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DETECT DIARRHEAGENIC STRAIN OF ESCHERICHIA COLI IN CHILDREN UNDER FIVE YEARS

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Abstract:
Aim: To determine the type of bacteria in human that cause diarrhea. using the multiplex pcr technique to identify strains
Material method: This study includes100 samples for children under the age of 5, collected from Tikrit Teaching Hospital, the samples were transferred to the laboratory, and all bacterial isolates were diagnosed based on microscopic and phenotypic characteristics and biochemical tests.
Results: E. coli bacteria was present, in addition to the presence of other types. The E. coli bacteria recorded the highest percentage of 59 (47%) of the bacterial isolates followed by Proteus spp. which recorded 36 (26%), while both bacterial isolates of Pseudomonas spp. and Salmonella enterica 18 (13%) and 8 (5%), respectively in the current study, about 78 (56%) stool samples from children under five years of age suffering from diarrhea were analyzed for the detection of E. coli strains. There were 56% of the isolates containing the uid A gene which confirms the detection and finding of E. coli. When determining the pathological patterns of the positive samples by cultural, biochemical tests, and by Multiplex PCR assay for the uid A gene, they were all positive. The type of strains causing diarrhea isolated from children's stool samples is shown in Figure (4-5), where the genes indicate that the strains of E. coli isolates causing diarrhea were ETEC and EHEC.
Among the E. coli isolates detected, 64 (82%) belonged to the ETEC strain and it is the most common. While only 14 (18%) belonged to the EHEC strains
Conclusion: Through these results, it was found that the most important type of bacteria that causes diarrhea in children. Prevalence of enterotoxogenic and shiga strains in Tikrit teaching hospital. The proportion of E. coli bacteria that causes diarrhea is higher than other types in animals.

Keywords: children, bacteria, diarrhea, E. coli.

Introduction

Diarrhea is defined as a pathological condition resulting from a functional defect in the digestive system (1). The normal rate in a watery or loose form leads to the loss of salts such as sodium and potassium from the body, which leads to an increase in the acidity of the blood and muscle contraction (2). The Enterobacter family is one of the most widespread germs in nature, They inhabit the intestines of humans and animals naturally and cause simple or severe infections such as Salmonella, Shigella , and cause gastroenteritis such as E.coli (3).

One of the most important pathogenic intestinal species, Enterobacter, Proteus ,Klebsiella, Salmonella, Yersinia, Escherichia (4).The E.coli germs live naturally in the intestines of humans and animals. At the same time, they are opportunistic germs that cause many diseases such as diarrhea and blood poisoning (5). They are among the most common types of germs that cause intestinal poisoning (6).

The pathogenicity of these germs is due to the possession of many virulence factors, and among these factors is the possession of Shiga Toxin, Siderophores and Cytotoxic Necritizing Factor, and the possession of surface structures such as flagella and capsules, Lipopolysaccharides (LPs) Which gives the bacteria antigenic characteristics, by producing flagellate antigen(H), somatic antigen(O), and capsular antigen(K), and also possesses pilli, fimbriae that help them adhere to the host tissues, thus giving them the ability to form a biofilm (7). During the last decades, E.coli has been associated with many foodstuffs, such as meat, milk and dairy products, eggs, and mayonnaise (8). Germs of E.coli are transmitted from ruminants to humans directly and indirectly through contaminated foods such as uncooked meat and unpasteurized milk, or through direct contact with the animal during slaughter (9). In his view, individual cases or herds, therefore, many studies indicated that the relationship between the E.coli and the host is symbiotic (10). Isolating and diagnosing E.coli that causes diarrhea in children.

Materials and methods

Chemical Materials

The laboratory equipment and supplies used in the present study are grose from Biolab(USA). Green Master mix from Intron Bio /Korea,Primers from Oligomer /Turky DNA extraction Kit from Chelex Bio Rad (USA), Alpha-naphthol from BDH(England). Methyl red from BDH(England) Ethanol from BDH(England),KOH from BDH(England), Red safe from BDH(England), Crystal violet from Biomerieux(Farance), Tetra methyle-para-phrn-dimine dihdrochloride from BDH(England), Urea from Fluka(Swizerland)

Culture Media

- Culture Media from Manufacture(Origin).
- Nutrient agar from Mast Diagnostic (UK).
- MacConkey agar from Mast Diagnostic (UK).
- Eosin Methylene Blue agar from Mast Diagnostic (UK).
- Brain heart infution agar fom Oxoid(UK).

