	0.00
<i>k. aeruginosa</i>	0	0.00	0	0.00	4	30.77	4	15.38
<i>E. cloacae</i>	0	0.00	3	7.32	0	0.00	0	0.00
<i>E. coli</i>	7	35.00	26	63.41	6	46.15	20	76.92
<i>K. pneumoniae</i>	6	30.00	6	14.63	3	23.08	2	7.69
<i>K. oxytoca</i>	4	20.00	3	7.32	0	0.00	0	0.00
<i>R. orinolithyca</i>	3	15.00	0	0.00	0	0.00	0	0.00
Total	20	20.0	41	41.0	13	13.0	26	26.0
CalX ² = 216.8 TabX ² = 28.87 DF= 18 p. value <0.01								

4.2.4. Prevalence of Pathogenic Bacteria in Pediatric Patients with Diarrhea According to Residency

According to the residency of patients, the results of the current study showed a significant difference ($p < 0.05$). Data recorded that the most infected patients were among residents of Nasiriyah (63.0%), followed by those of Shatrah (20.0%). Furthermore, the lowest percentage of infected patients was among those residing in Suq-Al-Shuykh (7.0%). Regarding the species of bacteria, the results showed that *E. coli* was the most isolated bacterium in both Shatrah and Rifai (70.0%), while *k. aeruginosa* was isolated in Suq-Al-Shuykh at a rate of (57.14%). In addition, non-isolated *C. freundii* and *R. orinholytica* in Shatrah, Rifai, and Suq-Al-Shuykh were (0.0%), and *k. aeruginosa* in Shatrah and Rifai was (0.0%) (Table 4.3).

Table 4-3: Prevalence of pathogenic bacteria in pediatric patients with diarrhea according to residency

Residency	Nasiriyah		Shatrah		Rifai		Suq Al-Shuyukh	
	No.	%	No.	%	No.	%	No.	%
<i>C. freundii</i>	3	4.76	0	0.00	0	0.00	0	0.00
<i>k. aeruginosa</i>	4	6.35	0	0.00	0	0.00	4	57.14
<i>E. cloacae</i>	3	4.76	0	0.00	0	0.00	0	0.00
<i>E. coli</i>	35	55.56	14	70.00	7	70.00	3	42.86
<i>K. pneumoniae</i>	10	15.87	4	20.00	3	30.00	0	0.00
<i>K. oxytoca</i>	5	7.94	2	10.00	0	0.00	0	0.00
<i>R. orinholytica</i>	3	4.76	0	0.00	0	0.00	0	0.00
Total	63	63.0	20	20.0	10	10.0	7	7.0
CalX ² = 254.9 TabX ² = 28.87 DF= 18 p. value <0.01								

4. 3. Antibiotic Sensitivity Test

4.3.1. Antibiotic Susceptibility of *C. freundii*

C. freundii bacteria revealed significant differences ($p < 0.05$) in the response to antibiotics with different mechanisms of action. The study showed that (100%) of bacteria were sensitive to most antibiotics. Furthermore, data showed that bacteria were (100.0%) resistant to the activity of Trimethoprim, Cefazoline, Cefuroxime, and Cefuroxime Axetil. Additionally, bacteria were (66.67%) resistant to Amoxicillin. Overall, bacteria were (72.55%) susceptible and (27.45%) resistant to the antibiotic (Table 4.4).

Table 4-4: Antibiotic susceptibility of *C. freundii*

<i>C. freundii</i>	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= 26 p. value <0.01						

4.3.2. Antibiotic Susceptibility of *K. aeruginosa*

The response of *k. aeruginosa* bacteria to antibiotics with different mechanisms of action showed significant differences ($p < 0.05$; Table 4.5). The data showed that *k. aeruginosa* bacteria were (100%) sensitive to the antibiotics Pip/Tazo, Cefepime, and Meropenem. Regarding ticarcillin, the *k. aeruginosa* bacteria showed an intermediate to moderate response (36.36%). Furthermore, the bacteria were resistant to Aztreonam (90.91%) and Piperacillin (72.73%). Overall, the data showed that *k.aeruginosa* was (58.79%) sensitive, (15.15%) intermediate, and (26.06%) resistant to all antibiotics.

Table 4-5: Antibiotic susceptibility of *k. aeruginosa*

<i>K. aeruginosa</i>	Sensitive		Intermediate		Resistance	
	No.	%	No.	%	No.	%
Pip/Tazo	11	100	0	0.00	0	0.00
Ceftazidime	9	81.82	2	18.18	0	0.00
Cefepime	11	100	0	0.00	0	0.00
Imipenem	8	72.73	2	18.18	1	9.09
Meropenem	11	100	0	0.00	0	0.00
Amikacin	7	63.64	2	18.18	2	18.18
Gentamycin	6	54.55	1	9.09	4	36.36
Ciprofloxacin	8	72.73	1	9.09	2	18.18
Trimethoprim	8	72.73	3	27.27	0	0.00
Aztreonam	0	0.00	1	9.09	10	90.91
Minocycline	9	81.82	0	0.00	2	18.18
Piperacillin	0	0.00	3	27.27	8	72.73
Ticarcillin	0	0.00	4	36.36	7	63.64
Ticarcillin /CA	2	18.18	3	27.27	6	54.55
Tobramycin	7	63.64	3	27.27	1	9.09
Susceptibility %	58.79		15.15		26.06	
CalX ² = 104.7 TabX ² = 41.34 DF= 28 p. value <0.01						

4.3.3. Antibiotic Susceptibility of *E. coli*

The *E. coli* bacteria's response to antibiotics with different mechanisms of action showed significant differences (**Table 4.6; $p < 0.05$**). The data showed that the sensitivity of *E. coli* bacteria to the activity of Meropenem and Ertapenem antibiotics was (100%) and (98.31%), respectively. Furthermore, of the bacteria were resistant to Cefazoline (89.83%) and Cefuroxime (88.14%).

Table 4-6: Antibiotic susceptibility of *E. coli*

<i>E. coli</i>	Sensitive		Intermediate		Resistance	
	No.	%	No.	%	No.	%
Pip/Tazo	46	77.97	5	8.47	8	13.56
Ceftazidime	14	23.73	11	18.64	34	57.63
Cefepime	10	16.95	5	8.47	44	74.58
Imipenem	54	91.53	5	8.47	0	0.00
Meropenem	59	100	0	0.00	0	0.00
Amikacin	48	81.36	3	5.08	8	13.56
Gentamycin	38	64.41	5	8.47	16	27.12
Ciprofloxacin	42	71.19	10	16.95	7	11.86
Trimethoprim	4	6.78	7	11.86	48	81.36
Ertapenem	58	98.31	1	1.69	0	0.00
Amoxicillin\CA	9	15.25	2	3.39	48	81.36
Cefazoline	2	3.39	4	6.78	53	89.83
Cefuroxime	3	5.08	4	6.78	52	88.14
Cefuroxime Axetil	4	6.78	6	10.17	49	83.05
Ceftriaxone	7	11.86	11	18.64	41	69.49
Susceptibility %	46.76		11.46		41.78	
CalX ² = 711.1 TabX ² = 45.98 DF= 28 p. value <0.01						

4.3.4. Antibiotic Susceptibility of *Klebsiella* Spp.

The susceptibility of *Klebsiella* Spp to antibiotics with different mechanisms of action revealed significant differences (**Table 4.7; $p < 0.05$**). Data displayed that *K. pneumoniae* bacteria were (100%) sensitive to

cefotaxime, Ceftazidime/av, Ceftazidime/taz, Meropenem, and Tigecycline. In contrast, its sensitivity to the Ceftazidime was weak to moderate (25.0%). Furthermore, the bacteria were resistant to Amp/Sulb (79.17%), Ceftazidime (62.50%), and Gentamycin (33.33%).

Table 4-7: Antibiotic susceptibility of *Klebsiella* Spp.

<i>K. pneumoniae</i>	Sensitive		Intermediate		Resistance	
	No.	%	No.	%	No.	%
Amp/Sulb	2	8.33	3	12.50	19	79.17
Pip/Tazo	18	75.0	2	8.33	4	16.67
Cefotaxime	24	100	0	0.00	0	0.00
Ceftazidime	3	12.5	6	25.0	15	62.50
Ceftazidime /av	24	100	0	0.00	0	0.00
Ceftazidime/taz	24	100	0	0.00	0	0.00
Cefepime	23	95.83	1	4.17	0	0.00
Imipenem	19	79.17	4	16.67	1	4.17
Meropenem	24	100	0	0.00	0	0.00
Amikacin	16	66.67	2	8.33	6	25.00
Gentamycin	14	58.33	2	8.33	8	33.33
Ciprofloxacin	21	87.50	1	4.17	2	8.33
Tigecycline	24	100	0	0.00	0	0.00
Trimethoprim	17	70.83	3	12.50	4	16.67
Susceptibility %	75.3		7.14		17.56	
CalX ² = 177.2 TabX ² = 38.89 DF= 26 p. value <0.01						

4.4. Molecular Results

4.4.1. Molecular detection of 16s rRNA of Gram-Negative Bacteria

PCR amplification of the target gene (16s rRNA) fragment was confirmed by agarose gel electrophoresis (Figure 4-3). A DNA ladder (lane M) served as a size marker. Clear bands of about (353) bp appeared in all tested samples (lanes 1-9), matching the expected amplicon size. There was no nonspecific amplification or primer-dimer formation, as evidenced by the

absence of extra bands or smearing. The consistency in band intensity across all samples shows successful and reliable amplification of the target fragment.

