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Research Article

Molecular Typing and Antibiotic-Resistance of Diarrheagenic *Escherichia coli* Isolated from Diarrhea-Diseased Children in Wasit Province, Iraq

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About Article

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ABSTRACT

Diarrheagenic Escherichia coli (*E. coli*) is a common zoonotic bacterial pathogen that becomes increasingly resistant to antibiotics, making treatment difficult. Worldwide, almost *E. coli* infections are associated with the consumption of contaminated food and water causing a potential threat to the health and welfare of both humans and animals. Isolation and molecular confirmation of pathogenic *E. coli* from the fecal samples of diarrhea-diseased children, and then, estimation of the antibiotic susceptibility of the study isolates to determinate the antibiotics resistance pattern. A total of 80 fecal samples were collected from diarrhea diseased-children in Wasit province (Iraq) during September to November (2024). All samples were cultivated on McConkey agar, and the pure isolates were sub cultured on Nutrient agar. Targeting the 16S rRNA gene, the fresh positive colonies were confirmed molecularly using the conventional polymerase chain reaction (PCR). Also, the colonies of *E. coli* were inoculated on the Mueller-Hinton agar, and antibiotic susceptibility testing was conducted using the Kirby-Bauer method. An overall 36.25% (29/80) isolates were identified in diarrhea-diseased children. Targeting the 16S rRNA gene, 40.74% (11/29) were shown a positive reaction to pathogenic *E. coli*. Antibiotic susceptibility testing for pathogenic *E. coli* isolates revealed their significant sensitivity to Colisitin, Nitofurantion, Azithromycin, Ciprofloxacillin, Ofloxacine, Imipenem, Meropenem, Levofloxacine, and Nalidixic acid. However, significant high resistance was recorded to Amoxicillin, Amikacin, Ampicillin, Ceftazidime, Ceftriaxone, Cefotaxime and Cefepime; while intermediate resistance was identified to Gentamicin. The findings of MDR revealed that there was significant resistance to one type of Aminoglycosides (Amikacin), two types of Penicillin (Amoxicillin and Ampicillin), and four types of Cephalosporins (Ceftazidime, Ceftriaxone, Cefotaxine, and Cefepime). There is marginal relationship between the isolated pathotypes were isolated in this study and the multiple drug resistance and there is no association between pathotypes, virulence factors, and multiple drug resistance.

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1. INTRODUCTION

The increasing antimicrobial resistance of *E. coli* worldwide demands immediate preventive measures to halt its transmission (Salleh *et al.*, 2022). The public health faces a growing threat because MDR and ESBL-producing diarrheagenic *E. coli* (DEC) strains can no longer be effectively treated by existing antibiotics (Abayneh *et al.*, 2020). DEC strains that produce MDR and ESBL belong to the category of superbugs which defend themselves against two different antibiotic classes while simultaneously breaking down Cefotaxime and Ceftazidime and Ceftriaxone and Cefuroxime and Cefepime and Aztreonam (Parvin *et al.*, 2020). The ESBL-producing DEC isolates mainly carry the blaTEM gene and blaSHV gene as well as blaCTX-M which have its highest occurrence in Enteroaggregative *E. coli* (EAEC) pathotype (Pishtiwan *et al.*, 2019; Dirar *et al.*, 2020). ESBL-producing and MDR DEC have become harder to manage because the transferable resistance genes easily move between bacterial species through mobile genetic elements (Michaelis *et al.*, 2023). These strains can become lethal because there are no currently available quality-assured antimicrobial treatments to combat them. Current information about DEC antimicrobial resistance plays a crucial role in effective treatment to decrease the death toll and increase survival rates among patients with diarrhea (Ayukekbong *et al.*, 2017; Salleh *et al.*, 2022).