- Brain heart infution broth fom Oxiod (UK).

Specimens Collection

In this study100 childen samples were collected infected, with ages ranging from one month to 5 years , and they were collected from tikrit Emergency hospital . The samples were collected in swabs containing a preservative for the growth of bacteria for a period of 24 hours, sterilized and transferred under refrigerated conditions to the laboratory for laboratory tests and bacterial culture.

Laboratory Diagnosis (Isolation and identification of E. coli)

One colony was taken from each positive culture on MacConkey agar, then it is identified depending on the morphology properties that include colony shape, color , nature of pigments, edge, elevation and texture .The specific shape, type of reaction, aggregation and staining bacteria with Gram stain for microscopic examination (11).The gram negative bacilli were transferred to MacConkey, eosin methylene blue agar (EMB) and chrom agar to clearly differentiate between the colonies of E.coli and other. All plates were incubated aerobically at 37ºC for 24 hrs..

Biochimcal test

The following tests were conducted.

***-Oxidase test**
***-Catalase test**
***-Methyle red test**
***-Indole test**
***-Voges –Proskauer test**
***-Citrate utilization test**
***-Urease test**
***-H2S test**

All bacterial isolates were diagnosed based on microscopic and phenotypic characteristics and biochemical tests after growing them in aerobic conditions in high altitude McConkey medium, EMP, and other media mentioned in a table (3-3) according to Mahon (2007) (11,12), in addition to diagnosis by biochemical tests.

Preservation and maintenance of bacterial isolates

After diagnosis, the bacterial isolates were preserved on slanted culture media from Nutrient Agar at a temperature of 4 °C, and the maintenance process continued on a monthly basis by renewing their culture on new media to ensure that they remain active throughout the study period. This preservation is short-term. As for the long-term preservation of the isolates, a brain-heart infusion broth medium was used to which glycerol was added at a rate of 15 without the possibility of losing some of its genetic characteristics, as a test tube containing 5 ml of the

medium was inoculated with one colony and the culture was incubated for 24 hours, then 0.85 ml of the culture was transferred to bottles With a tight-fitting cap containing 0.15 ml of sterile glycerol. The contents were mixed by turning the tube up and down several times and store the crops at -20 C until use.

Moleculer Biology Experiments

Extraction of DNA

The method used to extract bacterial DNA was Celex100, BIO Rad, USA. For every sample, a tiny quantity of bacterial colonies was extracted and put into a 0.6 mL tube with 200 µL of Celex100 and 100 µL of TE. After 10 minutes, the tubes were put in a water bath at 95 °C. After being eluted, the samples were centrifuged for ten minutes at 13000 rpm. Subsequently, the samples' upper aqueous layer containing DNA was carefully removed and transferred into 0.2 ml tubes. After that, it was kept in a refrigerator at -4 °C.

Multiplex PCR reaction Specific primers used in the

Then the identified genes were amplified by polymerase chain reaction (PCR) using five gene-specific primers (estA, uidA, eltB, vt2, LT, ST, EA, vt1) as previously described in the literature (Saeed et al., 2015; Svenungsson et al., 2000; Nguyen et al., 2005). To accurately identify the virulence factors related to the genotypes of the E. coli strains present in the samples, the study relied on the coding genes obtained by PCR technique. The genes encoding of virulence factors indicate the presence of pathological patterns resulting from E. coli isolates. These genes are: vt1 and vt2 resulting from the gene amplification process that detects type of the EHEC; uidA gene reveals the types of E. coli and ETEC; eltB gene reveals the presence of ETEC. In addition, gene amplification of estA, ST, and/or LT reveals the presence of the ETEC. Furthermore, to identify the types of E. coli strains, the PCR products were separated and the E. coli isolates were electrophoresis on a 1.5% agarose gel containing Ethidium Bromide (EtBr). Then picture taken under ultraviolet (UV) light and obtaining the results as shown in Figure (4-5), (4-6), and (4-7).

