Evaluation some virulance factor of Eschirichia coli that causes diarrhea in children by using polymerase chain reaction technique in kirkuk city

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Abstract

The presented study was carried out from May2015 to September 2015 in the Pediatric General Hospital in Kirkuk. A total of 102 samples from children detect some virulance factor of Escherichia coli ,. The identification of these bacteria is depended on several biochemical tests including (indole test , methyl red test , vogas proskaur test , citrate utilization test , urease test, oxidase test and motility test) and the identification was confirmed by using Analytical profile index (API 20 E system) . Antibiotic Susceptibility test was done for these isolates to detect the ability of this bacteria to drug resistance . Finally the genes that are responsible for diarrhea in E.coli isolates (eae gene for EPEC, ial gene for EIEC, lt gene for ETEC,stx gene for EHEC) detected using PCR

. E.coli were the most common pathogen isolated from children (30isolates), and pathogenic Ecoli was 11 isolated ,4 was ETEC, 4EIEC,3EPEC, and absent of EHEC.

Introduction

Infections of the gastrointestinal tract are among the world's leading causes of illness and death among children. Recently such infections were reported to cause more than 3.2 disease episodes per year in children under the age of five in developing countries (1). They have also been estimated to be the third most common cause of death by infectious diseases, only preceded by lower respiratory tract infections and HIV/AIDS (2). Globally 21% of all deaths in children under five years of age are estimated to be due to diarrhoeal infections (1). In recent years studies from several developing countries have shown that diarrhoeal diseases also cause considerable lasting disabilities both in physical growth and fitness, and in cognitive skills and school performance (3).

Escherichia coli was first described as Bacterium coli commune by the German paediatrician Theodore Escherich in 1885. This name was used until the genus Escherichia with the type species E. coli w E. coli strains associated with diarrhea have been classified into six groups, based on clinical, epidemiological and molecular criteria: enteropathogenic E. coli (EPEC), enterohaemorrhagic E. coli (EHEC), enteroinvasive E. coli (EIEC),enterotoxigenic E. coli (ETEC),enteroaggregative E. coli(EAggEC) and diffusely adherent E. coli (DAEC)(4)

The identification of DEC cannot be based only on cultural and biochemical criteria, since they are indistinguishable from the non-pathogenic E. coli commonly found in human feces. Moreover, specific serotyping

is not always correlated with pathogenicity. Since several virulence factors and DNA sequences of DEC have been identified, they can be determined by the presence of genes coding for specific virulence factors, which are absent in non-pathogenic strains. Polymerase chain reaction (PCR) is a commonly used method that gives rapid, reliable results, and shows high sensitivity and high specificity. Several PCR methods, with both single and multiple target genes, have been reported for detecting the different DEC pathotypes.

Aim of study

the aim of this study is to decrease the severity of diarrhea caused by Ecoli ,by detection of some virulance factors of the bacteria using PCR.

Materials and methods

patients

Patients were children with diarrhea attending Pediatric General Hospital in Kirkuk city between May2015 to Septemper 2015.

During the study period, 102 stool samples from children less than 5years old were investigated for DEC. Of them , 82 were with diarrhea and 20 without diarrhea (control group).

The selection criteria for inclusion of children patients with diarrhea was having at least 3 or more soft ,semi solid or liquid bloody faeces with in 24 hours.

Also, selection was made on basis of a questionnaire , providing information regarding other gastrointestinal disorder, frequency of diarrhea episodes, type of feeding , source of water , age and sex , place of residance, previous and current antibiotic treatment, and other associated diseases.

Methods

- **Specimen Collection**: 102 stool samples were collected by sterile cary-Blair transport mediam, from children under five years of age .

