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Prevalence of resistance gene from diarrheal patients caused by *EnteroToxigenic Esherichia coli* in Iraq

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Background: Enterotoxigenic*Escherichia coli* (ETEC) isolates are genetically diverse pathological variants of ETEC defined by the production of heat-labile (LT) and/or heat-stable (ST) toxins, ETEC strains are estimated to cause hundreds of millions of cases of diarrheal illness annually, however, it is not clear that all strains are equally equipped to cause disease, and asymptomatic colonization with ETEC is common in low- to middle-income regions lacking basic sanitation and clean water where ETEC are ubiquitous.

Materials and Methods: This study was carried out at University of Al-Qadsiyah, College of Biotechnology. Eighty samples were collected from child diarrheal Patients (under 6 years old) that has been diagnosed by using Vitek and PCR technique. It included stool samples from Iraqi patients with diarrhea.

Result: In bacterial isolate distribution according to sample collection it was showed that60 of specimens (75%) were Gram negative fermenting bacteria detected as *E.coli*, while 20 of specimens (25%) were another enteric bacteria .Antimicrobial susceptibility test of *E.coli* isolates showed that*E.coli* isolates were more sensitive to Amoxicillin (AMC), Piperacillin-Tazobactum (TZP), Entapenem (ERT), Imipenem (IPM) and Meropenem (MRP). While the

isolates were resist to Ampicillin (AMP), Piperacillin (PR), Ciprofloxacillin (CIP), Norfloxacillin (NOR), Trimethoprem –Sulfamethoxazole (TM-SMX) and Cefotaxime (CTX).The determination of LTI, STa and STb genes gene in *E.coli* isolates using mPCR technique showed that 50% isolates were positive for both LTI and STb genes, while no *Escherichia coli* isolates showed positivity for STa gene.

Conclusion: Our outcomes showed a moderate rate of LTI and STbgenes virulence factor in EnteroToxigenic*Esherichiacoli* which may play a significant role in antibiotic resistance.

Key words: EnteroToxigenic*Esherichiacoli*, Antibiotic sensitivity test, PCR, LTI, STa and STb genes.

1-Introduction

The family Enterobacteriaceae is part of the domain bacteria and there are over 30 genera and 120 species of Enterbacteriaceae but more than 95% of clinically significant strains fall into 10 genera and less than 25 species. Nearly all are facultative anaerobes, they ferment glucose, reduce nitrates to nitrites, and are oxidase negative with the exceptions of *Shigella* and *Klebsiella* which are nonmotile, these bacteria have peritrichous flagella, the *Enterobacteriaceae* include some of the normal inhabitants of the small and large gastrointestinal tracts and, therefore, are sometimes referred to as enterics. However, these terms are not synonymous as some of the species do not live in the gastrointestinal tract and many species in the gastrointestinal tract do not belong to the *Enterobacteriaceae*(1).

The most common species of Enterobacteriaceae are *Escherichia*, *Citrobacter*, *Enterobacter*, *Proteus*, *Hafnia*, *Klebsiella*, *Providencia*, and *Serratia*, and also some of the most important enteric pathogens, such as *Salmonella* spp., *Shigella* spp., *Yersinia enterocolitica*, and pathogenic *Escherichia coli (E. coli*) (2), it is a gram-negative, facultative anaerobic, rod-shaped, coliform bacterium of the genus *Escherichia* that is commonly found in the lower intestine of warm-blooded organisms, most *E.coli*strains are harmless, but some serotypes including enteropathogenicE.coli (EPEC),enterotoxigenicE.coli(ETEC), enteroinvasiveE.coli (EIEC), enteroaggregativeE.coli (EAEC), enterohemorrhagic (EHEC), that have been associated with Crohn's disease, there are also hybrid pathotypes, that carry associated virulence genes (3).