	Expected gene size	Primer sequence	Primer	Source
1	322	5-TCTCTATGTGCATACGGAGC-3	LT	Nguyen etal.,2005
		5-CCATACTGATTGCCGCAAT-3		
2	147	5-GCTAAACCAGTAGAGGTCTTCAAAA-3	ST	
		5-CCCGGTACAGAGCAGGATTACAACA-3		
3	376	5-TTCTTGGTGCTTGCGTGTCTTTT-3	BfpA	
		5-TTTTGTTTGTTGTATCTTTGTAA-3		
4	630	5-CTGGCGAAAGACTGTATCAT-3	EA	
		5-CAATGTATAGAAATCCGCTGTT-3		
5	542	5-GAACGTTGGTTAATGTGGGGTAA-3	DaaE	Vidal etal., 2005
		5-TATTCACCGGTCGGTTATCAGT-3		
6	130	5-GAAGAGTCCGTGGGATTACG-3	Vt1	Nguyen etal.,2005

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		5-AGCGATGCAGCTATTAATAA-3		
7	298	5-ACCGTTTTTCAGATTTTGACACATA-3	Vt2	
		5-TACACAGGAGCAGTTTCAGACAGT-3		
8	376	5-CACACGAATAAACTGACTAAAATG-3	Eae	
		5-AAAAACGCTGACCCGCACCTAAAT-3		
9	320	5-CTGGTAGGTATGGTGAGG-3	SHIG	
		5-CCAGGCCAACAATTATTTCC-3		
10	623	5-CCAAAAGCCAGACAGAGT-3	uidA	Moyo etal.,2007
		5-GCACAGCACATCAAAGAG-3		

Microgen south korea

Components of the PCR Brod kit used in multiplex polymerase chain reaction

PCR Prod	µL
Master mix	**25**
primer F	**1(10)**
primer R	**1(10)**
DNA	**3**
D.W	**2**
Total	**50**

.Screening for virulence factor genes using multiplex polymerase chain reaction technology

Gene	PCR Steps			
Uid **V1** **V2** **St** **LT** **BfpA** **EA** **Eae** **SHIG** **DaaE**	95	15 min	1x	
	95 58 72	40 s 1min 40s	35x	
	72	7min	1x	

Antibiotic sensitivity test using disc diffusion method.

Susceptibility testing of E. coli isolates against antibiotics (antimicrobial discs) was performed using the Diffusion Disk method described in reference (Charyeva et al., 2015). The most common antibiotics available in different concentrations were used (Gentamicin (GEN-10 µg), Amikacin (AM-25 µg), Ciprofloxacin (Cip-5 µg), Nitrofurointin (Nit-100 µg), Trimethoprim

(Tri-10 µg), and Rifampicin (Rif-30 µg)). All these antibiotics have been tested on E. coli isolates for ETEC and EHCE. Antibiotic sensitivity was determined and measured by the resistance of bacterial isolates to several different types of antibiotics. This was done by measuring the diameters of the inhibition zones surrounding each antibiotic disc placed on the surface agar for each bacterial isolate, as shown in Figure (4-8). The diameters of zones of inhibition measured according to a specific standard were interpreted as Sensitive or Resistant by comparing the results with the values of standard tables found in the Clinical and Laboratory Standards Institute manual [CLSI 2019].

Results

Identification of bacteria isolated from diarrhea samples

100 sample bacterial isolates were examined and obtained. According to the results of routine biochemical and microscopic tests used in the process of identifying bacteria, the types of bacteria were identified through their phenotypic characteristics of cultural colonies. The E. coli recorded the highest percentage 78 (56%) of bacterial isolates, followed by Proteus spp. which recorded 36 (26%), while both bacterial isolates of Pseudomonus spp. and Salmonella enterica 18 (13%) and 8 (5%), respectively, as shown in Figures (1) and (2).

