

OCCURRENCE OF AMPC, MBL, CRE AND ESBLs AMONG DIARRHEGENIC *E. COLI* RECOVERED FROM INFANTILE DIARRHEA, IRAQ.

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ABSTRACT: *Diarrhegenic E. coli (DEC) belongs to the one of the sex pathotypes: Enteropathogenic E. coli (EPEC), Enterohemorrhagic (or Shiga toxin-producing) E. coli (EHEC or STEC), Enterotoxigenic E. coli (ETEC), Enteroinvasive E. coli (EIEC), Enteraggregative E. coli (EAEC) and Diffusely adherent E. coli (DAEC). The risk increased upon acquisition of the β -lactamases like ESBL, AmpC, KPC and MBL. The current study aim to phenotypically investigation of different β -lactamases types among DEC. Fifty eight stool samples were collected from children undergo diarrhea and have fever (38°C- 41°C) with age between (3 months to 4 years). The methods of feeding (Breast feeding or artificial feeding) and geographic area were also recorded. Stool samples collected by transport swab with medium and cultured in the same day on nutrient agar plates. The results revealed high percentage of DEC isolation among stool samples collected from children artificial feeding 33 (78.6%), while 2 (16.7%) positive among breast feeding and this may be due to breast milk secretory IgA, oligosaccharides and lactoferrin have dynamic role preventing adhesion and augmenting the immune response against DEC. Antibiotic susceptibility results revealed high sensitivity of DEC toward amikacin, norfloxacin and netilmicin whereas showed high resistance for the others. For ESBL detection the results display approximate similarity for double disc synergy test (DDST) and ESBL Chromatic medium in which 19(48.7%) and 22(56.4%) positive for ESBL respectively. The positive results for carbapenemase resistant Enterobacteriaceae (CRE) were (10.3%) using MIC strip and (15.4%) when the CRE chromatic medium used. AmpC positive among DEC were (7.7%) while (10.3%) of isolates were positive for MBL using MIC strip test. The current study conclude importance of the natural breast milk feeding for the children to get rid the intestinal pathogens especially diarrhegenic *E. coli* and possessing of diarrhegenic *E. coli* for different types of β -lactamase leads to difficulties in treatments.*

KEYWORDS: ESBL, AmpC, KPC, MBL, Diarrhegenic *E. Coli*.

INTRODUCTION

At present, 6 pathotypes of DEC are commonly recognized. Enterotoxigenic *E. coli* (ETEC) which secrete the enterotoxins LT and/or ST and regards important cause of traveler's diarrhea and diarrhea in children living in developing countries. In order to adhere mucosa, ETEC ETEC express either the colonization factors CFA/I, CFA/II or CFA/IV [1]. The second pathotype is Enteropathogenic *E. coli* (EPEC) which cause prominent histopathology lesion called Attachment-and-effacement (A/E) lesion in the mucosa of the gut [2]. Bundle-forming pilus

(BFP) and intimin α facilitate initial and final adhesion respectively and play a pivotal role in the formation of A/E lesions. Enterohemorrhagic (or Shiga toxin-producing) *E. coli* (EHEC or STEC) express antigenically distinct variants of intimin (intimin γ) and colonize different intestinal sites but also cause A/E lesion [3]. Enteroinvasive *E. coli* (EIEC) cause bacillary dysentery characterized by the destruction of the colonic epithelium. Enteroaggregative *E. coli* (EAEC) is the second most common cause of travelers' diarrhea after ETEC. Intestinal mucosa colonization by EAEC occurs via aggregative adherence fimbriae (AAF). Diffusely adherent *E. coli* (DAEC) cause watery diarrhea that can persist in young children and the adherence to mucosa can be accomplished by two types of adhesions were collectively designated Afa-Dr adhesins [4].