2. LITERATURE REVIEW

The emergence of drug-resistant strains globally now poses the central obstacle in treating DEC infections because it reduces the available options for effective antimicrobial drugs for diarrhea treatment. ETEC has been identified as developing resistance to multiple first-line drugs including Ampicillin, Nalidixic acid, Tetracycline, Sulfonamides as well as Azithromycin according to Kariuki *et al.* (2022) and Pokharel *et al.* (2023). When treating non-shigatoxigenic *E. coli* (STEC) the Centers for Disease Control and Prevention (CDC) supports the use of Fluoroquinolones (Ciprofloxacin), Macrolides (Aazithromycin) and Rifaximin (or Rifampicin and other Rifamycin derivatives) although these medications remain contraindicated for STEC due to their possible role in causing hemolytic uremic syndrome (CDC, 2019; CDC, 2023). Many DEC isolates showed resistance against antibiotics in the penicillin classification. The presence of resistant DEC against penicillin antibiotics was substantial yet adding other antimicrobial drugs made DEC more sensitive to amoxicillin and ampicillin. Amoxicillin treatment using clavulanic acid produced a favorable effect by making DEC more responsive to the antibiotic (Urban-Chmiel *et al.*, 2022). Research has shown that DEC showed reduced resistance levels when exposed to ampicillin together with the drug sulbactam according to Madadi-Goli *et al.* (2017).

The treatment of DEC with Piperacillin showed resistance but when Piperacillin was combined with Tazobactam the resistance rates diminished significantly (Mekdad *et al.*, 2020). The use of multiple β-lactam inhibiting antibiotics for treating DEC appears to boost dramatically the pathogen's sensitivity to treatment. The excessive and unwarranted general medical antibiotic usage in most Asian countries leads to high levels of resistance toward bacteria strains challenged by Amoxicillin, Ampicillin and Piperacillin (Bush *et al.*, 2016; Salleh *et al.*, 2022; Narendrakumar *et al.*, 2023).

This study aims to isolate and molecular confirmation of pathogenic *E. coli* from the fecal samples of diarrhea-diseased children, and then, estimate the antibiotic susceptibility of the study isolates to determinate the antibiotics resistance pattern.

3. METHODOLOGY

3.1. Samples and bacterial isolation

A total of 80 fecal samples were collected from diarrhea diseased-children in Wasit province (Iraq) during September to November (2024). The bacteriological loop was soaked in the original bottle's solution before a quantity of the sample was used to create the first streak on MacConkey agar at 37°C for aerobic growth for 24 hours. The bacterial culture on MacConkey agar showed round-shaped, smooth surface colonies with pink color which were suspected to be coliforms so they received subculture on Nutrient agar plates.

3.2. Molecular examination

According to manufacturers' instructions of the Presto Mini gDNA Bacteria Kit (Geneaid, Taiwan), DNAs were extracted from the pus samples and examined by the Nanodrop System (Thermo Sientific, UK). Targeting the 16S rRNA gene, one set of primers [(F: 5´-AGT TGC AGA CTC CAA TCC GG-3´) and R: (5´-TGG TAG TCC ACG CCG TAA AC-3´)] were designed for the current study based on the NCBI-GenBank *E. coli* (LC848137.1) isolate to prepare the Mastermix tubes at a final volume of 20µl using the GoTaq Green Master Mix Kit (Promega, Korea). For PCR reaction, the following conditions of the Thermal Cycler system (BioRad, USA) were followed: 1 cycle for initial denaturation (95°C / 7 minutes); 35 cycles for denaturation (95°C / 45 seconds), annealing (52°C / 45 seconds) and extension (72°C / 45 seconds); and 1 cycle for final extension (72°C / 10 minutes). Electrophoresis of PCR products in Agarose-gel (1.5%) stained with Ethidium Bromide was conducted at 80Am and 100Volt for 90 minutes, and the product size of positive samples was identified under the UV illuminator (Clinx Science, China) at approximately 524bp.

3.3. Antibiotic sensitivity test

The disc diffusion (Kirby-Bauer) method for in vitro antibiotic susceptibility tests was done according to method described by Balouiri *et al.* (2016). The antibiotic disc agents, concentrations, and the interpretation of zones of inhibition for *E. coli* were performed according to the National Committee for Clinical Laboratory Standards (CLSI, 2022). The preparation of test was done by using Mueller-Hinton agar plate that inoculated by 0.5 McFarland tube dilution of bacterial culture which spread by sterile cotton swab. The antibiotics discs were included Amikacin (30µg), Amoxicillin (µg), Ampicillin (µg), Azithromycin (30µg), Cefepime (30µg), Cefotaxime (30µg), Ceftazidime (30µg), Ceftriaxone (30µg), Ciprofloxacillin (10µg), Colisitin (10µg), Gentamicin (10µg), Imipenem (10µg), Levofloxacine (5µg), Meropenem (10µg), Nalidixic acid (30µg), Nitofurantion (30µg), and Ofloxacin (10µg). Then the plate incubated at 37°C for 24 hours. Present or absence zone of inhibition around each of the

disc after the period of incubation was explained antibacterial action and the diameter of zone of inhibition produced by each antibiotic was measured to determinate patterns of antibiotic susceptibility.