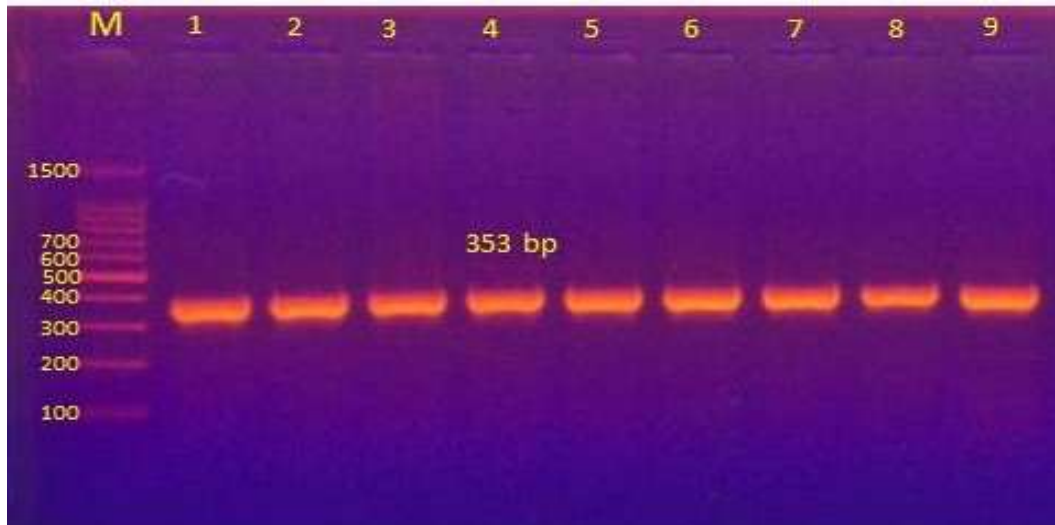


Figure 4.3: PCR products of the amplification of the partial region of gene 16s rRNA. The size of the PCR product is 353bp. The gel was 1.5% and the DNA dye is RedSafe (Intron, Korea). V: 90, Time: 45 minutes. M: DNA ladder.

4.4.2. Phylogenetic analysis of Bacterial Isolates

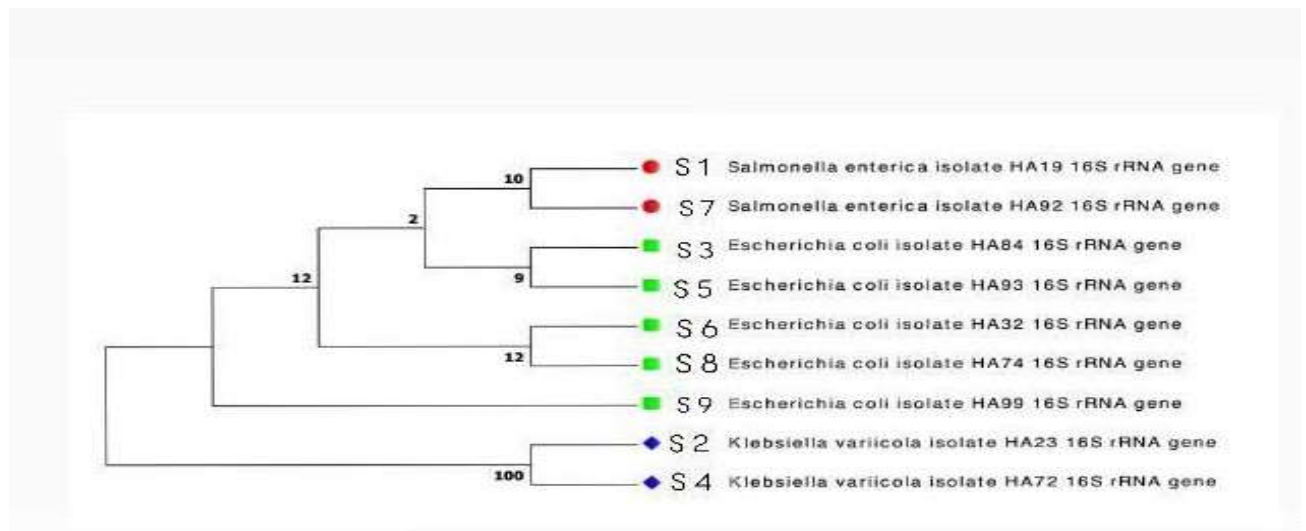
Sanger sequencing and BLAST analysis were used to identify each isolate. Table 1 summarizes the identification results. Among the nine samples, five were identified as *Escherichia coli* (S3, S5, S6, S8 and S9), two as *Salmonella enterica* (S1 and S7), and two as *Klebsiella variicola* (S2 and S4). Sequence alignment revealed single-nucleotide polymorphisms (SNPs) that helped to distinguish closely related strains.

To further examine the genetic relationships, a phylogenetic tree was built using the partial 16S rRNA sequences through the Neighbor-Joining method with (1,000) bootstrap replicates (Figure 4-4). The tree grouped the

nine isolates into three distinct clades based on their BLAST identifications. The *E. coli* isolates clustered together with strong bootstrap support (100%). The *K. variicola* isolates formed a separate clade with (98%) bootstrap support. The *S. enterica* isolates also formed a distinct clade, closely related to the reference strain CLSSen01, supported by bootstrap values of (99%).

The phylogenetic clustering matched the BLAST results. The SNPs found in the aligned sequences further differentiated closely related strains within each clade. This confirmed the presence of *E. coli*, *K. variicola*, and *S. enterica* among the tested samples and highlighted their genetic diversity.

Figure (4.4): Phylogenetic tree of bacterial isolates



● *Salmonella enterica*

◆ *Klebsiella variicola*

■ *Escherichia coli*

Table 4-8: Identification of bacterial samples using DNA sequences of gene 16S rRNA (Sanger Method)

Sample ID	Target DNA	Identification by DNA sequence
S1	16S rRNA	<i>Salmonella enterica</i>
S2	16S rRNA	<i>Klebsiella variicola</i>
S3	16S rRNA	<i>Escherihia coli</i>
S4	16S rRNA	<i>Klebsiella variicola</i>
S5	16S rRNA	<i>Escherihia coli</i>
S6	16S rRNA	<i>Escherihia coli</i>
S7	16S rRNA	<i>Salmonella enterica</i>
S8	16S rRNA	<i>Escherihia coli</i>
S9	16S rRNA	<i>Escherihia coli</i>

Chapter Five

Discussion

5. Discussion**5.1. Isolation and Identification of Bacteria**

Diarrhea continues to be a leading cause of illness and death in children under five, especially in low-resource areas. Each year, there are around (1.7) billion episode and more than (400,000) death (WHO, 2023). Young children are particularly at risk because they have a higher body water content, which makes them prone to fast dehydration. When diarrhea persists and is accompanied by fever, the clinical risk increases. This emphasizes the importance of early diagnosis and treatment (Daley and Avva, 2024).

The current study reveals that *E.coli* account for (59%) of cases, making it the leading bacterial cause of diarrheal in children. This finding aligns with global trends that highlight its role in diarrheal disease in areas with limited resources. The notable isolation rates of *Klebsiella spp* at (24%) and *klebsiella aerogenes* at (8%) highlight the variety of enteric pathogens in this group. The detection of *K. pneumoniae* at (17%) and *K. oxytoca* at (7%) is significant because these species are not typically the focus of research on diarrhea. Their presence might indicate hospital transmission, local antibiotic pressures, or specific patterns in the community.

Using MacConkey agar for initial differentiation, combined with automated identification (VITEK) and molecular confirmation, allowed for a clear distinction between closely related Gram-negative species. This method overcomes the challenges of traditional biochemical tests, particularly for closely related organisms such as *K.pneumoniae* and *Roultella orinithiolytica*. The fruity odor observed in *R. ornithinolytica*

supports earlier findings that volatile metabolites can provide diagnostic clues (Seng *et al.*, 2010).

5.2. Distribution of Bacteria According to Sex

Several studies have investigated the impact of gender on children's susceptibility to bacterial infections from contaminated food or water, but the results have varied. In Murang'a Country, Kenya, girls under five were found to have a higher risk of infections, especially with *E.coli* and *shigella*. This suggests that there may be immunological or environmental factors related to gender (Mutua *et al.*, 2020). Similar research in Ethiopia revealed that more than half of bacterial samples from diarrhea cases in children under five years old came from girls, although this association was not statistically significant (Adane *et al.*, 2023). On the other hand, studies from southern Tehran and Bangladesh found higher rates of bacterial diarrhea in boys, with *E.coli* infections being much more common in males under five (Zali *et al.*, 2006; Chisti *et al.*, 2019). These differences might be due to variation in cultural practices, eating habits, healthcare-seeking behaviors, or local environmental exposures. This highlights the importance of conducting specific epidemiological studies to understand how gender affects the risk of pediatric diarrhea.