There is a relationship between milk feeding method and infection with DEC. Breastfeeding has evidenced to be the most effective strategy for preventing diarrhea in children. Lactoferrin, aside from the immunoglobulin content, glycocompounds and oligosaccharides in breast milk play a critical role in the innate immunity against DEC [5]. B-lactamases regards a big problem when produced by DEC rendering the infection hard to be or untreatable. The arising of resistance toward extended-spectrum cephalosporins is most often due to hydrolyzing them by extended-spectrum β -lactamases (ESBLs) or due to plasmid-mediated or chromosomally hyperproduced AmpC [6]. AmpC β -lactamases are clinically important cephalosporinases encoded on the chromosomes of many of the Enterobacteriaceae and mediate resistance to cephalothin, cefazolin, cefoxitin, most penicillins, and beta-lactamase inhibitor-beta-lactam combinations. [7].

Emergence of the transmissible metallo- β -lactamases (MBLs) can in itself confer an MDR phenotype, since these β -lactamases can hydrolyze all β -lactams except aztreonam. Also, due to the collocation of genes encoding MBLs and aminoglycoside modifying enzymes on mobile genetic elements, such isolates are also frequently resistant to aminoglycosides [8].

MATERIALS AND METHODS

Samples

Fifty eight stool samples were collected from children resident in Babylon Hospital for Children and Gynecology during a period from October 2013 - January 2014. All children undergo diarrhea and have fever (38°C - 41°C), the age between (3 months to 4 years). The methods of feeding (Breast feeding or artificial feeding) and geographic area were also recorded. Stool samples collected by transport swab with medium and cultured in the same day on nutrient agar plates.

Bacterial Diagnosis

All swabs were cultured on nutrient agar at the same day of collection and incubated at 37°C overnight. Gram stain used for primary diagnosis of the grown isolates and then suspected colonies finally tested for oxidase test and the only oxidase positive isolates then cultured on MacConkey agar for lactose fermentation testing. The lactose fermenter isolates (Pink colony) transferred to EMB agar plates to check isolates with green metallic shin colony which confirmed as *E.coli*. Further confirmation achieved with identification system using Enterosystem 18R kit according to the manufacturer instructions (Liofilchem/Italy).

Antibiotics Susceptibility Test (AST) and Double-Disc Synergy Test (DDST):

Antibiotics susceptibility test were done for all antibiotics disc used in this study according to the protocol of Clinical Laboratory Standards Institute 2013 (CLSI, 2013) [9]. The detection of ESBL was performed according to CLSI (2013) using double-disc synergy test (initial screening test and confirmatory test) as mentioned in the table (1).

Table (1): Screening and Confirmatory test for ESBL detection.

Method	Initial screen test	Phenotypic confirmatory test
Medium	Muller-Hinton agar	Muller-Hinton agar
Disc concentration	Cefotaxime 30µg Or Ceftazidime 30µg Or Cefepime 30µg	Cefotaxime 30µg and Cefotaxime-clavulanic acid 30/10µg Or Ceftazidime 30µg and Ceftazidime-clavulanic acid 30/10µg Or Cefepime 30µg and Cefepime - clavulanic acid 30/10µg
Incubation condition	Standard disc diffusion recommendations	
Results	Cefotaxime ≤ 27mm Ceftazidime ≤ 22mm Cefepime ≤ 14mm Zone above my indicate ESBL production	A ≥ 5mm increase in zone diameter for either antimicrobial tested in combination with clavulanic acid versus its zone when tested alone =ESBL

Chromatic ESBL and Chromatic CRE:

Chromatic ESBL and Chromatic CRE are chromogenic medium used to detect extend spectrum β-lactamase (ESBL) and carbapenemase resistance enterobacteria (CRE) producing isolates. The E. coli that produce ESBL and CRE give pink to red colonies on these media (Liofilchem / Italy).