3.4. Statistical analysis

Data were analyzed using the One-Way ANOVA in the GraphPad Prism Software (8.0.2), and significant differences were detected at $p<0.05$ (Al-Taee *et al.*, 2023).

4. RESULTS AND DISCUSSION

Among a totally of 80 fecal samples cultures and subcultured on traditional agars, 29 (36.25%) isolates were identified in diarrhea-diseased children (Figure 1). Targeting the 16S rRNA gene, 40.74% (11/29) were shown a positive reaction to pathogenic *E. coli* (Figure 2).

The present study examined the susceptibility of DEC isolates to antibiotics using disc diffusion method. Significantly, the findings revealed that the isolates were resistant to Amoxicillin, Amikacin, Ampicillin, Ceftazidime, Ceftriaxone, Cefotaxime, and Cefepime; while intermediate resistance was identified with Gentamicin. However, significant sensitivity was recorded to Colisitin, Nitofurantion, Azithromycin, Ciprofloxacillin, Ofloxacine, Imipenem, Meropenem, Levofloxacine, and Nalidixic acid. However, the findings of multidrug resistance revealed that there was significant resistance to two types of Penicillin [Amoxicillin (15%) and Ampicillin (15%)], four types of Cephalosporins [Ceftazidime (15%), Ceftriaxone (13%), Cefotaxime (15%), and Cefepime (15%)], and 1 type of Aminoglycosides [Amikacin (12%)], However there is marginally relationship between the isolated pathotypes were isolated in this study and the multiple drug resistance at p-value = 0.074, and there is no association between pathotypes, virulence factors and multiple drug resistance (Table 1, Figure 3).

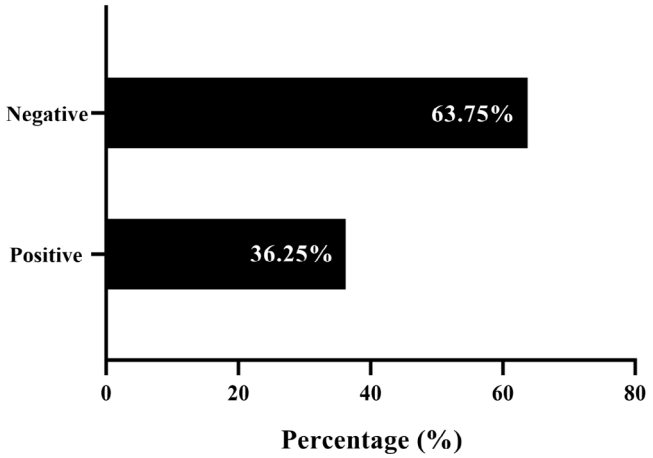

Figure 1. Total results for isolation of *E. coli* from totally 80 fecal samples

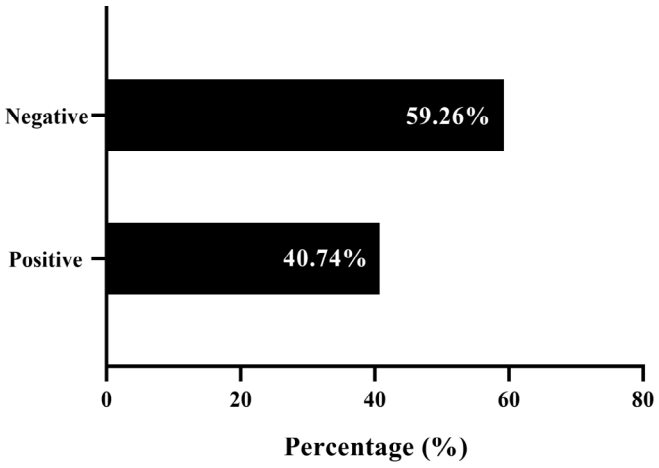

Figure 2. Total results of PCR assay for positive pathogenic *E. coli* from totally 29 study isolates

