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Research Article

Molecular Mechanisms of Antibiotic Resistance in Diarrheagenic *Escherichia coli* Isolated from the Pediatric Department Zagazig University Hospitals

Nagwa Shawky[1], Ghada El-Akad[2], Eman El-Moghazy[3], Youmna Montaser[4] and Rania Ahmed Ghonaim[5]*

[1]Professor of Clinical Pathology, Department of Clinical Pathology, Zagazig University Hospitals, Egypt

[2]Assistant Professor of Clinical Pathology, Department of Clinical Pathology, Zagazig University Hospitals, Egypt

[3]Professor of Pediatric, Department of Pediatric, Zagazig University Hospitals, Egypt

[4]Master of Clinical Pathology, Department of Clinical Pathology, Zagazig University Hospitals, Egypt

[5]Assistant Professor of Clinical Pathology, Department of Clinical Pathology, Zagazig University Hospitals, Egypt

***Corresponding Author:** Rania Ahmed Ghonaim, Assistant Professor of Clinical Pathology, Department of Clinical Pathology, Zagazig University Hospitals, Egypt.

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Abstract

Background: Diarrhea is considered the second most common cause of mortality in infants worldwide, however due to its neglected clinical manifestations, labor-intensive microbiological diagnosis and epidemiology, it is still a common problem.

The Aim of this Work: Performing multiplex PCR assay to detect the main pathotypes of DEC. Detect the resistance of DEC to beta lactam antibiotics and the molecular mechanism responsible for this resistance.

Patient and Method: All stool samples were plated and the yielded bacterial isolates were identified as *E. coli* using Maldi-Tof (Vitac MS). The identified *E. coli* strains were then exposed to Multiplex PCR to identify the DEC strains genotypically. DEC strains were tested for their antibiotic susceptibility by Vitec-MS and then evaluated for the presence of TEM and SHV genes by conventional PCR.

Results: DEC represented 56 out of 196 *E. coli* strains (28.5%). The most sensitive antibiotics were Imipenem and Aztreonam (96.5% and 89.3% respectively), while the most resistant antibiotics were Amoxicillin and Unasyn showing resistance of (91.1% and 78.6% respectively). TEM gene was positive in 28.6% of cases, while SHV gene was positive in 7.1% of cases..

Limitations for the Study: Including cases above or below the required age. The child has any disease other than diarrhea. The required organism to be tested is *E. coli*.

Conclusion: DEC patho-type distribution was, ETEC: 37.5%, EAEC: 30.4% and EPEC: 32.6%. The most sensitive antibiotics were IPM and ATM (96.5% and 89.3% respectively), while the most resistance antibiotics were AMC and SAM (91.1% and 78.6% respectively). TEM gene was positive in 28.6% of cases and SHV gene was positive in 7.1% of cases.

Keywords: Antibiotics; Resistance; IPM; ATM; AMC; SAM; TEM Gene; SHV Gene; PCR

Abbreviations

DEC: Diarrheagenic *Escherichia coli*; EPEC: Enteropathogenic *E. coli*; ETEC: Enterotoxigenic *E. coli*; MALDI-TOF MS Vitek MS: Matrix-assisted Laser Desorption Ionization–time of Flight Mass Spectrometry

Introduction

Diarrhea remains an important cause of pediatric morbidty and mortality globally especially in developing countries [1]. Although *Escherichia coli* is a commensal bacteria that is a member of intestinal microflora of a variety of animals and humans, some strains are pathogenic and can cause serious and even lethal diseases in human **[2].**

It's difficult to fully distinguish pathogenic *E. coli* from commensal strains using conventional microbiological testing available in developing countries. The sure method is using molecular methods for identification of different pathogenic strains of *E. coli* depending on different chromosomal or plasmid encoded virulence genes, which are absent in the commensal *E. coli* [3].

Inspite of their pathogenesis and virulence, *E. coli* **s**trains also acquire resistance over time. Mobile DNA elements, temperate bacteriophage and transmissible plasmid have all served as carriers for antibiotic resistance genes in *E. coli* [4]. The emergence of antibiotic-resistant bacteria is a major cause of treatment failure in infected newborns [5].

Strains of DEC can be put into six main pathogenic categories. Enteropathogenic *E. coli* (EPEC), its adherence factor (EAF) plasmid is the main virulence detector, its absence classified the genotype as atypical EPEC (a-EPEC). Any *E. coli* strain producing a toxin (Stx) are called "Shiga toxin-producing *E. coli* (STEC)", when containing the locus of enterocyte effacement (LEE), are named Enterohemorrhagic *E. coli* (EHEC) [6].

Enterotoxigenic *E. coli* (ETEC) is another pathogenic category, whose strains are the main causative of watery diarrhea among children in developing countries. In addition to Enteroaggregative *E. coli* [7].