5.3. Distribution of Bacteria According to Age

In this study, the highest infection rate was found in infants aged (7 - 12) months at (41.0%). The (19 - 24) month group had a follow-up rate of (26.0%). The lowest rate, at (13.0%), was observed in the (13 - 18) months group. This pattern reflects the weaning period. During this time, children are exposed to more contaminated food, water, and environments, while

maternal antibody protection decrease. *Escherichia coli* was the most common pathogen in all age groups. It had the highest prevalence in the (19-24) month group, at (76.9%) , and in the (7-12) month group, at (63.4%). These results align with findings from Babylon Governorate in Iraq, where diarrheagenic *E. coli* was found to be very common in crowded households (Al-Sayagh *et al.*, 2023).

Klebsiella pneumoniae showed the highest prevalence in infants under six months at (30.0%) , indicating either vertical transmission or early hospital exposure. This finding partly matches neonatal studies in Al-Anbar Governorate, Iraq, where *K. pneumoniae* was responsible for nearly one-third of septicemia cases, suggesting early hospital-acquired infections (Shaw and Thapaliyal, 2007). Other bacteria, such as *Citrobacter freundii* and *Raoultella ornithinolytica*, appeared infrequently. This means they likely have a limited or occasional role in treating pediatric diarrhea. *klebsiella aerogenes* was not detected in infants under six months but was found in the (13-24) month range, possibly indicating postnatal environmental or hospital-related exposures.

Although infection rates unexpectedly dropped in the (13- 18) month old group , regional studies report a consistent rise in diarrheal infections with age. For example (Al Mamory *et al.*, 2022) noted an ongoing increase in acute bloody diarrhea among children under five in Babylon Province ,Iraq. These findings highlight the combined impact of age-related mobility, environmental exposure, and maternal immunity on the risk of diarrhea in children.

5.4. Antimicrobial Susceptibility Test for Bacteria Causing Diarrhea

The study revealed varying levels of antibiotic resistance among Gram-negative bacteria isolated from pediatric diarrhea cases in Thi-Qar province. This creates challenges for treatment ($p < 0.01$). Resistance patterns varied by species, reflecting both natural and acquired resistance mechanisms.

Citrobacter freundii isolates showed complete resistance (100%) to trimethoprim, cefazoline, cefuroxime, and cefuroxime axetil, with (66.7%) resistance to amoxicillin/clavulanic acid. However, they were fully susceptible to carbapenems (imipenem, meropenem, ertapenem), fluoroquinolones, and aminoglycosides, resulting in an overall susceptibility rate of (72.6%). These findings confirm earlier reports that *C. freundii* produces chromosomal AmpC β -lactamases, resulting in resistance to many β -lactams while remaining sensitive to carbapenems and aminoglycosides (Zurfluh *et al.*, 2017; WHO, 2023).

Enterobacter spp. showed high sensitivity to piperacillin/tazobactam, cefepime, meropenem, imipenem, and ciprofloxacin, but exhibited significant resistance to aztreonam (90.9%) and moderate resistance to ticarcillin and piperacillin. This resulted in an overall susceptibility of (58.8%). This finding aligns with previous research indicating that the *Enterobacter cloacae* complex possess natural β -lactam resistance due to the production of AmpC β -lactamase (Matsuo *et al.*, 2025).

E. coli exhibited excellent susceptibility to carbapenems (100% for meropenem, 98.3% for ertapenem) and good sensitivity to aminoglycosides (81% for amikacin). However, there was high resistance against cefazolin (89.8%), cefuroxime (88.1%), and trimethoprim (81.4%). These results

reflect global trends in diarrheagenic *E. coli*, where ESBL production and improper antibiotic use lead to widespread resistance, affecting the effectiveness of first-line oral treatments in children (Kotloff *et al.*, 2013; Momtaz *et al.*, 2013). *K. pneumoniae* isolates were fully susceptible to cefotaxime, ceftazidime/avibactam, meropenem, and tigecycline. Moderate to high resistance was observed for ampicillin/sulbactam (79.2%) and ceftazidime (62.5%), raising global concerns about ESBL and carbapenemase-producing strains (El-Sawalhi *et al.*, 2023).

Overall, carbapenems, particularly meropenem, ertapenem, and imipenem, demonstrated the highest effectiveness against all Gram-negative isolates. Piperacillin/tazobactam also demonstrated vigorous activity and may be helpful in non-severe or oral treatment options. On the other hand, antibiotics such as trimethoprim, cefazolin, cefuroxime, and amoxicillin/clavulanic acid displayed high resistance and should be avoided in empirical therapy, particularly in areas with high resistance, such as southern Iraq. These findings underlying the urgent need for local antibiogram-guided therapy and continuous monitoring to improve treatment outcomes in pediatric diarrhea.

5.5. Confirmation of Gram-negative bacteria by PCR

5.5.1. Detection of Gram-negative bacteria by 16S rRNA

The 16S rRNA gene, universally presents in bacteria, serves as reliable target for sequence-based identification, allowing for discrimination at both the genus and species levels (Clarridge III, 2004).

While some regions of the 16S rRNA gene mutate rapidly , facilitating differentiation among closely related species , other conserved regions allow resolution at higher taxonomic levels(Clayton *et al.*, 1995).

In this study , the molecular identification of *E.coli* corroborates its well established role as the leading bacterial cause of pediatric diarrhea, particularly in low-resource settings.This finding is consistent with those feom the Global Enteric Multicenter study (GEMS) ,which identified *E.coli* as the leading bacterial pathogen in children under five across multiple developing countries (Kotloff *et al.*, 2013).

The detection of *salmonella enterica* in two isolates aligns with regional data parts of Asia , where non-typhoidal *salmonella* strains are emerging contributors to pediatric gastroenteritis, as confirmed by molecular diagnostic methods (Feasey *et al.*, 2015). Interestingly, two isolates initially identified phenotypically as *klebsiella pneumoniae* were classified molecularly as *k.vriicola* , highlighting the limitation of conventional biochemical systems . This observation reinforces prior reports emphasizing the need for genetic discrimination *between k. pnumoniae , k.variicola , k.quasipneumoniae* , which share overlapping biochemical characteristics but differ in pathogenicity (Brisse *et al.*, 2014). These findings demonstrate the critical role of molecular tools in accurately identifying enteric bacterial pathogens and avoiding misclassification that may impact epidemiological assessments and treatment strategies..

5.5.2 Comparison between biochemical and molecular diagnostics

Discrepancies between automated biochemical identification and molecular methods align with earlier studies, which show that systems like

VITEK rely on metabolic profiles that can be highly similar among members of the Enterobacteriaceae family. Species such as *Klebsiella variicola*, *K. pneumoniae*, and *K. oxytoca* have overlapping biochemical traits. This often leads to misidentification (Brisse *et al.*, 2014). The case of identifying salmonella enterica in two isolates previously labeled as *k.pneumoniae* and *E.coli* highlights the risk of false-negative clinical interpretations. Misdiagnosing salmonella can delay crucial infection control measures and lead to incorrect antibiotic treatment (Janda and Abbott, 2007).

There are also instances where a sample identified as *k.pneumoniae* by VITEK was found to be *E.coli* by PCR. This may indicate co-infection, plate contamination, or the presence of non-target organisms during the culture process. These situations highlight the importance of molecular confirmation when culture-based results do not align with clinical symptoms. These observations point to either phenotypic misclassification by automated system or the presence of multiple pathogens in diarrheal stool samples. Molecular techniques have proven crucial for clarifying taxonomic uncertainties and identifying co-infections. Prior studies support the use of both biochemical and molecular diagnostics, especially for enteric infections where mixed pathogens are frequent (Zhou *et al.*, 2016).

•5.3. Phylogenetic Tree analysis

The evolutionary relationships among the bacterial isolates were reconstructed using the Neighbour-Joining (NJ) method. This distance-based approach is widely used for phylogenetic studies (Saitou and Nei, 1987). It estimates the evolutionary divergence between sequences and creates a tree

that displays the shortest overall branch lengths, which facilitates the analysis of closely related bacterial groups.

To test the reliability of the inferred relationships, a bootstrap analysis with 500 replicates was performed. The resulting bootstrap consensus tree displays confidence levels for each branch. Branches supported by fewer than (50%) of replicates were collapsed to reduce uncertainty in the phylogenetic reconstruction (Felsenstein, 1985). Evolutionary distance were calculated using the Jukes-Cantor model. This model assumes equal substitution rates among nucleotides and measures divergence as the number of base substitution per site (Jukes and Cantor, 1969). It is suitable for sequences with moderate divergence and gives a straightforward estimate of evolutionary distance among bacterial strains.