Table 1. Results of antibiotic susceptibility to DEC

Anti-Biotic Group (Family)	Types of antibiotic	Total No. of tested isolates	Standard zone of inhibition		Susceptibility pattern			p-value
			Resistant	Sensitive	Resistant	Intermediate	Sensitive	
Penicillin (Amino-penicillin)	Amoxicillin	12	≤ 15	≥ 20	12 (100%) **	0 (0%)	0 (0%)	0.009 S
Penicillin (Amino-penicillin)	Ampicillin	12	≤ 10	≥ 14	12 (100%) **	0 (0%)	0 (0%)	0.009 S

Nitrofurans	Polymyxins	Macrolides	Aminoglycosides	Aminoglycosides	Cephalosporin	Cephalosporin	Cephalosporins	Cephalosporins
Nitrofurantoin	Colistin	Azithromycin	Gentamicin	Amikacin	Cefepime	Cefotaxime	Ceftriaxone	Ceftazidime
12	12	12	12	12	12	12	12	12
≤ 14	≤ 10	≤ 12	≤ 11	≤ 14	≤ 17	≤ 17	≤ 14	≤ 20
≥ 17	≥ 11	≥ 15	≥ 15	≥ 17	≥ 25	≥ 22	≥ 20	≥ 21
0 (0%)	0 (0%)	1 (8.33%)	0 (0%)	10 (83.33%) *	12 (100%) **	12 (100%) **	11 (91.67%) *	12 (100%) **
3 (25%)	0 (0%)	2 (16.67%)	9 (75%) *	2 (16.67%)	0 (0%)	0 (0%)	1 (8.33%)	0 (0%)
9 (75%) *	12 (100%) **	9 (75%) *	3 (25%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)
0.0361 S	0.009 S	0.0249 S	0.0361 S	0.0188 S	0.009 S	0.009 S	0.0113 S	0.009 S

p-value	Carbapenems	Carbapenems	Quinolones (Fluoroquinolones)	Quinolones (Fluoroquinolones)	Quinolones (Fluoroquinolones)	Monocarboxylic acid (Quinolone)
	Imipenem	**Meropenem**	**Levofloxacine**	**Ofloxacine**	**Ciprofloxacillin**	**Nalidixic acid**
	12	12	12	12	12	12
	≤ 19	≤ 14	≤ 16	≤ 12	≤ 21	≤ 13
	≥ 23	≥ 18	≥ 21	≥ 16	≥ 26	≥ 19
0.0097 **	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)
0.0082 **	1 (8.33%)	0 (0%)	1 (8.33%)	0 (0%)	0 (0%)	0 (0%)
0.0092 **	11 (91.67%) *	12 (100%) **	11 (91%) *	12 (100%) **	12 (100%) **	12 (100%) *
-	0.0113 S	0.009 S	0.0113 S	0.009 S	0.009 S	0.009 S

S: Significance *

4.1. Discussion

Antibiotics are very useful against pathogenic bacteria and as such the use of these drugs has decreased the rate of death from bacterial infection globally (Nayak *et al.*, 2016). However, because of the misuse and incorrect prescription of antibiotics, genetic factors and environmental factors, the rate of antibiotic resistance is increasing and so is the threat to health (Talebi *et al.*, 2014). Medical professionals do not recommend antibiotic treatment for DEC but knowing antibiotic sensitivities remains essential because *E. coli* in the intestine harbors antibiotic resistance genes (Imdad *et al.*, 2018). Antimicrobial medications considered appropriate when treating children with diarrhea caused by DEC following diagnosis and for persistent diarrhea cases. Research studies by Seidman *et al.* (2016) parallel these