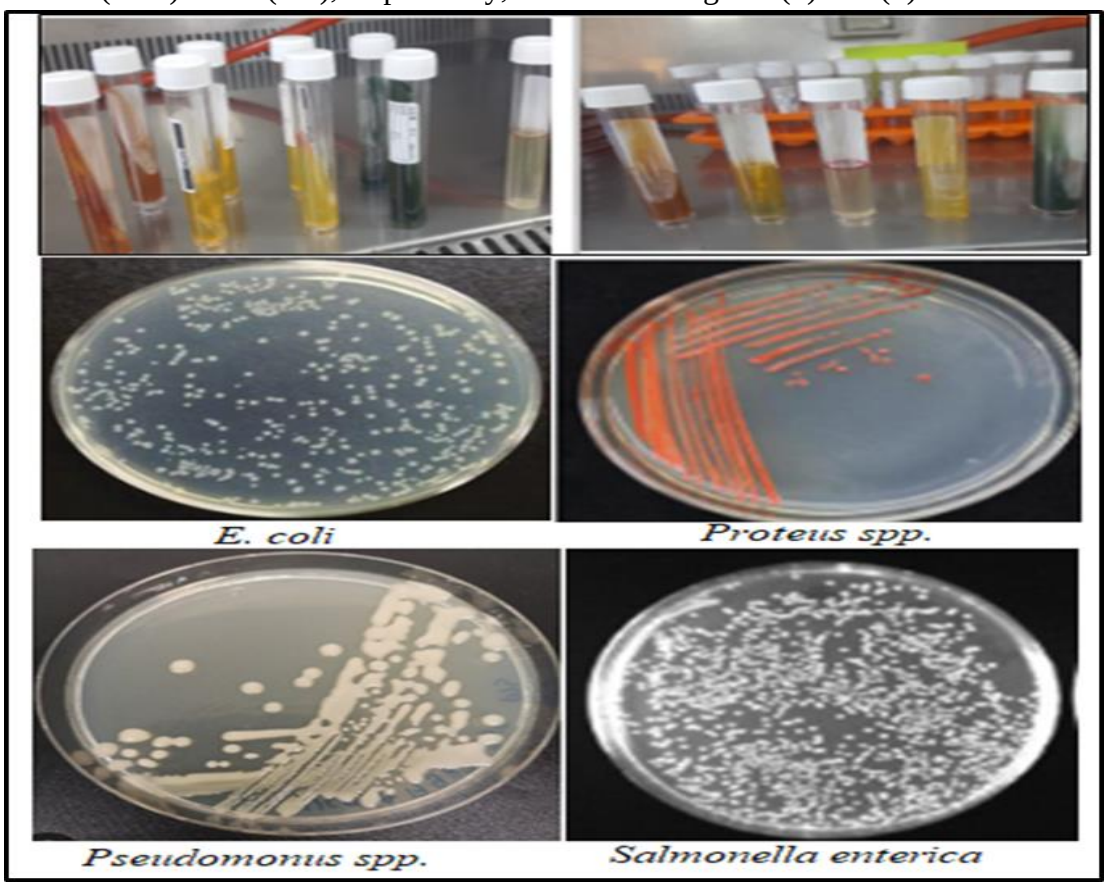

Figure (1): The different colonies of bacteria isolated from diarrhea samples.