General Stool Examination (GSE): This test involves two steps, macroscopic and microscopic examinations. The macroscopic examination of stool sample is done visually according to [7], for consistency (formed, unformed 'soft', or liquid), color (white, yellow, brown, or black), and presence of any abnormal components (mucus or blood). While the Microscopic examination of stool sample is done to demonstrate RBCs, pus cells, Monillia, bacteria, intestinal protozoa, fatty drops, undigested food, and normally small to moderate epithelial cells. The presence of large number of epithelial cells indicates that the intestinal mucosa is irritated [3].

A Stool Culture: Culture of fresh stool specimens remains the standard for determining an etiologic diagnosis [4]. The sample will inoculated on differential culture media (MacConkey agar',, Salmonella – Shigella agar neutrient agar), through the using of streaking plate method technique,followed by overnigh incubation at 37Ċ for 24 hours.

Purification of bacterial isolates

The selection of bacterial colonies fermented sugar lactose that growth on MacConky agar and planning on nutrient agar for the purpose of purification.

Identification of bacterial isolates

1- Microscopic examination

The smear was prepared and fixed on clean glass slide and stained with gram stain by added crystal violet for 30 seconds then smear washed with distilled water. Gram's iodine was added for 10 seconds then the smear was washed with water and decolorized with 95% Acetone alcohol and finally safranin a secondary dye was added to the smear for 30 seconds washed with water, air dried and observed under oil immersion objective(100X).

2-Morphological characteristics

Observed colonies according to size,shape and color and ferment or not ferment lactose.

3-Biochemical tests

Analytical Profile Index (API 20 E System) test:

API 20 E (Analytical profile index) is a standardized identification system for *Enterobacteriaceae* and other non-fastidious, Gram negative rods which uses 21 miniaturized biochemical tests and a database. The complete list of those organisms that it is possible to identify with this system is given in the identification.

Preservation and Maintenance of bacterial isolates:

After identification of bacterial isolates and identification if *Ecoli* , preserved Ecoli by slant method in tube contains nutrient agar (Washington *et al*., 2006). Which commonly used for G+ve bacteria and some G-ve bacteria, and repeated every month for the purpose of active survival for the duration of the study

Antimicrobial Susceptibility Testing:

Disk diffusion test method:

The disk diffusion susceptibility method is simple and practical and has been well-standardized. The test is performed by applying a bacterial inoculum of approximately $1–2\times10^{8}$CFU/mL to the surface of a large (150 mm diameter) Mueller-Hinton agar plate. Up to 12 commercially-prepared, fixed concentration, paper antibiotic disks are placed on the inoculated agar surface. Plates are incubated for 16–24 h at 35°C prior to determination of results. The zones of growth inhibition around each of the antibiotic disks are measured to the nearest millimeter.

Molecular diagnosis of *Escherichia coli*

Genomic DNA isolation

Wizard Genomic purification DNA kit from (PROMEGA , USA) provide fast and easy method for purification DNA for PCR test .

Agarose gel electrophoresis:

It is a method of gel electrophoresis used in biochemistry , molecular biology, and clinical chemistry to separate a mixed population of DNA or proteins in a matrix of agarose (Sambrook *et al*., 2001) .

PCR amplification:

Primer selection The primers were selected to detect several different gene for Ecoli,lt

gene for ETEC,ial gene for EIEC , stx gene for EHEC , eae and bfp gene for EPEC(Elsabeth *et al*,2003),these primers create according to the manufacturer's instructions(Bioner –Korea)

Specific genes, primers, and expected products for PCR assays for analysis of diarrheagenic *Escherichia coli shown table (1).*

PCR premix kit

It is contains DNA polymerase, dNTPs, a tracking dye and reaction buffer in a premixed format, freeze-dried into a pellet. The patented chemical stabilizer of this product enables to maintain the activity of pre mixture for over a month even when stored at room temperature (25oC), over 2 years in the freezer.