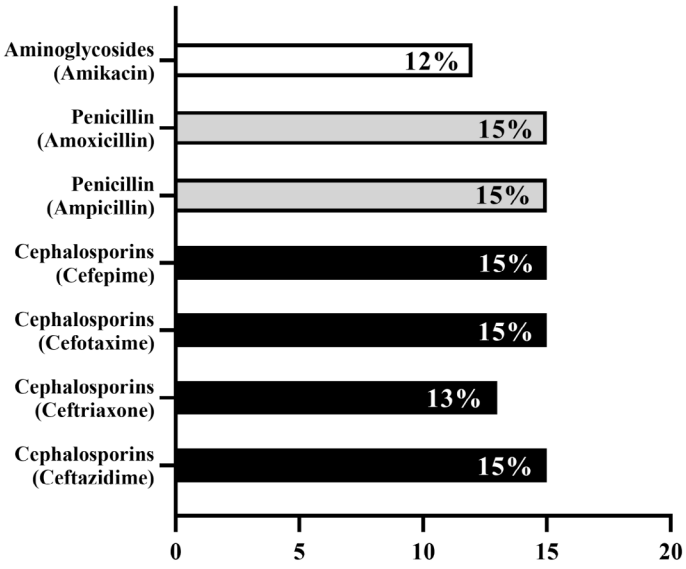

Figure 3. Results of MDR among the diarrheagenic *E. coli* isolates of the current study

findings as DEC becomes resistant to two times more antibiotics (Seidman *et al.*, 2016). This corresponds to laboratory results that demonstrate most DEC isolates show resistance to multiple antibiotics used for pediatric infections (Galindo-Méndez, 2020; Puvača & de Llanos Frutos, 2021; Sora *et al.*, 2021).

These high levels of drug resistance have previously been documented as emerging issues among DEC strains collected from developing nation children and multiple other bacteria species globally, which indicates antibiotic use and such selective pressures have likely driven resistant bacteria evolution (Putnam *et al.*, 2014; Faure, 2023). According to Roy *et al.* (2013) *E. coli* demonstrated significant antimicrobial drug resistance toward treatment agents used clinically to manage diarrhea. All isolated bacteria showed resistance to Ampicillin combined with Imipenem alongside Cotrimoxazole yet remained sensitive to the antibiotics Amikacin. A high percentage of tested *E. coli* isolates demonstrated resistance against Ampicillin, Chloramphenicol, Cotrimoxazole, Imipenem and Nalidixic acid, Norfloxacin according to our study results. The extensive unregulated use of basic and affordable antibiotics throughout our country seems to be the main reason behind this phenomenon.

Resistance to ampicillin and Imipenem occurs through beta-lactamases enzymes whereas resistance to Cotrimoxazole happens mainly because of plasmid-encoded, variant diamino-pyrimidine folate reductase enzymes (Georges *et al.*, 2014). Relevant resistance genes exist either on the chromosome or on plasmids. The emergence of third-generation Cephalosporin resistance represents a critical matter which can be observed in this research. The studies by Taneja *et al.* (2014) show that multidrug-resistant strains are rising in number throughout the years in India. Serving diarrhea with suitable antibiotic treatments both lessens patient death risks and decreases symptom duration. The high occurrence of drug-resistant *E. coli* strains demonstrates an essential problem because health care systems now need expensive second-line medications for effective treatment (Siciliano *et al.*, 2020).

Duy (2018) mentioned that Azithromycin resistant strains were also more frequent in Southeast Asia/India than in Africa and Latin America, with resistance rates of 33.3%, 25%, and 9.1%, respectively, for EAEC and 28.6%, 11.1%, and 0%, respectively, for ETEC. Research data showed that among the Indian and Southeast Asian patient group who visited India, 75% and 71.4% of EAEC strains while 43% and 28.6% of ETEC strains demonstrated resistance to Nalidixic acid and ciprofloxacin and azithromycin, respectively. Statistical tests were not feasible because the sample became too small when strains were divided by pathotype and geographical origin.

During the last decade the increased use of human medicine Colistin emerged because of the development of multidrug resistant Pseudomonas, Klebsiella and Acinetobacter spp. (Gupta *et al.*, 2019; Boyen *et al.*, 2020). Colistin proves to be an antiseptic agent often used in medical treatments and preventive practices against *E. coli* infections because of its solid intrinsic active properties and low resistance development rates as well as its inadequate oral absorption capabilities (Gresse *et al.*, 2021). Only sporadically had *E. coli* strains developed acquired Colistin resistance before yet such occurrences have become more frequent in recent years (Harada *et al.*, 2005; Wang *et al.*, 2008).

5. CONCLUSIONS

Diarrheagenic *E. coli* isolates characterized here were highly resistant to Amoxicillin, Amikacin, Ampicillin, Ceftazidime, Ceftriaxone, Cefotaxime and Cefepime. There is marginally relationship between the isolated pathotypes were isolated in this study and the multiple drug resistance and there is no association between pathotypes, virulence factors and multiple drug resistance.

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