E-test (for MIC test)

The same procedure as mention above was also used for E-test according to the CLSI 2013 protocol and according to the manufacturer instructions (Liofilchem /Italy). The MIC detection systems used to investigate antibiotics degrading enzyme were:

1-MIC Test Strip for AmpC detection: (CEFOTETAN / CEFOTETAN+CLOXACILLIN) (0.5-32/0.5-32 µg/mL) (CTT/CXT) When the CTT/CXT ratio ≥ 8 the result is positive for AmpC production while when the CTT/CXT ratio < 8 the result is negative.

2- MIC Test Strip for MBL detection: (MEROPENEM / MEROPENEM + EDTA) (0.125-8 / 0.032-2 µg/mL) (MRP/MRD). When the MRP/MRD ratio ≥ 8 the result is positive for MBL production while when the MRP/MRD ratio < 8 the result is negative.

3- MIC Test Strip for KPC (or CRE) detection: (ERTAPENEM / ERTAPENEM + BORONIC ACID) (0.125-8 / 0.032-2 µg/mL) (ETP/EBO). When the ETP/EBO ratio ≥ 8 the result is positive for KPC (or CRE) production while when the ETP/EBO ratio < 8 the result is negative.

RESULTS AND DISCUSSION

Feeding Method and Bacterial Infection:

The results display that 39 (67.2%) of stool samples give positive culture for diarrhegenic *E. coli* (DEC) while 19 (32.8%) negative and this may be due to other diarrheal causes such as viral or parasite. Three types of feeding were recorded in this study: artificial, breast and mixed (artificial and breast) feeding. Children with artificial feeding were the largest group 42(72.4%) followed by breast 12(20.7%) and mixed feeding 4(6.9%). Among artificial feeding 33 (78.6%) of stool samples give positive results for DEC isolation while 9 (21.4%) give negative results. 2 (16.7%) isolates give positive culture while 10 (83.3%) give negative culture among breast feeding while all stool samples 4 (100%) of mixed feeding were positive for DEC table(2). Our results were in agreement with those accomplished in Jordan by [10] who found that 59% of the infants carried DEC in their intestine and 71.7% of infant with milk formula (artificial) feeding give positive results for DEC while 47.9% of those with breast feeding were positive for DEC.

Table (2) Distribution of diarrhegenic *E. coli* according to feeding method.

Feeding type	children	Bacterial Culture	
		Positive	Negative
Artificial	42 (72.4%)	33 (78.6%)	9 (21.4%)
Breast	12 (20.7%)	2 (16.7%)	10 (83.3%)
Mixed	4 (6.9%)	4 (100%)	0 (0.00%)
Total	58 (100%)	39 (67.2%)	19 (32.8%)

The result revealed high percentage of DEC isolation among children with artificial feeding while low percentage among those with breast feeding and these results in accordance with those gathered from other studies [11]. The breast milk components such as secretory IgA, oligosaccharides and lactoferrin play vital role in preventing adhesion and augmenting the immune response against DEC [12].

Antibiotics Susceptibility Test results:

The results display high percentage of resistance of DEC for ampicillin (97.4%), carbenicillin (90%), cefoperazone(89.7%), cefotaxime (89.7%), cefuroxime (84.5%), co-trimoxazole (82%), ceftazidime (79.5%), nalidixic acid (71.8%), cefepime (69.2%), nitrofurantion(61.5%) while show intermediate resistance to gentamicin(51.3%) and tobramycin(38.5%). The low levels of resistance (high sensitivity) of DEC isolates were exhibited to netilimicin(17.9%), norfloxacin(15.8%) and amikacin(12.8%) as shown in figure (1). The results of current study were in similar with those done by [10] in Jordan. Also these results were in agreement with [13] in Iraq.