Enterotoxigenic*Escherichia coli* (ETEC) isolates are genetically diverse pathological variants of ETEC defined by the production of heat-labile (LT) and/or heat-stable (ST) toxins, ETEC strains are estimated to cause hundreds of millions of cases of diarrheal illness annually, however, it is not clear that all strains are equally equipped to cause disease, and asymptomatic colonization with ETEC is common in low- to middle-income regions lacking basic sanitation and clean water where ETEC are ubiquitous (4).

The polymerase chain reaction (PCR) is a method widely used to rapidly make millions to billions of copies of a specific DNA in bacterial and human sample, allowing scientists to take a very small sample of DNA and amplify it to a large enough amount to study in detail, PCR is fundamental to many of the procedures used in genetic testing and research, including analysis of ancient samples of DNA and identification of infectious agents, PCR is now a common and often indispensable technique used in medical laboratory research for a broad variety of applications such as detection of virulence genes (5). Recent molecular epidemiology studies using polymerase chain reaction (PCR) have revealed a significant association between strains that produce toxins, it was demonstrate that LT stimulates production of toxins by goblet cells in human small intestine, enhancing the protective barrier between pathogens and enterocytes (4, 6).

2-Materials and methods:

Sample collection

This study was carried out at University of Al-Qadsiyah, College of Biotechnology, Department of Health AL-Qadsiyah. Eighty samples were collected from child diarrheal Patients (under 6 years old) that has been diagnosed by using Vitek and PCR technique. It included stool samples from Iraqi patients with diarrhea. The study was conducted during the period from January 2022 to March, 2023 to investigate the prevalence of EnteroToxigenic*Esherichiacoli* bacteria in Al-Hussein Hospital and from Child and pregnancy women Hospital. Samples were transported to laboratory using special transport media. Growing samples were preserved after that at -20 °C in deep freeze .

Molecular study

Multiplex PCR

The m PCR technique was performed for detection of *E. coli* bacteriafrom samples. This method was carried out according to following steps:

DNA Extraction (7)

DNA from samples were extracted by using Presto™ Bacteria DNA Extraction Kit

Primers

The PCR primers for detection of *Escherichia coli* were designed in this study using NCBI-Gene bank (AB608092.1- MK635343.1) and primer 3 plus design. These primers was provided from Scientific Resercher. Co. Ltd, Iraq as shown in table (1).

Table (1): primers used in this study

Primers	Sequence 5'-3'		Product size
LTI gene	F	**TATCCTCTCTATATGCACAG**	480 bp
	R	**CTGTAGTGGAAGCTGTTATA**	
STa gene	F	**TCTTTCCCCTCTTTAGTCAG**	166bp
	R	**ACAGGCCGGATTACAACAAAG**	
STb gene	F	**GCCTATGCATCTACACAATC**	278 bp
	R	**TGAGAAATGGACAATGTCCG**	

3- Results

Antimicrobial susceptibility test of *E.coli* isolates

A.S.T gave susceptibility of 60 isolates of *E.coli* to 11 different antibiotics. The results were that*E.coli* isolates were more sensitive to the antibiotics of Amoxicillin (AMC), Piperacillin-Tazobactum (TZP), Entapenem (ERT), Imipenem (IPM) and Meropenem (MRP) in a number and percentage of 34(56.7%), 40 (66.7%), 30(50%), 56(93.3%) respectively. While the isolates were resist to antibiotics of Ampicillin (AMP), Piperacillin (PR), Ciprofloxacillin (CIP), Norfloxacillin (NOR), Trimethoprem –Sulfamethoxazole (TM-SMX) and Cefotaxime (CTX)in a number and percentage of 50 (83.3%), 38(63.3%), 32 (53.3%), 58(96.7%), 34(56.7%) and 30(50%) respectively.Few isolates revealed intermediate susceptibility test included Amoxicillin (AMC), Piperacillin-Tazobactum (TZP) and Cefotaxime (CTX) in a number and percentage of 10(16.7%), 4(6.7%) and 2(3.3%) respectively, as showed in table (2).