The dataset comprised nine nucleotide sequences from the studied isolates. The codon positions analyzed were the first, second, third, and noncoding positions, ensuring a thorough assessment of both conserved and variable regions. Position with gaps or missing data were excluded to prevent introducing errors into the analysis, leading to final alignment of 313 nucleotide positions

All evolutionary analysis, including distance calculations, tree building, and visualization, were carried out using MEGA7 software (Kumar *et al.*, 2016). The phylogenetic trees generated helped identify clades corresponding to specific bacterial species, clarified the genetic relationships among isolates, and provided insights into potential misclassifications seen in phenotypic identification system like VITEK. By combining molecular and phylogenetic data, this approach provides a

deeper understanding of bacterial classification , evolutionary divergence , and potential relationships among pediatric diarrheal pathogens .

The phylogenetic analysis not only supports the identification of species like *E.coli*, *salmonella enterica* ,and *klebsiella spp.* but also helps clarify doubts in cases where traditional biochemical systems fall short. For example, isolates initially labeled as *K. pneumoniae* or *E. coli* but later identified as *K. variicola* or *S. enterica* through 16S rRNA sequencing clustered correctly within their clades on the phylogenetic tree. This show the value of integrating molecular phylogenetic into diagnostic proccess.

Conclusions and Reconendation

Conclusions

1. Major contributors Gram-negative bacteria, particularly *E. coli* and *Klebsiella* species, are the leading causes of diarrhea in children under the age of five.
2. *E. coli* was the most common pathogen found, accounting for (59%) . It was followed by *Klebsiella* spp at (32%) .
3. sex distribution Infections were almost evenly split between females and males .
4. Antibiotic effectiveness:
 - Highly effective: Meropenem demonstrated 100% sensitivity, comparable to imipenem and piperacillin/tazobactam.
 - High resistance: Trimethoprim, cefazolin, cefuroxime, and cefuroxime axetil showed high resistance, highlighting multidrug resistance.

Recommendations

1. Use antibiotics rationally prescribe based on susceptibility testing; start broad-spectrum empirically only when necessary, and adjust according to culture results.
2. Enhance diagnostics and surveillance by utilizing rapid tools, such as the VITEK HN card, maintaining unified health records, and monitoring antimicrobial resistance genes to inform treatment and public health interventions.
3. Promote prevention encourage exclusive breastfeeding, good nutrition, hand washing, safe sanitation, and access to clean water.
4. Educate caregivers and communities by raise awareness on diarrhea prevention, early symptom recognition, and timely medical care.
5. Standardize treatment by implementing evidence-based guidelines for managing childhood diarrhea, including oral rehydration therapy and prudent antibiotic use.

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Appendix

Appendix I: Test Substrates on GN Card.

Well	Test	Mnemonic	Amount/Well
1.	Ala-phe-Pro-ARYLAMIDASE	APPA	0.0384 mg
2.	ADONITOL	ADO	0.1875 mg
3.	L-Pyrrolyl-ARYLAMIDASE	PyrA	0.018 mg
4.	L-ARABITOL	IARL	0.3 mg
5.	D-CELLOBIOSE	dCEL	0.3 mg
6.	BETA-GALACTOSIDASE	BGAL	0.036 mg
7.	H ₂ S PRODUCTION	H ₂ S	0.0024 mg
8.	BETA-N-ACETYL-GLUCOSAMINDASE	BNAG	0.0408 mg
9.	Glutamyl Arylamidase pNA	AGLTp	0.0324 mg
10.	D-GLUCOSE	dGLU	0.3 mg
11.	GAMMA-GLUTAMYL-TRANSFERASE	GGT	0.0228 mg
12.	FERMENTATION /GLUCOSE	OFF	0.45 mg
13.	BETA-GLUCOSIDASE	BGLU	0.036 mg
14.	D-MALTOSE	dMAL	0.3 mg
15.	D-MANNITOL	dMAN	0.1875 mg
16.	D-MANNOSE	dMNE	0.3 mg
17.	BETA-XYLOSIDASE	BXYL	0.0324 mg
18.	BETA-Alanine arylamidase	BAlap	0.174 mg
19.	L-Proline ARYLAMIDASE	ProA	0.0234 mg
20.	LIPASE	LIP	0.0192 mg
21.	PALATINOSE	PLE	0.3 mg
22.	Tyrosine ARYLAMIDASE	TyrA	0.0276 mg

23.	UREASE	URE	0.15 mg
24.	D-SORBITOL	dSOR	0.1875 mg
25.	SACCHAROSE/SUCROSE	SAC	0.3 mg
26.	D-TAGATOSE	dTAG	0.3 mg
27.	D-TREHALOSE	dTRE	0.3 mg
28.	CITRATE(SODIUM)	CIT	0.054 mg
29.	MALONATE	MNT	0.15 mg
30.	5-KETO-D-GLUCONATE	5KG	0.3 mg
31.	L-LACTATE alkalisation	ILATk	0.15 mg
32.	ALPHA-GLUCOSIDASE	AGLU	0.0306 mg
33.	SUCCINATE alkalisation	SUCT	0.15 mg
34.	Beta-N-ACETYL-GALACTOSAMINIDASE	NAGA	0.036 mg
35.	ALPHA-GALACTOSIDASE	AGAL	0.036 mg
36.	PHOSPHATASE	PHOS	0.0504 mg
37.	Glycine ARYLAMIDASE	GlyA	0.012 mg
38.	ORNITHINE DECARBOXYLASE	ODC	0.3 mg
39.	LYSINE DECARBOXYLASE	LDC	0.15 mg
40.	L-HISTIDINE assimilation	IHISa	0.087 mg
41.	COUMARATE	CMT	0.162 mg
42.	BETA-GLUCORONIDASE	BGUR	0.0378 mg
43.	O/129RESISTANCE (comp vibiro.)	O129R	0.0105 mg
44.	Glu-Gly-Arg-ARYLAMIDASE	GGAA	0.0576 mg
45.	L-MALATE assimilation	IMLTa	0.042 mg
46.	ELLMAN	ELLM	0.03 mg
47.	L-LACTATE assimilation	ILATa	0.186 mg

Appendix II

Salmonella enterica strain CLSSen01 16S ribosomal RNA gene, partial sequence

Sequence ID: [MF682400.1](#) Length: 1445 Number of Matches: 1

Range 1: 1108 to 1433 [GenBank](#) [Graphics](#)

▼ [Next Match](#) ▲ [Previous Match](#)

Score	Expect	Identities	Gaps	Strand
590 bits(319)	5e-164	324/326(99%)	1/326(0%)	Plus/Minus
Query 1	TGCTACGACTTC-CCCCAGTCATGAATCACAAAGTGGTAAGCGCCCTCCCGAAGGTTAAG	59		
Sbjct 1433	TGCTACGACTTCACCCAGTCATGAATCACAAAGTGGTAAGCGCCCTCCCGAAGGTTAAG	1374		
Query 60	CTACCTACTTCTTTTGAACCCACTCCCATGGTGTGACGGGCGGTGTGTACAAGGCCCGG	119		
Sbjct 1373	CTACCTACTTCTTTTGAACCCACTCCCATGGTGTGACGGGCGGTGTGTACAAGGCCCGG	1314		
Query 120	GAACGTATTACCGTGGCATTCTGATCCACGATTACTAGCGATTCCGACTTCATGGAGTC	179		
Sbjct 1313	GAACGTATTACCGTGGCATTCTGATCCACGATTACTAGCGATTCCGACTTCATGGAGTC	1254		
Query 180	GAGTTGCAGACTCCAATCCGGACTACGACGACTTTATGAGGTCCGCTTGCTCTCGCGAG	239		
Sbjct 1253	GAGTTGCAGACTCCAATCCGGACTACGACGACTTTATGAGGTCCGCTTGCTCTCGCGAG	1194		
Query 240	GTCGCTTCTCTTTGTATGCGCCATTGTAGCACGTGTGTAGCCCTGGTCGTAAGGGCCATG	299		
Sbjct 1193	GTCGCTTCTCTTTGTATGCGCCATTGTAGCACGTGTGTAGCCCTGGTCGTAAGGGCCATG	1134		
Query 300	ATGACTTGACGTCATCCCCACCATCC	325		
Sbjct 1133	ATGACTTGACGTCATCCCCACCTTCC	1108		

(A)

Klebsiella variicola strain IARI-PC4-59 16S ribosomal RNA gene, partial sequence

Sequence ID: [KT149751.1](#) Length: 1453 Number of Matches: 1

Range 1: 1121 to 1449 [GenBank](#) [Graphics](#)

▼ [Next Match](#) ▲ [Previous Match](#)