Cultural characters and biochemical criteria are not enough for the identification of DEC. Molecular identification of different strains of DEC is the main method distinguishing DEC from commensal *E. coli*, PCR is the highly specific and sensitive method that gives rapid, reliable results. PCR methodology targeting multiple genes is successfully applied [8].

Aim of the Work

The aim of this research work is to investigate beta lactams antibiotics resistance in Diarrheagenic *E. Coli* isolated from pediatric patients having diarrhea and to identify the molecular mechanisms of this resistance.

Patients and Methods

This study was carried out in the microbiology laboratory in the Clinical Pathology department together with the Pediatric department. 196 children suffering from acute diarrhea (sudden occurrence of three or more, loose watery stool or at least one bloody loose stool in a 24 hour) were recruited in this study and diagnosed to be *E. coli* during the period from March 2016 to January 2017. Their ages ranged from 6 months to 8 years. The patient group included 106 (54.1%) males and 90 (45.9%) females.

Clinical samples

Stool samples collected were processed by routine microbiological tests. MacConkey's agar was used to isolate the pathogens, *E. coli* colonies were elected and identified by using Matrix-assisted laser desorption ionization–time of flight mass spectrometry (MALDI-TOF MS) Vitek MS (bioMerieux, Marcy l'Etoile, France). O157:H7 was screened by sorbitol-Mac. Antibiotic susceptibility test was done by detecting the appropriate antibiotics together with clarifing the existance of resistance using the Vitic.

PCR

Specimens identified as *E. coli* were exposed to real-time muliplex PCR to determine DEC pathotypes genotypically in addition to evaluating the TEM and SHV genes. The optical density (OD) of each sample was measured at 260 nm and 280 nm wavelengths. DNA has a maximum absorbance at 260 nm as the resonance structures of pyrimidine and purines bases are responsible for this absorbance. Proteins absorb maximally at 280 nm due to the presence of tyrosine, phenylalanine and tryptophan. The absorbance at this wavelength is used for detection of protein in DNA samples. If 260/280 ratio is between 1.8 to 1.9, this indicates presence of pure double-stranded DNA. While, if impurities such as protein were present, the ratio would be less than 1.8 [9].

An absorbance of 1.0 at 260 nm gives DNA concentration 50 µg/ml. So, concentration of DNA in sample was calculated according to the following equation given by [10].

Concentration of DNA= OD at 260 nm x 50 µg/ml x dilution factor (100).

The amplified PCR products were visualized by agarose gel electrophoresis as described by [11].

Results

The patient group included 196 patient 106 males (54.1%) and 90 females (45.9%), their ages ranged from 6 months to 8 years with Mean ± SD (2.93 ± 1.72) and median (2.5). DEC represented 56 out of 196 *E. coli* strains (28.57%).

Variable Pathogens	DEC +ve cases (56)
ETEC	21 (37.5%)
EAEC	17 (30.4%)
EPEC	18 (32.1%)

Table 1: Multiplex PCR among the Diarrheagenic *Escherichia coli* cases.

Variable No		(n = 56)	
		%	
AMC	R	51	91.1
	S	5	8.9
SAM	R	44	78.6
	S	12	21.4
CAZ	R	16	28.6
	S	40	71.4
CTX	R	16	28.6
	S	40	71.4
CRO	R	15	26.8
	S	41	73.2
FOX	R	15	26.8
	S	41	73.2
FEP	R	8	14.3
	S	48	85.7
CXM	R	16	28.6
	S	40	71.4
IPM	R	2	3.5
	S	54	96.5
ATM	R	6	10.7
	S	50	89.3

Table 2: Antibiogram among the DEC cases.

This table shows that the most sensitive antibiotics were IPM and ATM (96.5% and 89.3% respectively), while the most resistant antibiotics were AMC and SAM showing resistance (91.1% and 78.6% respectively).

Variable	(n=56)	
	No	%
TEM	16	28.6
SHV	4	7.1
Non TEM non SHV	36	64.3

Table 3: Distribution of TEM and SHV genes among the DEC cases.

Figure 1: Multiplex PCR products on agarose gel stained with Ethedium Bromide.

Lane 1: Molecular size marker (10 bands) ranging from 100-1000 bp.

Lane 2: Negative control (no bands).

Lane 3: Shows 450 bp band of amplified LT gene of ETEC and 900 bp band of amplified PhoA internal control gene.

Lane 4, 6,11: Shows 630 bp band of amplified AA gene of EAEC and 900 bp band of amplified PhoA internal control gene.

Lane 5, 8: Shows 900 bp band of amplified PhoA internal control gene.

Lane 7: Shows 384, 324 bp bands of amplified EAE and BFP genes of EPEC and 900 bp band of amplified PhoA internal control gene.