Table (2): Antibiotics resistance patterns of ***E.coli***isolates

No	Antibiotic disc	Sensitive No (%)	Resist No (%)	Intermediate No (%)
.١	Ampicillin (AMP)	10 (16.7%)	50 (83.3%)	-
.٢	Amoxicillin (AMC)	34 (56.7%)	16 (26.7%)	**10 (16.7%)**
.٣	Piperacillin (PR)	22 (36.7%)	38 (63.3%)	-
.٤	Entapenem (ERT)	30 (50%)	-	-
.٥	Imipenem (IPM)	56 (93.3%)	4 (6.7%)	-
.٦	Meropenem (MRP)	56 (93.3%)	4 (6.7%)	-
.٧	Ciprofloxacillin (CIP)	28 (46.7%)	32 (53.3%)	-
.٨	Norfloxacillin (NOR)	2 (3.3%)	58 (96.7%)	-
.٩	Trimethoprem –Sulfamethoxazole (TM-SMX)	26 (43.3%)	34 (56.7%)	-

.١٠	Piperacillin-Tazobactum (TZP)	40 (66.7%)	16 (26.6%)	**4 (6.7%)**
.١١	Cefotaxime (CTX)	28 (46.7%)	30 (50%)	**2 (3.3%)**

Determination of LTI, STa and STb genes in *E.coli* isolates using PCR technique

In present study sixty isolates of *E.coli* has been tested for the presence of LTI, STa and STb genes, which coding for bacterial resistance, results indicated that from those tested 60 isolates only approximately 50% isolates were positive for both LTI and STb genes, while no *Escherichia coli* isolates showed positivity for STa gene, figure (1).

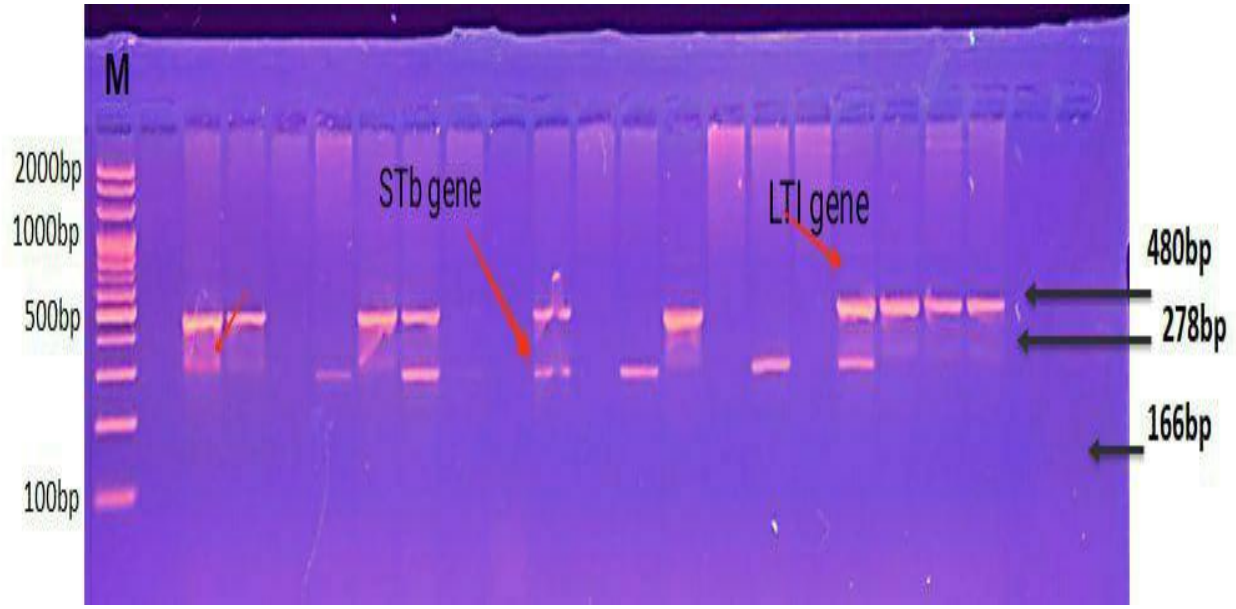

Figure (1): Agarose gel electrophoresis image that showed PCR product analysis of heat labile and heat stable enterotoxin gene in Enterotoxigenic*Escherichia coli* isolates. M (Marker ladder 2000-100bp). Only LTI and STb genes were showed positive in some *Escherichia coli* isolates at (480bp and 278bp PCR product size)respectively.No *E.coli* isolate were showed positive for STa at 166 bp PCR product size