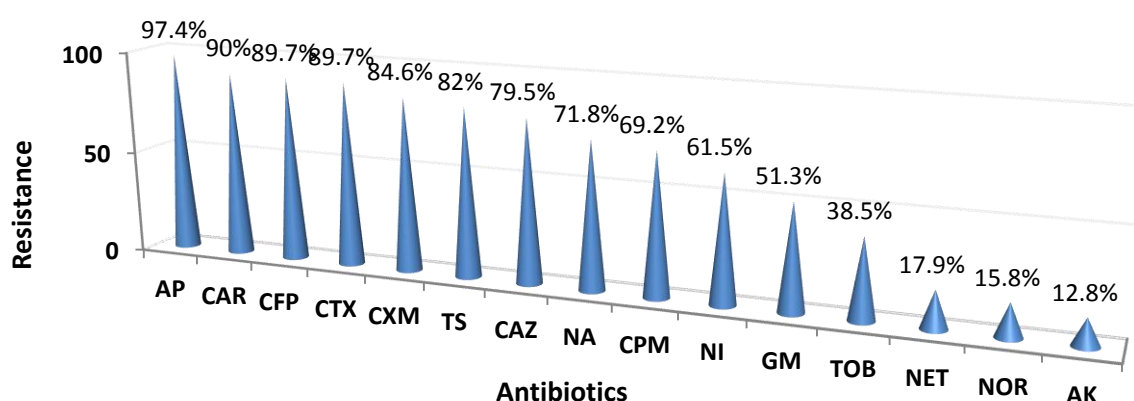


Figure (1) Antibiotics resistance percentage among DEC.

AP= ampicillin (10ug), CAR= carbenicillin(100ug), CFP=cefoperazone(30ug), CTX= cefotaxime (30ug), CXM= cefuroxime (30ug), TS= co-trimoxazole (25ug), CAZ= ceftazidime (30ug), NA= nalidixic acid (30ug), CPM=Cefepime (30ug), NI= nitrofurantion(300ug), GM=gentamicin(10ug), TOB=tobramycin(10ug), NET=netilimicin(30ug), NOR=norfloxacin(10ug), AK=amikacin(30ug).

The resistance to cefotaxime, ceftazidime, cefuroxime and cefoperazone are most often due to the breakdown of the extended-spectrum cephalosporin by extended-spectrum β -lactamases (ESBLs), but it may also be due to plasmid-mediated or chromosomally hyperproduced AmpC. Also this resistance could be interpreted depending on the fact that many strains of *E. coli* have acquired plasmids conferring resistance to one or more than one type of antibiotics. The resistance to Fluoroquinolones, ampicillin, cotrimoxazole, tetracycline and nalidixic acid were also recorded [14].

ESBL Investigation Results

The results revealed that 19(48.7%) were positive for ESBL while 20(51.3%) were negative. The result of ESBL Chromatic medium was 22(56.4%) give red colonies (positive for ESBL) while 17(43.6%) were inhibited (no growth= negative for ESBL) figure (2).

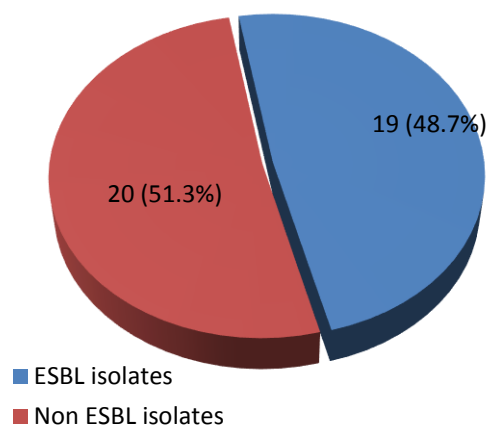


Figure (2) ESBLs distribution among DEC.

Our study results show high percentage of ESBL producing DEC isolates and in accordance with those achieved in the Jordan who found that High incidence of CTX-M ESBL-producing *E. coli* (94.2%) were found in association with fluoroquinolones-resistance and Class I integrons colonizing the intestine of Jordanian infants [10]. The acquisition of resistance genes by horizontal transfer is currently thought to play a major role in the development of multidrug resistant (MDR) strains [15].