PCR procedure:

The PCR consists of a series of 20-40 repeated temperature changes, called cycles, with each cycle commonly consisting of 2-3 discrete temperature steps, usually three, The cycling is often preceded by a single temperature step at a high temperature (> 90 °C), and followed by one hold at the end for final product extension or brief storage. The temperatures used and the length of time they are applied in each cycle depend on a variety of parameters. These include the enzyme used for DNA synthesis, the concentration of divalent ions and dNTPs in the reaction, and the melting temperature of the primers (Rychlik *et al.*, 1990) These steps are: Initialization step,Denaturation step,Annealing step,Extension/elongation step, Final elongation ,Final hold:

Results

Study sample was 102 subject: acute diarrhea 61(59.8%), chronic diarrhea21(20.6%), and non- diarrheal 20(19.6%).

Distribution of the study groups according to direct stool examination results.

Direct stool examination show that Entamoeba H found among 26(31.7%) of the cases as compared with 3(15%) of the control group, Giardia found among 5 (6.1%) of cases and non of the control group, this relation was statistically not significant , as shown in table 2.

Distribution of the study groups according to stool culture results.

Most common infection among cases was E.Coli 28(34.1%), followed by Klebsila 17(20.7%), while among controls was Klebsila 10(50%), followed by no growth 5(2%, this relation was statistically not significant as shown in table 2,3.

Distribution of the study groups according to E coli infection and and age

Among cases about 10(35.7%) of the infected was from 1-12 months, among cases about 6(21.4%) of the infected was from the age group 13-24 months versus 9(16.7%) of the non- infected with E coli, this relation was statistically not significant as shown in table 6.Among controls about 1(50%) of the infected was from the age group 25-36 months versus 5(27.8%) of the non- infected with E coli, and 1(50%) of the infected was from the age group 37-48 months versus 1(5.6%) of the

non- infected with E coli this relation was statistically not significant as shown in table 4.

Distribution of the study groups according to E coli infection gender

Among cases about 18(64.3%) of the infected was female versus 20(37%) of the non- infected with E coli, this relation was statistically significant as shown in table 7.Among controls about 1(50%) of the infected was female versus 6(33.3%) of the non- infected with E coli, and 1(50%) of the infected male versus 12(66.7%) of the non-infected with E coli this relation was statistically not significant as shown in table 5.

Distribution of the study groups according to E coli infection and feeding

Among cases about 13(46.4%) of the infected was bottle feed versus 15(27.8%) of the non- infected with E coli, this relation was statistically not significant as shown in table 8.Among controls 2 (100%) of the infected was mixed feed versus 7(38.9%) of the non- infected with E coli, , this relation was statistically not significant as shown in table 6.

Distribution of the study groups according to PCR results

Among cases ETEC was commonly detected 4(14.3%), followed by EPEC and EIEC 3(10.7%) for each one, while among the controls EIEC was detected 1(50%) and 1(50%) non- pathogenic E coli was detected, this relation was statistically not significant as shown in table 7.

Sensitivity test results of the isolated E coli

Cefotaxime was the most drug that the bacteria sensitive to 20(66.7%), followed by Amikacin 18(60%), while the organism was resistant to Amoxicillin 28(93.3%) and Ampicillin 27(90%) , as shown in table 8

Discussion

Diarrheal diseases remain a leading cause of preventable death,espicially among childreen under five years in developing countries.

Distributio of the study groups according to direct stool examination

The direct stool examination show that *Entamoeba histolytica* and *Giardia lamblia* in 28.4% and 4.9% respectively . E.histolytica was the most common enteric protozoan found in the present study 28.4% in cases and control groups, this result in close to reports from Erbil[5,6and7].which was 39.1% ,39.7% and 6.4% respectively

Distribution of the study groups according to stool culture results

In stool culture result the most frequent isolated pathogen was *Ecoli* 29.40% and Klebsila 26.5%, this result is agreement with study in Chad [8] which was the isolation rate for Ecoli 34.3%, similar result reported in Iran [9,10].and also in senegal[11].