4- Discussion

The results in this study reported that*E.coli* isolates were more sensitive to Amoxicillin (AMC), Piperacillin-Tazobactum (TZP), Entapenem (ERT), Imipenem (IPM) and Meropenem (MRP). While the isolates were resist to Ampicillin (AMP), Piperacillin (PR), Ciprofloxacillin (CIP), Norfloxacillin (NOR), Trimethoprem –Sulfamethoxazole (TM-SMX) and Cefotaxime (CTX).

These records were compatible to Iraqi study conducted by Taha *et al.,*, (2023) and showed that E. coli (ETEC) were characterized and determined their antibiotic resistance in Duhok and Zakho Province. The Isolates of E. coli identified by culture methods were confirmed as ETEC by multiplex PCR of the identified virulence genes, the disk diffusion method performed the susceptibility of antibiotics on the isolated ETEC. Out of 130 examined samples, 39 (30%) isolates of E. coli and 16 (12.3%) ETEC were detected. A high antibiotic resistance rate was observed with total resistance to Amoxicillin/clavulanate, Clarithromycin, Doxycycline, Erythromycin, and Clindamycin. Isolates showed a higher resistance rate when compared with the other sample types ($P \leq 0.05$). Multi-drug resistance was noticed in all ETEC isolates. RTE meat products sold in our area have a high rate of clonally heterogeneous carrying multi-drug resistant ETEC and may constitute a significant public health risk. Additionally the results were i In O consistence with Obasi *et al.,*, (2019) study the analyses of the profile of resistance to *ETEC* bacterial isolates, the profile showed that overall percentage resistance ranges from 0% (ertapenem) to 70.1% (sulfamethoxazole/trimethoprim), susceptibility was low for tigecycline (16.7%) and high for meropenem (94.8%). For the Enterobacteriaceae, the antibiotic-resistance ranged from 34.6% (cefotaxime and ceftazidime) to 75% (ampicillin). And also, for the nonfermenters, the antibiotic-resistance ranged from 1.6% (levofloxacin) to 85.7% (sulfamethoxazole/trimethoprim). All seven *K. pneumoniae* isolates were resistant to ampicillin, piperacillin, ciprofloxacin, gentamicin, and sulfamethoxazole/trimethoprim but remained susceptible to carbapenems (ertapenem, imipenem, meropenem), amikacin, and colistin. Resistance to piperacillin/tazobactam, cefpodoxime, cefotaxime, and ceftazidime was detected in six *K. pneumoniae* isolates. The resistance profile for *E. cloacae* complex isolates ($n = 6$) showed that all were resistant to ampicillin and cefpodoxime, while two were resistant to ciprofloxacin but all were susceptible to β-lactam and β-lactamase inhibitor combination (piperacillin/tazobactam), tigecycline, cephalosporins (cefotaxime, cefuroxime, ceftazidime), aminoglycosides (gentamicin), and carbapenems (ertapenem, imipenem, meropenem).

Afolayan *et al.,*, (2021) indicated that *Escherichia coli* infections are typically attributed to a limited number of lineages that carry virulence factors associated with invasion and, in recent years, invasive *E. coli* are increasingly multiply antimicrobial resistant. *E. coli* is a common cause of infections but the identity of circulating clones is largely unknown