Score	Expect	Identities	Gaps	Strand
608 bits(329)	1e-169	329/329(100%)	0/329(0%)	Plus/Minus
Query 1	GGCTCTTGTTACGACTTCACCCAGTCATGAATCACAAAGTGGTAAGCGCCCTCCCGAAG	60		
Sbjct 1449	GGCTCTTGTTACGACTTCACCCAGTCATGAATCACAAAGTGGTAAGCGCCCTCCCGAAG	1390		
Query 61	GTTAAGCTACCTACTTCTTTTGAACCCACTCCCATGGTGTGACGGGCGGTGTGTACAAG	120		
Sbjct 1389	GTTAAGCTACCTACTTCTTTTGAACCCACTCCCATGGTGTGACGGGCGGTGTGTACAAG	1330		
Query 121	GCCCCGGAACGTATTACCGTAGCATTCTGATCTACGATTACTAGCGATTCCGACTTCAT	180		
Sbjct 1329	GCCCCGGAACGTATTACCGTAGCATTCTGATCTACGATTACTAGCGATTCCGACTTCAT	1270		
Query 181	GGAGTCGAGTTGCAGACTCCAATCCGGACTACGACATACTTTATGAGGTCCGCTTGCTCT	240		
Sbjct 1269	GGAGTCGAGTTGCAGACTCCAATCCGGACTACGACATACTTTATGAGGTCCGCTTGCTCT	1210		
Query 241	CGCGAGGTCGCTTCTCTTTGTATATGCCATTGTAGCACGTGTGTAGCCCTGGTCGTAAGG	300		
Sbjct 1209	CGCGAGGTCGCTTCTCTTTGTATATGCCATTGTAGCACGTGTGTAGCCCTGGTCGTAAGG	1150		
Query 301	GCCATGATGACTTGACGTCATCCCCACCT	329		
Sbjct 1149	GCCATGATGACTTGACGTCATCCCCACCT	1121		

(B)

Escherichia coli strain RM315 16S ribosomal RNA gene, partial sequenceSequence ID: [OR018552.1](#) Length: 950 Number of Matches: 1Range 1: 612 to 935 [GenBank](#) [Graphics](#)[▼ Next Match](#) [▲ Previous Match](#)

Score	Expect	Identities	Gaps	Strand
599 bits(324)	9e-167	324/324(100%)	0/324(0%)	Plus/Minus
Query 1	CTTGTACGACTTCACCCCAGTCATGAATCACAAAGTGGTAAGCGCCCTCCCGAAGGTTAA	60		
Sbjct 935	CTTGTACGACTTCACCCCAGTCATGAATCACAAAGTGGTAAGCGCCCTCCCGAAGGTTAA	876		
Query 61	GCTACCTACTTCTTTTGCAACCCACTCCCATGGTGTGACGGGCGGTGTGTACAAGGCCCG	120		
Sbjct 875	GCTACCTACTTCTTTTGCAACCCACTCCCATGGTGTGACGGGCGGTGTGTACAAGGCCCG	816		
Query 121	GGAACGTATTACCGTGGCATTTCTGATCCACGATTACTAGCGATTCCGACTTCATGGAGT	180		
Sbjct 815	GGAACGTATTACCGTGGCATTTCTGATCCACGATTACTAGCGATTCCGACTTCATGGAGT	756		
Query 181	CGAGTTGCAGACTCCAATCCGGACTACGACGCACCTTATGAGGTCCGCTTGCTCTCGCGA	240		
Sbjct 755	CGAGTTGCAGACTCCAATCCGGACTACGACGCACCTTATGAGGTCCGCTTGCTCTCGCGA	696		
Query 241	GGTCGCTTCTCTTTGTATGCGCCATTGTAGCACGTGTGTAGCCCTGGTCGTAAGGGCCAT	300		
Sbjct 695	GGTCGCTTCTCTTTGTATGCGCCATTGTAGCACGTGTGTAGCCCTGGTCGTAAGGGCCAT	636		
Query 301	GATGACTTGACGTCATCCCCACCT	324		
Sbjct 635	GATGACTTGACGTCATCCCCACCT	612		

(C)

Klebsiella variicola strain IARI-PC4-59 16S ribosomal RNA gene, partial sequenceSequence ID: [KT149751.1](#) Length: 1453 Number of Matches: 1Range 1: 1123 to 1449 [GenBank](#) [Graphics](#)[▼ Next Match](#) [▲ Previous Match](#)

Score	Expect	Identities	Gaps	Strand
604 bits(327)	2e-168	327/327(100%)	0/327(0%)	Plus/Minus
Query 1	GGCTCTTGTTACGACTTCACCCCAGTCATGAATCACAAAGTGGTAAGCGCCCTCCCGAAG	60		
Sbjct 1449	GGCTCTTGTTACGACTTCACCCCAGTCATGAATCACAAAGTGGTAAGCGCCCTCCCGAAG	1390		
Query 61	GTTAAGCTACCTACTTCTTTTGCAACCCACTCCCATGGTGTGACGGGCGGTGTGTACAAG	120		
Sbjct 1389	GTTAAGCTACCTACTTCTTTTGCAACCCACTCCCATGGTGTGACGGGCGGTGTGTACAAG	1330		
Query 121	GCCCCGGGAACGTATTACCGTAGCATTCTGATCTACGATTACTAGCGATTCCGACTTCAT	180		
Sbjct 1329	GCCCCGGGAACGTATTACCGTAGCATTCTGATCTACGATTACTAGCGATTCCGACTTCAT	1270		
Query 181	GGAGTCGAGTTGCAGACTCCAATCCGGACTACGACATACCTTATGAGGTCCGCTTGCTCT	240		
Sbjct 1269	GGAGTCGAGTTGCAGACTCCAATCCGGACTACGACATACCTTATGAGGTCCGCTTGCTCT	1210		
Query 241	CGCGAGGTCGCTTCTCTTTGTATATGCCATTGTAGCACGTGTGTAGCCCTGGTCGTAAGG	300		
Sbjct 1209	CGCGAGGTCGCTTCTCTTTGTATATGCCATTGTAGCACGTGTGTAGCCCTGGTCGTAAGG	1150		
Query 301	GCCATGATGACTTGACGTCATCCCCAC	327		
Sbjct 1149	GCCATGATGACTTGACGTCATCCCCAC	1123		

(D)

Escherichia coli partial 16S rRNA gene, isolate ASI-2

Sequence ID: [LN558637.1](#) Length: 1384 Number of Matches: 1

Range 1: 20 to 332 [GenBank](#) [Graphics](#)

[Next Match](#) [Previous Match](#)

Score	Expect	Identities	Gaps	Strand
579 bits(313)	1e-160	313/313(100%)	0/313(0%)	Plus/Plus
Query 1	ACTTCCCCCAGTCATGAATCACAAAGTGGTAAGCGCCCTCCCGAAGGTTAAGCTACCTAC	60		
Sbjct 20	ACTTCCCCCAGTCATGAATCACAAAGTGGTAAGCGCCCTCCCGAAGGTTAAGCTACCTAC	79		
Query 61	TTCTTTTGCAACCCACTCCCATGGTGTGACGGGCGGTGTGTACAAGGCCCGGGAACGTAT	120		
Sbjct 80	TTCTTTTGCAACCCACTCCCATGGTGTGACGGGCGGTGTGTACAAGGCCCGGGAACGTAT	139		
Query 121	TCACCGTGGCATTCTGATCCACGATTACTAGCGATTCCGACTTCATGGAGTCGAGTTGCA	180		
Sbjct 140	TCACCGTGGCATTCTGATCCACGATTACTAGCGATTCCGACTTCATGGAGTCGAGTTGCA	199		
Query 181	GACTCCAATCCGGACTACGACGCACTTTATGAGGTCCGCTTGCTCTCGCGAGGTCGCTTC	240		
Sbjct 200	GACTCCAATCCGGACTACGACGCACTTTATGAGGTCCGCTTGCTCTCGCGAGGTCGCTTC	259		
Query 241	TCTTTGTATGCGCCATTGTAGCACGTGTGTAGCCCTGGTCGTAAGGGCCATGATGACTTG	300		
Sbjct 260	TCTTTGTATGCGCCATTGTAGCACGTGTGTAGCCCTGGTCGTAAGGGCCATGATGACTTG	319		
Query 301	ACGTCATCCCCAC	313		
Sbjct 320	ACGTCATCCCCAC	332		

(E)

Escherichia coli strain DSM 112119 16S ribosomal RNA gene, partial sequence

Sequence ID: [QM658579.1](#) Length: 1479 Number of Matches: 1

Range 1: 1151 to 1478 [GenBank](#) [Graphics](#)

[Next Match](#) [Previous Match](#)