AmpC, KPC and MBL MIC (E-test) investigation results:

The results revealed that (2.6%) of isolates were positive for AmpC, (7.7%) of isolates were positive for KPC (CRE) and (10.3%) of isolates were positive for MBL figure (3). CRE chromatic medium result was (15.4%) give red colonies.

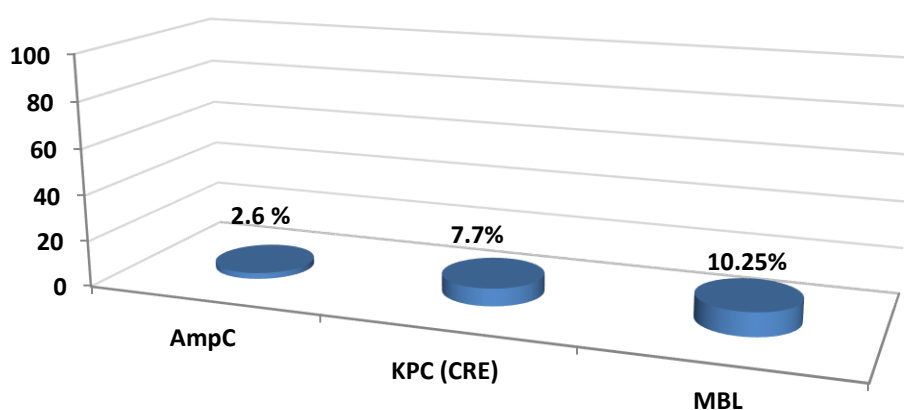
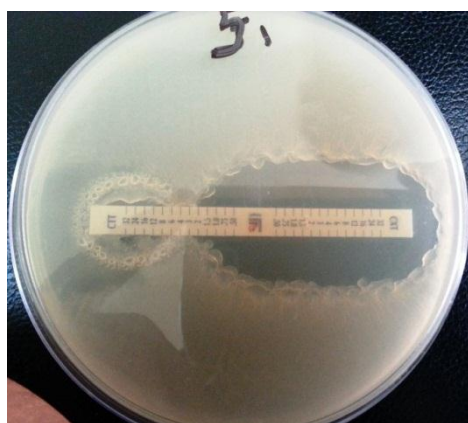


Figure (3) Percentage of AmpC, KPC and MBL among DEC.

AmpC is inducible via a system involving ampD, ampG, ampR, and intermediates in peptidoglycan recycling [16]. The ampC gene of *E. coli* is normally expressed at a low level, regulated by a growth rate-dependent attenuation mechanism but not by induction, since ampR is missing.

Although initial reports described that carbapenem resistance among Enterobacteriaceae was due to overproduction of Amp C-mediated β -lactamases or extended-spectrum β -lactamases (ESBLs) in organisms with porin mutations [17], carbapenemases have now become another mechanism for carbapenem resistance among CRE in the United States. Gram-negative bacteria can sequester and/or evade, eluding the actions of carbapenems and other β -lactams and the common form of resistance is either through lack of drug penetration (i.e., outer membrane protein [OMP] mutations and efflux pumps), hyper production of an AmpC-type β -lactamase, and/or carbapenem-hydrolyzing β -lactamases. Based on molecular studies, two types of carbapenem-hydrolyzing enzymes have been described: serine enzymes possessing a serine moiety at the active site, and metallo- β -lactamases (MBLs), requiring divalent cations, usually zinc, as metal cofactors for enzyme activity [18].

The current study conclude importance of the natural breast milk feeding for the children to get rid the intestinal pathogens especially diarrhegenic E. coli and possessing of diarrhegenic E. coli for different types of β -lactamase leads to difficulties in treatments.



MIC test for AmpC detection (CTT/CXT) MIC Test for CRE detection (ETP/EPO)



MIC test for MBL detection (MRP/MRD)



Double-Disc Synergy Test for ESBL

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