and surveillance of their antimicrobial resistance has been limited. It was verified and whole genome-sequenced 68 bloodstream *E. coli* isolates recovered between 2016 and 2018 at three sentinel sites in southwestern Nigeria and susceptibility tested 67 of them. Resistance to antimicrobials commonly used in Nigeria was high, with 67(100%), 62 (92.5%), 53 (79%) and 37(55%) showing resistance to trimethoprim, ampicillin, ciprofloxacin and aminoglycosides, respectively. All the isolates were susceptible to carbapenems and colistin. Results Of the 383 bacterial isolates, 175 were identified as E. coli. Among the E. coli isolates, Enteroaggregative E. coli (19%; 34/175), Enterohaemorrhagic E. coli (5%; 9/175), and Enteropathogenic E. coli (3%; 5/175). Overall, DEC isolates were resistant to cefazolin (90%; 43/48), ampicillin (83%; 40/48), ampicillin-sulbactam (77%; 37/48), and trimethoprim-sulfamethoxazole (83%; 40/48) but susceptible to amikacin, tigecycline, and carbapenems (100%) (8).

While our findings were disagreed to Edwards *et al.,*, (2020) recorded that resistance to piperacillin/tazobactam (TZP) in *Escherichia coli* has predominantly been associated with mechanisms that confer resistance to third-generation cephalosporins. Recent reports have identified *E. coli* strains with phenotypic resistance to piperacillin/tazobactam but susceptibility to third-generation cephalosporins (TZP-R/3GC-S). The putative resistance mechanisms were equally diverse, including hyperproduction of TEM-1, either via strong promoters or gene amplification, carriage of inhibitor-resistant β-lactamases, and an S133G *bla* $_{\text{CTX-M-15}}$ mutation detected for the first time in clinical isolates. Several of these mechanisms were present at a lower abundance in the TZP-S/3GC-S isolates from the UK-wide collection, but without the associated phenotypic resistance to TZP. Eleven (19%) of the isolates had no putative mechanism identified from the genomic data. Our findings highlight the complexity of this cryptic phenotype and the need for continued phenotypic monitoring, as well as further investigation to improve detection and prediction of the TZP-R/3GC-S phenotype from genomic data, This work highlights the phylogenetic diversity of the TZP-R/3GC-S phenotype in *E. coli* and the variety of the putative resistance mechanisms involved, including β-lactamase hyperproduction via gene amplification and promoter mutations, inhibitor-resistant TEM-1 and CTX-M-15 variants.. An extended spectrum β-lactamase (ESBL) production, mediating resistance to third-generation cephalosporins (3GCs) and other β-lactam antibiotics (9), is of particular concern. ESBLs were recorded in

approximately 11% of *E. coli* isolated from bloodstream infections in the UK in 2018 (10). This differences in outcomes may due to the differences of antibiotics used in each country.

This prevalence of multidrug resistance recorded in this study could be as a result of mutations and the dynamic ability of *E. coli* to exchange genetic-resistance genes through horizontal gene transfer according to (11). An inadequate knowledge of antibiotics and the inappropriate prescription of antibiotics to patients could also be contributory factors for the surge in multidrug resistance in *E. coli* in this study (12). The susceptibility of the *E. coli* isolates to nitrofurantoin and ciprofloxacin might be a result of the reduced prescription and usage of these antimicrobials (13).

In present study sixty isolates of *E.coli* has been tested for the presence of LTI, STa and STb genes, from those tested 60 isolates only approximately 50% isolates were positive for both LTI and STb genes, while no *Escherichia coli* isolates showed positivity for STa gene. To examine secretion of LT, pSTa, and STb by porcine-specific ETEC strains in CAYE, Diff, IEC-conditioned medium, and heat-treated IEC-conditioned medium, bacterial culture supernatants and periplasmic extracts were collected. LT, pSTa, and STb levels were assessed by GM1-ELISA, a competitive ELISA, and Western blotting, respectively, as described, to assess transcriptional changes in ETEC induced by secreted epithelial factors, several genes were evaluated by RT-qPCR(14).