Score	Expect	Identities	Gaps	Strand
606 bits(328)	5e-169	328/328(100%)	0/328(0%)	Plus/Minus
Query 1	ACGACTTCACCCAGTCATGAATCACAAAGTGGTAAGCGCCCTCCCGAAGGTTAAGCTAC	60		
Sbjct 1478	ACGACTTCACCCAGTCATGAATCACAAAGTGGTAAGCGCCCTCCCGAAGGTTAAGCTAC	1419		
Query 61	CTACTTCTTTTGCAACCCACTCCCATGGTGTGACGGGCGGTGTGTACAAGGCCCGGGAAC	120		
Sbjct 1418	CTACTTCTTTTGCAACCCACTCCCATGGTGTGACGGGCGGTGTGTACAAGGCCCGGGAAC	1359		
Query 121	GTATTACCGTGGCATTCTGATCCACGATTACTAGCGATTCCGACTTCATGGAGTCGAGT	180		
Sbjct 1358	GTATTACCGTGGCATTCTGATCCACGATTACTAGCGATTCCGACTTCATGGAGTCGAGT	1299		
Query 181	TGCAGACTCCAATCCGGACTACGACGCACTTTATGAGGTCCGCTTGCTCTCGCGAGGTCG	240		
Sbjct 1298	TGCAGACTCCAATCCGGACTACGACGCACTTTATGAGGTCCGCTTGCTCTCGCGAGGTCG	1239		
Query 241	CTTCTCTTTGTATGCGCCATTGTAGCACGTGTGTAGCCCTGGTCGTAAGGGCCATGATGA	300		
Sbjct 1238	CTTCTCTTTGTATGCGCCATTGTAGCACGTGTGTAGCCCTGGTCGTAAGGGCCATGATGA	1179		
Query 301	CTTGACGTCATCCCCACCTTCCTCCAGT	328		
Sbjct 1178	CTTGACGTCATCCCCACCTTCCTCCAGT	1151		

(F)

Salmonella enterica strain CLSSen01 16S ribosomal RNA gene, partial sequenceSequence ID: [MF682400.1](#) Length: 1445 Number of Matches: 1Range 1: 1108 to 1435 [GenBank](#) [Graphics](#)[▼ Next Match](#) [▲ Previous Match](#)

Score	Expect	Identities	Gaps	Strand
601 bits(325)	2e-167	327/328(99%)	0/328(0%)	Plus/Minus
Query 1	CTTGCTACGACTTCACCCCAGTCATGAATCACAAAGTGGTAAGCGCCCTCCCGAAGGTTA	60		
Sbjct 1435	CTTGCTACGACTTCACCCCAGTCATGAATCACAAAGTGGTAAGCGCCCTCCCGAAGGTTA	1376		
Query 61	AGCTACCTACTTCTTTTGAACCCACTCCCATGGTGTGACGGGCGGTGTGTACAAGGCC	120		
Sbjct 1375	AGCTACCTACTTCTTTTGAACCCACTCCCATGGTGTGACGGGCGGTGTGTACAAGGCC	1316		
Query 121	GGGAACGTATTACCGTGGCATTCTGATCCACGATTACTAGCGATTCCGACTTCATGGAG	180		
Sbjct 1315	GGGAACGTATTACCGTGGCATTCTGATCCACGATTACTAGCGATTCCGACTTCATGGAG	1256		
Query 181	TCGAGTTGCAGACTCCAATCCGGACTACGACGCACCTTTATGAGGTCCGCTTGCTCTCGCG	240		
Sbjct 1255	TCGAGTTGCAGACTCCAATCCGGACTACGACGCACCTTTATGAGGTCCGCTTGCTCTCGCG	1196		
Query 241	AGGTCGCTTCTCTTTGTATGCGCCATTGTAGCACGTGTGTAGCCCTGGTCGTAAGGGCCA	300		
Sbjct 1195	AGGTCGCTTCTCTTTGTATGCGCCATTGTAGCACGTGTGTAGCCCTGGTCGTAAGGGCCA	1136		
Query 301	TGATGACTTGACGTCATCCCCACCGTCC	328		
Sbjct 1135	TGATGACTTGACGTCATCCCCACCTTCC	1108		

(G)

Escherichia coli strain OXEC-381 chromosome, complete genomeSequence ID: [CP165483.1](#) Length: 5008277 Number of Matches: 7Range 1: 3878829 to 3879166 [GenBank](#) [Graphics](#)[▼ Next Match](#) [▲ Previous Match](#)

Score	Expect	Identities	Gaps	Strand
625 bits(338)	1e-174	338/338(100%)	0/338(0%)	Plus/Minus
Query 1	ACCTTGTTACGACTTCACCCCAGTCATGAATCACAAAGTGGTAAGCGCCCTCCCGAAGGT	60		
Sbjct 3879166	ACCTTGTTACGACTTCACCCCAGTCATGAATCACAAAGTGGTAAGCGCCCTCCCGAAGGT	3879107		
Query 61	TAAGCTACCTACTTCTTTTGAACCCACTCCCATGGTGTGACGGGCGGTGTGTACAAGGC	120		
Sbjct 3879106	TAAGCTACCTACTTCTTTTGAACCCACTCCCATGGTGTGACGGGCGGTGTGTACAAGGC	3879047		
Query 121	CCGGGAACGTATTACCGTGGCATTCTGATCCACGATTACTAGCGATTCCGACTTCATGG	180		
Sbjct 3879046	CCGGGAACGTATTACCGTGGCATTCTGATCCACGATTACTAGCGATTCCGACTTCATGG	3878987		
Query 181	AGTCGAGTTGCAGACTCCAATCCGGACTACGACGCACCTTTATGAGGTCCGCTTGCTCTCG	240		
Sbjct 3878986	AGTCGAGTTGCAGACTCCAATCCGGACTACGACGCACCTTTATGAGGTCCGCTTGCTCTCG	3878927		
Query 241	CGAGGTCGCTTCTCTTTGTATGCGCCATTGTAGCACGTGTGTAGCCCTGGTCGTAAGGGC	300		
Sbjct 3878926	CGAGGTCGCTTCTCTTTGTATGCGCCATTGTAGCACGTGTGTAGCCCTGGTCGTAAGGGC	3878867		
Query 301	CATGATGACTTGACGTCATCCCCACCTTCCCTCCAGTT	338		
Sbjct 3878866	CATGATGACTTGACGTCATCCCCACCTTCCCTCCAGTT	3878829		

(H)

Escherichia coli strain P10 16S ribosomal RNA gene, partial sequence

Sequence ID: [MN700062.1](#) Length: 694 Number of Matches: 1

Range 1: 364 to 688 [GenBank](#) [Graphics](#)

▼ [Next Match](#) ▲ [Previous Match](#)

Score	Expect	Identities	Gaps	Strand
595 bits(322)	1e-165	324/325(99%)	0/325(0%)	Plus/Minus
Query 1	CCTTTGCTACGACTTCACCCAGTCATGAATCACAAAGTGGTAAGCGCCCTCCCGAAGGT	60		
Sbjct 688	CCTTTGTTACGACTTCACCCAGTCATGAATCACAAAGTGGTAAGCGCCCTCCCGAAGGT	629		
Query 61	TAAGCTACCTACTTCTTTTGCAACCCACTCCCATGGTGTGACGGGCGGTGTGTACAAGGC	120		
Sbjct 628	TAAGCTACCTACTTCTTTTGCAACCCACTCCCATGGTGTGACGGGCGGTGTGTACAAGGC	569		
Query 121	CCGGGAACGTATTACCGTGGCATCTCTGATCCACGATTACTAGCGATTCCGACTTCATGG	180		
Sbjct 568	CCGGGAACGTATTACCGTGGCATCTCTGATCCACGATTACTAGCGATTCCGACTTCATGG	509		
Query 181	AGTCGAGTTGCAGACTCCAATCCGGACTACGACGCACTTTATGAGGTCCGCTTGCTCTCG	240		
Sbjct 508	AGTCGAGTTGCAGACTCCAATCCGGACTACGACGCACTTTATGAGGTCCGCTTGCTCTCG	449		
Query 241	CGAGGTCGCTTCTCTTTGTATGCGCCATTGTAGCACGTGTGTAGCCCTGGTTCGTAAGGGC	300		
Sbjct 448	CGAGGTCGCTTCTCTTTGTATGCGCCATTGTAGCACGTGTGTAGCCCTGGTTCGTAAGGGC	389		
Query 301	CATGATGACTTGACGTCATCCCCAC	325		
Sbjct 388	CATGATGACTTGACGTCATCCCCAC	364		

(I)

Appendix II Captions

Figure A: Nucleotide blast of the partial region of gene 16S rRNA of isolate HA19 against 16S rRNA sequences deposited in the NCBI database showed a high identity to *Salmonella enterica* species.

Source: (<https://blast.ncbi.nlm.nih.gov/Blast>)

Figure B: Nucleotide blast of the partial region of gene 16S rRNA of isolate HA23 against 16S rRNA sequences deposited in the NCBI data base showed a high identity to *Klebsiella variicola* species.

Source: (<https://blast.ncbi.nlm.nih.gov/Blast>)..

Figure C: Nucleotide blast of the partial region of gene 16S rRNA of isolate HA32 against 16S rRNA sequences deposited in the NCBI database showed a high identity to *Escherichia coli* species.

Source: (<https://blast.ncbi.nlm.nih.gov/Blast>).