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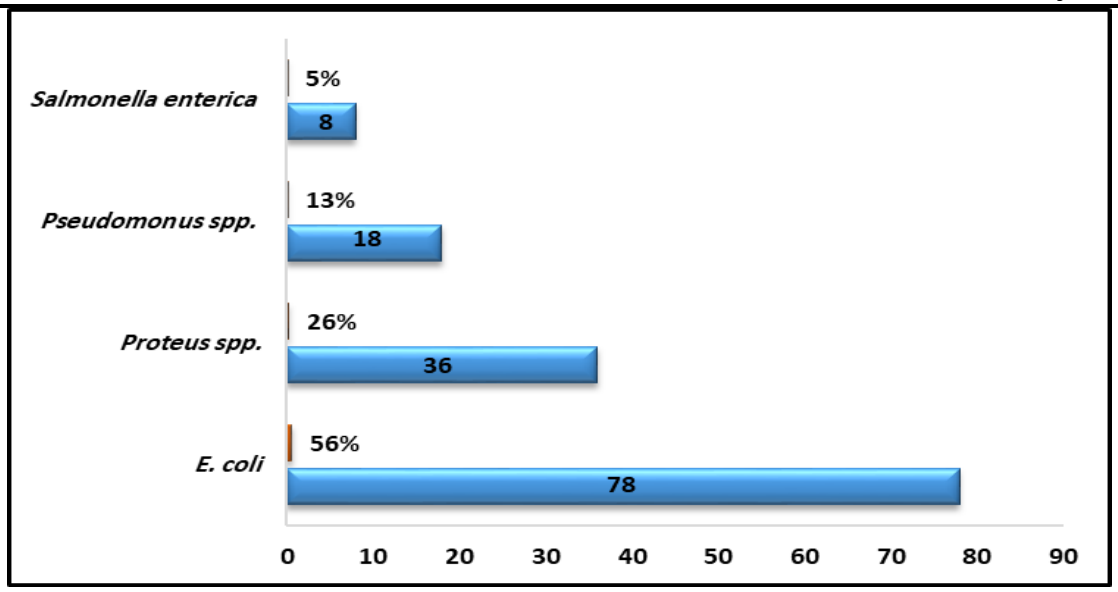

Figure (2): The percentages distribution of bacterial types isolated from Analysis of the table 1 reveals that samples were examined for four different bacterial species. Among these samples, E. coli was the most prevalent at 56%, with 78 isolates. Proteus spp. followed as the second most prevalent at 26%, with 36 isolates. Pseudomonas spp. ranked third at 13%, with 18 isolates. Lastly, Salmonella enterica was the least prevalent at 5%, with only 8 isolates.

Table 1: The distribution of bacterial isolates
diarrhea samples.

Bacterial Species	Number of Isolates	Percentage of Total Isolates
E. coli	78	56%
Proteus spp.	36	26%
Pseudomonas spp.	18	13%
Salmonella enterica	8	5%

Detection of diarrheagenic E. coli strains in in children using Multiplex PCR technique

In the current study, about 78 (56%) stool samples from children under five years of age suffering from diarrhea were analyzed for the detection of E. coli strains. There were 56% of the isolates containing the uidA gene which confirms the detection and finding of E. coli. When determining the pathological patterns of the positive samples by cultural, biochemical tests, and by Multiplex PCR assay for the uidA gene, they were all positive. The type of strains causing diarrhea isolated from children's stool samples is shown in Figure (4-5), where the genes indicate that the strains of E. coli isolates causing diarrhea were ETEC and EHEC.

Among the E. coli isolates detected, 64 (82%) belonged to the ETEC strain and it is the most common. While only 14 (18%) belonged to the EHEC strains as shown in Figure (4-4). Thus, the ETEC was the most common strain, and this result is consistent with the results of previous studies, including a study conducted in Ethiopia and another study conducted in Kenya, where

they reported that the most common strain of E. coli isolates detected in children with diarrhea is the ETEC (Belete et al., 2022; Makobe et al., 2012). This the predominant E. coli strain in children under five years of age in middle- and low-income countries (Roy et al., 2014; Guerra et al., 2014; Belete et al., 2022). In addition, these results were also consistent with the results of many previous studies, as the results they obtained confirmed that there is a possibility of detecting multiple types of E. coli strains that cause diarrhea in the stool samples of children under five years of age (Saeed et al., 2015; Nguyen et al., 2005; Albert et al., 1995; Simuyandi et al., 2019; Omotade et al., 2023; Belete et al., 2022).