Distribution of the study groups according to Ecoli infection and age

Their was prevalence of diarrhea in early age group in children aged 0-2 years which was more than 50% as reported in Baghdad [12,13].

Similar to Palestinian report [14] ,our study showed that bacterial infection was significantly higher in children less than two years old .

Distribution of the study groups according to Ecoli infection and and gender

In this study aslight higher microbial infection rate were recorded among boys than girls 53.7% and 46.3% ,this result agreed with other studies performed in Erbil [15] and Saudia Arabia [16], this slightly increasing may be due to boys more involved in out and indoor activities than girls.

no difference between male and female in infected with Ecoli ,that agreement with other studies done in Bagdad and India.[17,18].

Distribution of the study groups according to Ecoli infection and and feeding

In the present study ,out of 30 infected cases and control with Ecoli , about 15 of these cases and control with mixed feeding ,these finding therefore corroborate findings from previous study in [19,20] .

Distribution of the study groups according to pathogenic Ecoli and PCR results.

Results showed prevalence of pathogenic Ecoli 11(36.7 %) of the total 30 infected with Ecoli , this result is similar with other reported in Oman [6] and in Iraq [17]. India, Chile and Peru [18,21,22]

Most common type of diarrheagenic *E. coli* incases was the ETEC with elt gene 4 (14.3%). This study is approximately similar to those reported by Qadri[23], in which they found that the rate of ETEC was 18.5 and 18%, respectively.

The prevalence of EPEC in this study study with eae gene was 3(10.7%) among all patients. Result agreed with study in Iraq , 12.5% by Al-Kaissi [24]and 13 % by Tawfeek[25]

The prevelance of EIEC in this study with ial gene was 3(10.7%)in cases and 1 in control ,this result agreed with other study in Kenya[26]

Sensitivity test results of the isolated Ecoli.

The treatment with antibiotics can lead to shorter disease period in children with diarrhea , in this study ,most antibiotic resistance to Ecoli was amoxicillin 93.3%, ampicillin 90.0 %, ofloxacine 76.7% and sensitive to amikacin 60.% and cefotaxime 66.7% . this study is similler to study done by Turkey in Anbar [27], which found that all isolation strains were sensitive to amikacin and imipenem antibiotics while it was found that all the isolation strains were resistance to ampicillin 83% , amoxicillin 97% .

Similarly to Mitra in Iran [28] , which found that *E.coli* was resist 100% to ampicillin , 84.3% to cephalothin , and 74.5% to ceftrixone .

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Table (1) Specific genes, primers, and expected products for PCR assays for analysis of diarrheagenic *Escherichia coli*

Pathotype	specific gene	primers(5'→3')	Product size
EPEC	eaeA	F: GAC CCG GCA CAA GCA TAA GC, R: CCA CCT GCA GCA ACA AGA GG	384
ETEC	Lt	F: GGC GAC AGA TTA TAC CGT GC R: CGG TCT CTA TAT TCC CTG TT	450
EHEC	Stx	F: CTG GAT TTA ATG TCG CAT AGT G R: AGA ACG CCC ACT GAG ATC ATC	150
EIEC	Ial	F: CTGGATGGTATGGTGAGG R: GGA GGC CAA TTA TTT CC	320

Table 2 Distribution of the study groups according to direct stool examination results.

Direct stool examination		Study groups		Total
		case	control	
	Entamoeba H	26 31.7%	3 15.0%	29 28.4%
	Giardia	5 6.1%	0 0.0%	5 4.9%
	Normal	51 62.2%	17 85.0%	68 66.7%
Total		82 100.0%	20 100.0%	102 100.0%

X2= 4.05, df=2, P value>0.05 NS

Table 3 Distribution of the study groups according to stool culture results.

stool culture	Study groups		Total
	case	control	
Klebsila	17 20.70%	10 50.00%	27 26.50%
Proteus	9 11.00%	2 10.00%	11 10.80%
E. coli	28 34.10%	2 10.00%	30 29.40%
No growth	17 20.70%	5 25.00%	22 21.60%
Staph. areus	3 3.70%	0 0.00%	3 2.90%
Shigella	1 1.20%	0 0.00%	1 1.00%
Psedomonous	7 8.50%	1 5.00%	8 7.80%
Total	82 100.00%	20 100.00%	102 100.00%

X2= 9.77, df=6, P value>0.05 NS

Table 4 Distribution of the study groups according to E coli infection and study groups and age.