Figure D: Nucleotide blast of the partial region of gene 16S rRNA of isolate HA72 against 16S rRNA sequences deposited in the NCBI database showed a high identity to *Klebsiella variicola* species.

Source: (<https://blast.ncbi.nlm.nih.gov/Blast>).

Figure E: Nucleotide blast of the partial region of gene 16S rRNA of isolate HA74 against 16S rRNA sequences deposited in the NCBI database showed a high identity to *Escherichia coli* species.

Source: (<https://blast.ncbi.nlm.nih.gov/Blast>).

Figure F: Nucleotide blast of the partial region of gene 16S rRNA of isolate HA84 against 16S rRNA sequences deposited in the NCBI database showed a high identity to *Escherichia coli* species.

Source: (<https://blast.ncbi.nlm.nih.gov/Blast>).

Figure G: Nucleotide blast of a partial region of gene 16S rRNA of isolate HA92 against 16S rRNA sequences deposited in the NCBI database showed a high identity to *Salmonella enterica* species.

Source: (<https://blast.ncbi.nlm.nih.gov/Blast>).

Figure H: Nucleotide blast of the partial region of gene 16S rRNA of isolate HA93 against 16S rRNA sequences deposited in the NCBI database showed a high identity to *Escherichia coli* species.

Source: (<https://blast.ncbi.nlm.nih.gov/Blast>).

Figure I: Nucleotide blast of the partial region of gene 16S rRNA of isolate HA99 against 16S rRNA sequences deposited in the NCBI database showed a high identity to *Escherichia coli* species.

Source: (<https://blast.ncbi.nlm.nih.gov/Blast>).

Appendix III: Examination report by the VITEK2 device on the sample under study.

bioMérieux Customer:

Microbiology Chart Report

Printed November 2, 2024 8:24:51 AM CDT

Patient Name: S7, .

Patient ID: 53

Location:

Physician:

Lab ID: 53

Isolate Number: 1

Organism Quantity:

Selected Organism : Citrobacter freundii

Source: Stool

Collected: Oct 30, 2024

Comments:	

Identification Information	Analysis Time: 4.83 hours	Status: Final
Selected Organism	98% Probability	Citrobacter freundii
ID Analysis Messages	Bionumber: 4417610555521211	

Biochemical Details																	
2	APPA	-	3	ADO	-	4	PyrA	+	5	IARL	-	7	dCEL	-	9	BGAL	+
10	H2S	+	11	BNAG	-	12	AGLTp	-	13	dGLU	+	14	GGT	+	15	OFF	+
17	BGLU	-	18	dMAL	+	19	dMAN	+	20	dMNE	+	21	BXYL	-	22	BAlap	-
23	ProA	-	26	LIP	-	27	PLE	-	29	TyrA	+	31	URE	-	32	dSOR	-
33	SAC	+	34	dTAG	-	35	dTRE	+	36	CIT	+	37	MNT	-	39	5KG	+
40	ILATk	+	41	AGLU	-	42	SUCT	+	43	NAGA	-	44	AGAL	+	45	PHOS	-
46	GlyA	+	47	ODC	-	48	LDC	-	53	IHISa	-	56	CMT	+	57	BGUR	-
58	O129R	+	59	GGAA	-	61	IMLTa	-	62	ELLM	(+)	64	ILATa	-			

bioMérieux Customer:

Microbiology Chart Report

Printed November 5, 2024 10:04:51 AM CST

Patient Name: S33, .

Patient ID: 59

Location:

Physician:

Lab ID: 59

Isolate Number: 1

Organism Quantity:

Selected Organism : Klebsiella pneumoniae ssp pneumoniae

Source: Stool

Collected: Nov 4, 2024

Comments:	

Identification Information	Analysis Time: 3.85 hours	Status: Final
Selected Organism	96% Probability	Klebsiella pneumoniae ssp pneumoniae
ID Analysis Messages	Bionumber: 2605734653524610	

Biochemical Details																	
2	APPA	-	3	ADO	+	4	PyrA	-	5	IARL	-	7	dCEL	+	9	BGAL	+
10	H2S	-	11	BNAG	-	12	AGLTp	-	13	dGLU	+	14	GGT	-	15	OFF	+
17	BGLU	+	18	dMAL	+	19	dMAN	+	20	dMNE	+	21	BXYL	+	22	BAlap	-
23	ProA	-	26	LIP	-	27	PLE	+	29	TyrA	-	31	URE	+	32	dSOR	+
33	SAC	+	34	dTAG	-	35	dTRE	+	36	CIT	+	37	MNT	+	39	5KG	-
40	ILATk	+	41	AGLU	-	42	SUCT	+	43	NAGA	-	44	AGAL	+	45	PHOS	-
46	GlyA	-	47	ODC	-	48	LDC	+	53	IHISa	-	56	CMT	+	57	BGUR	+
58	O129R	+	59	GGAA	-	61	IMLTa	-	62	ELLM	-	64	ILATa	-			

bioMérieux Customer:

Microbiology Chart Report

Printed November 5, 2024 10:05:21 AM CST

Patient Name: S11, .

Patient ID: 60

Location:

Physician:

Lab ID: 60

Isolate Number: 1

Organism Quantity:

Selected Organism : Enterobacter aerogenes

Source: Stool

Collected: Nov 4, 2024

Comments:	

Identification Information	Analysis Time: 9.47 hours	Status: Final
Selected Organism	Enterobacter aerogenes	
ID Analysis Messages	Bionumber: 6607735773567752	

Biochemical Details																	
2	APPA	=	3	ADO	+	4	PyrA	+	5	IARL	=	7	dCEL	+	9	BGAL	+
10	H2S	=	11	BNAG	=	12	AGLTp	=	13	dGLU	+	14	GGT	+	15	OFF	+
17	BGLU	+	18	dMAL	+	19	dMAN	+	20	dMNE	+	21	BXYL	+	22	BAlap	=
23	ProA	+	26	LIP	=	27	PLE	+	29	TyrA	+	31	URE	+	32	dSOR	+
33	SAC	+	34	dTAG	+	35	dTRE	+	36	CIT	+	37	MNT	+	39	5KG	=
40	ILATk	+	41	AGLU	=	42	SUCT	+	43	NAGA	=	44	AGAL	+	45	PHOS	+
46	GlyA	+	47	ODC	+	48	LDC	+	53	IHISa	+	56	CMT	+	57	BGUR	+
58	O129R	+	59	GGAA	=	61	IMLTa	+	62	ELLM	=	64	ILATa	+			

bioMérieux Customer:

Microbiology Chart Report

Printed November 5, 2024 10:03:29 AM CST

Patient Name: S22, .

Patient ID: 56

Location:

Physician:

Lab ID: 56

Isolate Number: 2

Organism Quantity:

Selected Organism : Escherichia coli

Source: Stool

Collected: Nov 4, 2024

Comments:	

Susceptibility Information	Analysis Time: 15.55 hours	Status: Final
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Antimicrobial	MIC	Interpretation	Antimicrobial	MIC	Interpretation
Amoxicillin/Clavulanic Acid	>= 32	R	Imipenem	1	S
Piperacillin/Tazobactam	<= 4	S	Meropenem	<= 0.25	S
Cefazolin	>= 64	R	Amikacin	2	S
Cefuroxime	>= 64	R	Gentamicin	<= 1	S
Cefuroxime Axetil	>= 64	R	Ciprofloxacin	0.5	S
Ceftazidime	32	R	Fosfomycin	<= 16	S
Ceftriaxone	>= 64	R	Nitrofurantoin	64	I
Cefepime	16	R	Trimethoprim/ Sulfamethoxazole	>= 320	R
Ertapenem	0.25	S			

AES Findings	
Confidence:	Consistent

bioMérieux Customer:

Microbiology Chart Report

Printed November 5, 2024 10:05:46 AM CST

Patient Name: S44, .

Patient ID: 61

Location:

Physician:

Lab ID: 61

Isolate Number: 1

Organism Quantity:

Selected Organism : Raoultella ornithinolytica

Source: Stool

Collected: Nov 4, 2024

Comments:	

Identification Information	Analysis Time: 5.78 hours	Status: Final
Selected Organism	86% Probability Raoultella ornithinolytica	
ID Analysis Messages	Bionumber: 6617735777567611	

Biochemical Details																	
2	APPA	-	3	ADO	+	4	PyrA	+	5	IARL	-	7	dCEL	+	9	BGAL	+
10	H2S	+	11	BNAG	(-)	12	AGLTp	-	13	dGLU	+	14	GGT	+	15	OFF	+
17	BGLU	+	18	dMAL	+	19	dMAN	+	20	dMNE	+	21	BXYL	+	22	BAlap	-
23	ProA	+	26	LIP	-	27	PLE	+	29	TyrA	+	31	URE	+	32	dSOR	+
33	SAC	+	34	dTAG	+	35	dTRE	+	36	CIT	+	37	MNT	+	39	5KG	+
40	ILATk	+	41	AGLU	-	42	SUCT	+	43	NAGA	-	44	AGAL	+	45	PHOS	+
46	GlyA	+	47	ODC	+	48	LDC	+	53	IHISa	-	56	CMT	+	57	BGUR	+
58	O129R	+	59	GGAA	-	61	IMLTa	-	62	ELLM	+	64	ILATa	-			

bioMérieux Customer:

Microbiology Chart Report

Printed November 19, 2024 2:57:25 PM CST

Patient Name: S-72, .