These outcomes were in compatible with Sheikh *et al.,*, (2020) confirmed that ETEC molecular pathogenesis can be viewed as the sum of events that enable these bacteria to engage epithelial cells and ultimately deliver their LT and/or ST toxin payloads. In Jia *et al.,*, (2022) study the 21 *E. coli* strains were classified into 15 ST types, indicating that *E. coli* of Hohhot origin are characterized by genetic diversity and distant affinity among strains. Because of the limited number of tested strains in this study, it was not possible to accurately describe the association between each ST type category and the germline taxa. Genetic target genes of the corresponding pathogenicity species of IPEC were identified for E1–E21, *eaeA*, and *bfp* genes for EPEC; *STa*, *STb*, and *LTI* and *LTII* genes for ETEC. This indicates that the main prevalent pathogenic type of *E. coli*. The strain of Afolayan *et al.,*, (2021) set included isolates from globally disseminated high risk clones including those belonging to ST12 (n=2), ST131 (n=12) and ST648 (n = 4). Twenty-three (33.82%) of the isolates clustered within two

clades. The first of these consisted of ST131 strains, comprised of O16:H5 and O25:H4 sub-lineages. In addition to pandemic lineages, particularly ST131, these include a previously undescribed lineage carrying an O-antigen cluster previously only reported from *Klebsiella*. Genomic surveillance is valuable for tracking these and other clones and for outbreak identification. An array of toxins was detected in *E. coli* clinical isolates including Heat-stable enterotoxins ST-I group b (*sta2)* in 23 isolates (15.33%), EAST1 (*astA*) in 61 isolates (40.67%), Shiga toxin II (*stx2)* in 85 isolates (56.67%), Cytotoxic necrotizing factor I *(cnf1)* in 63 isolates (42%), Vacuolatingautotransporter toxin (*vat)* in 10 isolates (6.67%), and Intimin *(eae)* in 23 isolates (15.33%). None of the isolates harbored Heat-stable enterotoxins ST-I group a (*sta1*), Heat-labile enterotoxin (*eltA)*, or Shiga toxin I (*stx1)*(15).

While present outcomes were not compatible to Vereecke *et al.,*, (2023) showed that ETEC/STEC strains can be distinguished from general *E. coli* by the presence of different host colonization factors (e.g., F4 and F18 fimbriae) and various toxins (e.g., LT, Stx2e, STa, STb, EAST-1). Increased resistance against a wide variety of antimicrobial drugs, such as paromomycin, trimethoprim, and tetracyclines, has been observed. Nowadays, diagnosing an ETEC/STEC infection requires culture-dependent antimicrobial susceptibility testing (AST) and multiplex PCRs, which are costly and time-consuming, the heat-stable enterotoxins STa (*estIa* gene) and STb (*stb* gene) represented a 98%/98% (46/46 true positives/negatives and one false positive and negative each) and 95%/96% (42/48 true positives/negatives and two false positive and negative each) predictive power, respectively . Casey *et al.,*, (1998) conclude that STb does not significantly contribute to diarrhea caused by enterotoxigenic *E. coli* in neonates. In the same way it was recorded that The STb enterotoxin is prevalent in *E. coli* strains isolated from pigs with diarrhea but is rarely found in *E. coli*strains isolated from humans or cattle (16).

Epidemiological study by imply that strains producing ST and/or LT elicit the most severe diarrhea among children in developing countries (17). Two ST variants, *STIa* (*STaI* or *STp*) and *STIb* (*STaII* or *STh*), were found in human ETEC strains (18). Enteroaggragative stable toxin (EAST1) was detected in 40.67% of the tested isolates. EAST1 has been infrequently associated with incidences of diarrhea in animals and humans (19). Moreover, variants of *astA* gene were detected in ExPEC from human and avian origins (20).

It was conducted that predictive estimates for alpha-hemolysis, fimbriae (F4 and F18), and enterotoxins (LT, STa, STb, and Stx2e) were high. The lowest specificity measure (88%) was observed for the hemolysis genes, which might be explained by the fact that alpha-hemolysis is not always easy to interpret on blood agar plates. It is highly dependent on the metabolic state of the strain, which is impacted by repeated passaging and extended incubation times (21). Or the differences in virulence gene percentage may belongs to the site of infection in which sample was taken (22, 23).

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