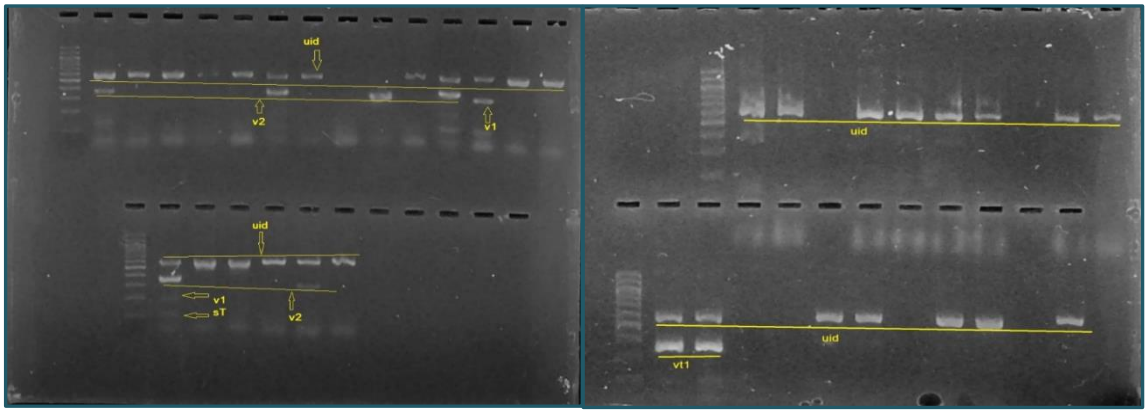

Figure (4-5): Electrophoresis on agarose gel by 1.5% concentration for them PCR reaction product for E. coli isolates from children's samples.

4.8. Antibiotic susceptibility test

The results of the current study showed that the ETEC and EHCE strains of E. coli isolates were 100% resistant to three types of antibiotic drugs (ciprofloxacin, trimethoprim, and rifampicin) as shown in Table (4-1) and Figure (4-8). This means that these bacterial strains have multiple resistance to several antibiotics.

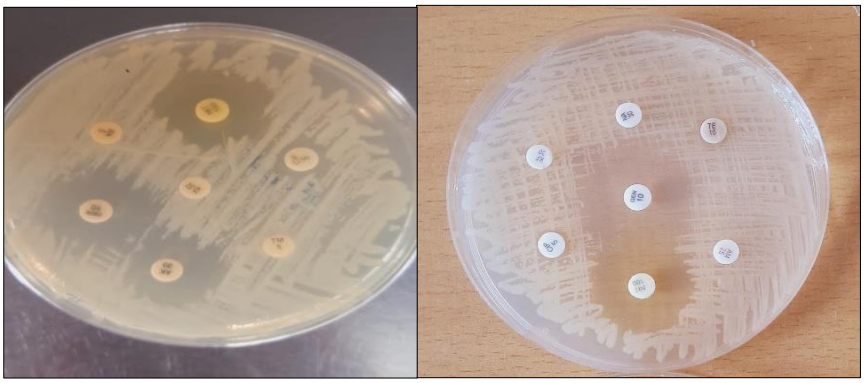

Figure (4-8): Shows the patterns of susceptibility and resistance of E. coli isolates to antibiotics using the Diffusion Disk test.

The results also showed that the ETEC and EHCE strains of E. coli isolates were 85% sensitive to two types of antibiotics (gentamicin, nitrofurontin), while the ETEC strain was 80% sensitive to one type of antibiotic (amikacin). Through these results, it is clear that E. coli isolates (ETEC,

EHCE) have resistance to some of the antibiotics included in the study. These results were consistent with the results of several previous studies that demonstrated the E. coli isolates from diarrhea samples
in children have resistance to many types of antibiotics (Langendorf et al., 2015; Salihi et al., 2023; Omotade et al., 2023; Afum et al., 2022).

Table (4-1): Shows the results of the antibiotic susceptibility test for E. coli isolates using the Diffusion Disk method.

Strains of E. coli isolate	Gentamicin GEN-10 µg	Amikacin AM-25µg	Ciprofloxacin Cip-5 µg	Nitrofurointin Nit-100 µg	Trimethoprim Tri-10 µg	Rifampicin Rif-30 µg
ETEC	24 mm	22 mm	R	25 mm	R	R
EHCE	26 mm	R	R	24 mm	R	R

Finally, the importance of focusing on studying the resistance and sensitivity of E. coli bacteria to antibiotics is evident in the revealing the phenomenon of bacterial resistance to antibiotic drugs. Which may lead to the emergence of many health problems, especially those related to inflammatory diseases and diarrhea. Therefore, the study focused on and search for the factors that cause this type of bacteria to be resistant to many antibiotics

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