Patient ID: 113

Location:

Physician:

Lab ID: 113

Isolate Number: 1

Organism Quantity:

Selected Organism : Klebsiella oxytoca

Source: Stool

Collected: Nov 18, 2024

Comments:	

Identification Information	Analysis Time: 9.97 hours	Status: Final
Selected Organism	Klebsiella oxytoca	
ID Analysis Messages	Bionumber: 6607735777567753	

Biochemical Details																	
2	APPA	-	3	ADO	+	4	PyrA	+	5	IARL	-	7	dCEL	+	9	BGAL	+
10	H2S	-	11	BNAG	-	12	AGLTp	-	13	dGLU	+	14	GGT	+	15	OFF	+
17	BGLU	+	18	dMAL	+	19	dMAN	+	20	dMNE	+	21	BXYL	+	22	BAlap	-
23	ProA	+	26	LIP	-	27	PLE	+	29	TyrA	+	31	URE	+	32	dSOR	+
33	SAC	+	34	dTAG	+	35	dTRE	+	36	CIT	+	37	MNT	+	39	5KG	+
40	ILATk	+	41	AGLU	-	42	SUCT	+	43	NAGA	-	44	AGAL	+	45	PHOS	+
46	GlyA	+	47	ODC	+	48	LDC	+	53	IHISa	(+)	56	CMT	+	57	BGUR	+
58	O129R	+	59	GGAA	-	61	IMLTa	+	62	ELLM	+	64	ILATa	+			

Organism Quantity:

Selected Organism : Enterobacter cloacae complex

Source: Stool

Collected: Nov 18, 2024

Comments:	

Identification Information	Analysis Time: 4.85 hours	Status: Final
Selected Organism	95% Probability	Enterobacter cloacae complex
	Bionumber:	0627735753532010
ID Analysis Messages		

Biochemical Details																	
2	APPA	-	3	ADO	-	4	PyrA	-	5	IARL	-	7	dCEL	+	9	BGAL	+
10	H2S	-	11	BNAG	+	12	AGLTp	-	13	dGLU	+	14	GGT	+	15	OFF	+
17	BGLU	+	18	dMAL	+	19	dMAN	+	20	dMNE	+	21	BXYL	+	22	BAlap	-
23	ProA	+	26	LIP	-	27	PLE	+	29	TyrA	+	31	URE	+	32	dSOR	+
33	SAC	+	34	dTAG	-	35	dTRE	+	36	CIT	+	37	MNT	+	39	5KG	-
40	ILATk	+	41	AGLU	-	42	SUCT	+	43	NAGA	+	44	AGAL	+	45	PHOS	-
46	GlyA	-	47	ODC	+	48	LDC	-	53	IHISa	-	56	CMT	-	57	BGUR	-
58	O129R	+	59	GGAA	-	61	IMLTa	-	62	ELLM	-	64	ILATa	-			

الخلاصة

يعد الإسهال من أهم المشكلات الصحية التي تواجه الأطفال دون سن الخامسة، وخاصة في الدول النامية، حيث يُسهم بشكل رئيسي في زياده معدلات المراضة والوفيات. وتكمن خطورة الإسهال في ما يسببه من جفاف واختلال في توازن الأملاح والسوائل، إضافة إلى تأثيراته السلبية على النمو والتغذية والمناعة. ورغم إمكانية الوقاية من هذه المشكلة وعلاجها بوسائل بسيطة نسبياً مثل الرضاعة الطبيعية والمعالجات ومحاليل الإماهة الفموية، إلا أنها ما تزال تمثل عبئاً كبيراً على الصحة العامة، خصوصاً مع محدودية الوصول إلى المياه النقية ووسائل الصرف الصحي المناسبة.

هدفت هذه الدراسة إلى تحديد العوامل البكتيرية المسببة للإسهال عند الأطفال، مع التركيز على الجراثيم سلبية الغرام، وذلك باستخدام طرق الزرع التقليدية والاختبارات الكيميائية الحيوية (VITEK II)، إلى جانب الاعتماد على الوسائل الجزيئية PCR (لجين ١٦٥ rRNA وتسلسل الحمض النووي) من أجل تأكيد الهوية البكتيرية للعزلات. كما شملت الدراسة إجراء اختبارات الحساسية للمضادات الحيوية لتحديد أنماط المقاومة البكتيرية ورصد السلالات متعددة المقاومة.

جمعت (١٣٢) عينة براز من الأطفال المصابين بالإسهال، استُبعد منها (٣٢) عينة بسبب التلوث أو وجود عدوى طفيلية مثبتة، وبذلك تم تحليل (١٠٠) عينة صالحة للزراعة البكتيرية. أظهرت النتائج وجود سبعة أنواع من البكتيريا سلبية الغرام، فكان أكثرها شيوعاً الإشريكية القولونية (٥٩٪)، تلتها الكلبسيلا (٣٢٪) وتشمل (*K. pneumoniae* بنسبة ١٧٪ و *K. oxytoca* بنسبة ٧٪، كلبسيلا أيروجينس ٨٪)، في حين ظهرت نسب أقل من سيتروبوكتريز فريندي وروتلا أورينتوليتكا. أما من حيث التوزيع بين الجنسين فقد كان متقارباً (٥١٪ أنثى، ٤٩٪ ذكور)، مما يشير إلى أن العدوى لا ترتبط بعامل الجنس وإنما يعوامل بيئية وصحية.

أكدت التحاليل الجزيئية صحة النتائج المخبرية، حيث أظهرت PCR شريطاً واضحاً بحجم يقترب من (٣٥٣) زوج قاعدة بينما أظهرت نتائج تسلسل الحمض النووي تطابقاً كبيراً مع قاعدة بيانات NCBI، مما يعزز دقة التشخيصي. أما اختبارات الحساسية للمضادات الحيوية فقد أظهرت نتائج مثيرة للانتباه، إذ تبين أن الميروبيونيم كان الأكثر فعالية بنسبه ١٠٠٪ ضد جميع العزلات الجرثومية، تلاه الإيميبينيم وبايبراسيلين/تازوباكتام. في المقابل ظهرت معدلات مقاومة مرتفعة للغاية تجاه بعض المضادات الشائعة الاستخدام مثل التريميثوبريم (١٠٠٪ مقاومة في *C. freundii* و ١٨٪

في *E. coli*)، بالإضافة إلى مقاومة واسعة النطاق ضد السيفالوسبورينات من الجيل الأول والثاني (سيفازولين، سيفوروكيسم سيفوروكيسم اكسيتيل) بنسبة تراوحت بين ٨٨٪ و ١٠٠٪.

أوضحت نتائج هذه الدراسة أن الجراثيم السالبة لصيغة غرام وبشكل خاص الإشريكية القولونية والكلبيسيك، ما تزال من أبرز مسببات الإسهال عند الأطفال، كما تكشف عن التزايد الملحوظ في انتشار السلالات متعددة المقاومة للأدوية (MDR)، مما يهدد فعالية العديد من المضادات الحيوية والتقليدية ويصعب من خيارات العلاج. بناءً على ما سبق، توصي الدراسة بضرورة اعتماد برامج وطنية وإقليمية لرصد أنماط المقاومة البكتيرية بشكل دوري، وتعزيز سياسات الاستخدام الرشيد للمضادات الحيوية داخل المؤسسات الصحية، إضافة إلى توعية المجتمع بخطورة الاستخدام العشوائي للأدوية كمل تؤكد النتائج على أهمية التركيز على التدابير الوقائية المستدامة مثل التشجيع على الرضاعة الطبيعية، وتحسين الوضع الغذائي للأطفال، رفع مستوى الوعي الصحي لدى الأمهات، ضمان الوصول إلى مياه شرب آمنه وتطوير شبكات الصرف الصحي. إن تطبيق هذه الإجراءات المتكاملة يشكل حجر الأساس للحد من انتشار الإسهال والوفيات الناتجة عنه وكذلك لمواجهة التحديات المتصاعدة التي تفرضها البكتيريا متعددة المقاومة.



جمهورية العراق
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جامعة الشطرة
كلية الطب البيطري
فرع الاحياء المجهرية



التشخيص الجزيئي والكيميائي الحيوي للبكتيريا سالبة الغرام لدى الأطفال الرضع المصابين بالإسهال في محافظة ذي قار

رسالة ماجستير

رسالة ماجستير مقدمة الى مجلس كلية الطب البيطري/جامعة الشطرة وهي جزء من متطلبات
الحصول على شهادة الماجستير في اختصاص علوم الطب البيطري (الأحياء المجهرية)

من قبل

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