Lane 9: Shows 450, 190 bp bands of amplified LT and ST genes of ETEC and 900 bp band of amplified PhoA internal control gene.

Lane 10,12: Shows 384 bp band of amplified eae gene of aEPEC and 900 bp band of amplified PhoA internal control gene.

Lane 13: Shows 190 bp band of amplified ST gene of ETEC and 900 bp band of amplified PhoA internal control gene.

Figure 2: TEM gene amplification product detection on agarose gel stained with Ethedium Bromide.

Lanes 1, 3, 4 and 6 shows the 1150 bp band of TEM gene.

Figure 3: SHV gene amplification product detection on agarose gel stained with Ethedium Bromide.

Lanes 2 and 3 shows the 795 bp band of SHV gene.

Discussion and Conclusion

Diarrhea is one of the most common causes of disease and death affecting infants and children especially in developing countries [12]. Many pathogens can be incriminated in infectious diarrhea as viruses, bacteria and parasites. DEC is the most commonly encountered agent among the bacterial pathogens and the most common cause of epidemic diarrhea worldwide [13].

In our study DEC represented 56 out of 196 *E. coli* strains (28.57%), which were positive for DEC virulence factors genes as proved by multiplex PCR. Studies were conducted in India and Texas showing near results to ours (21% and 19% resp.) [7,14]. Our results are also consistent with the results reported by the study conducted in Zagazig city by Allam and his colleagues in 2006, who reported a prevalence of 24% [15]. However, an Indian study show a different prevalence of 6.8% justified by the researchers themselves by the remote location and sparse population of the island [16].

In our study the differential prevalence of DEC pathotypes showed that the most prevalent pathotype was the ETEC (37.5%) followed by EPEC (32.1%) and lastly the EAEC (30.4%). The same findings were reported by the Egyptian studies in 2013 [17] and in 2006 [15]. These results were different from those done by [14,16,18]. It seems that the geographical location and the nature of population greatly affect the differential prevalence of each pathotype.

Meropenem and Imipenem are both members of carbapenems used in the treatment of Multidrug–Resistant bacterial infections [5]. The antibiotic sensitivity profile of the DEC isolates in the current study showed the top sensitive antibiotics were Imipenem (96.4%) and Aztreonam (89.3%). On the other hand the antibiotics to which most of the isolates showed resistance were Amoxicillin-Clavulanic acid (89.3%) and Ampicillin-Sulbactam (78.6%). Additionally there is low prevalence of resistance to Chephalosporins especially the fourth generation drugs, Cefipime, the results suggest a low level of ESBL enzymes production. Same results were reported from studies done in [23] Iran, [19] India, [22] Kenya and [24] Iraq.

Our results are consistent with results reported by different studies, India [19,20,21] Iran, all the three studies reported almost zero% resistance to Imipinem. Regarding aztreonam a study from

Kenya reported low prevalence of resistance among the isolates (15%) [22].

Additionally there is low prevalence of resistance to Chephalosporins especially the fourth generation drugs, Cefipime, the results suggest a low level of ESBL enzymes production. Same results were reported from studies done in [23] Iran, [19] India, [22] Kenya and [24] Iraq.

There is also some disparity between our results and that reported by [20], revealing high prevalence of resistance to some Cephalosporins, namely Cefotaxime and Ceftriaxone (94.7% and 87.9% respectively), which was justified by the researchers themselves as the highest isolation rate of DEC was for EAEC, which is known for its tendency to biofilm formation both *in vivo* and *in vitro* increasing the ability of antibiotic resistance [25].

From the molecular point of view, on exposing DEC isolates in the current study to molecular characterization of ESBL genes, namely TEM and SHV genes, we found that 28.6% of the isolates expressed the TEM gene. These results are consistent with the result reported by the Egyptian study conducted in 2015 that reported 22.5% [26]. Studies conducted in China, South African and Iran reported different results (17% , 17% and 19% resp.) [27-29].

However other studies reported higher prevalence for TEM gene expression in DEC isolates, for example prevalence of 32% in Iran [23], 51% in India [30], 50.9% in China [31], 37.7% in Egypt [32], and 52.6% in Iran [33]. All these studies had something common in their methodology, which is conducting phenotypic testing for ESBL production before testing for the genotypic presence of ESBL genes, unlike the current study, where we exposed all the DEC isolates to genotypic testing directly.

SHV gene expression prevalence in the DEC isolates in our study was 7.1%. This result is almost comparable to other studies' results as an Egyptian study that reported a prevalence of 10% [26] and 7.8% in a Chinese study [34] and 5% in Iran [33]. However an Indian study reported a prevalence of 44.4% for SHV expression [30]. This study also performed phenotypic testing for ESBl production before genotypic testing.

A recent study done in Morocco [35], showed that the high rate of resistance was noted against ampicillin (100%), amoxicillin-

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