Study groups			culture code			X2, P value
			Infected with E. coli	Not infected	Total	
case	age in months	1-12	10 35.7%	21 38.9%	31 37.8%	1.3, > 0.05 NS
		13-24	6 21.4%	9 16.7%	15 18.3%	
		25-36	5 17.9%	13 24.1%	18 22.0%	
		37-48	4 14.3%	8 14.8%	12 14.6%	
		49-60	3 10.7%	3 5.6%	6 7.3%	
	Total		28 100.0%	54 100.0%	82 100.0%	
control	Age	1-12	0 .0%	3 16.7%	3 15.0%	5.2, > 0.05 NS
		13-24	0 .0%	5 27.8%	5 25.0%	
		25-36	1 50.0%	5 27.8%	6 30.0%	
		37-48	1 50.0%	1 5.6%	2 10.0%	
		49-60	0 .0%	4 22.2%	4 20.0%	
	Total		2 100.0%	18 100.0%	20 100.0%	

Table 5 Distribution of the study groups according to E coli infection and study groups and gender

Study groups		culture code			X2, P value
		Infected with E. coli	Not infected	Total	
case	Male	10 35.7%	34 63.0%	44 53.7%	5.5, < 0.05 S
	Female	18 64.3%	20 37.0%	38 46.3%	
	Total	28 100.0%	54 100.0%	82 100.0%	
control	Male	1 50.0%	12 66.7%	13 65.0%	0.2, > 0.05 NS
	Female	1 50.0%	6 33.3%	7 35.0%	
	Total	2 100.0%	18 100.0%	20 100.0%	

Table 6 Distribution of the study groups according to E coli infection and study groups and feeding

Study groups			culture results			X2, P value
			Infected with E. coli	Not infected	Total	
case	Feeding	Bf	4 14.3%	17 31.5%	21 25.6%	6.1, > 0.05 NS
		bottle	4 14.3%	14 25.9 %	18 22.0%	
		Mixed	13 46.4%	15 27.8%	28 34.1%	
		Solid	7 25.0%	8 14.8%	15 18.3%	
	Total		28 100.0%	54 100.0%	82 100.0%	
control	Feeding	bottle	0 0.0%	4 22.2%	4 20.0%	2.7, > 0.05 NS*
		Mixed	2 100.0%	7 38.9%	9 45.0%	
		Solid	0 .0%	7 38.9%	7 35.0%	
	Total		2 100.0%	18 100.0%	20 100.0%	

Table 7 Distribution of the study groups according to PCR results

PCR	Study groups				Total
	case	gene	control	gene	
EPEC	3	eae	0		3
	10.70%		0.00%		10.00%
ETEC	4	elt	0		4
	14.30%		0.00%		13.30%
EIEC	3	ial	1	ial	4
	10.70%		50.00%		13.30%
Negative	18		1		19
	64.30%		50.00%		63.30%
Total	28		2		30
	100.00%		100.00%		100.00%

Likelihood Ratio= 2.36,df=3,P value> 0.05

Table 8 Sensitivity test results of the isolated E coli

Antibiotic	Dissk code	disk potency in Mcg	Zone size in mm	Resistant		Intermediate No. (%)		Sensitive	
				No.	(%)	No	(%)	No	(%)
Ciprofloxacine	cip	10	23	3	10.0	10	33.3	17	56.7
Ampicillin	AM	10	0	27	90.0	3	10.0	0	0.0
Ofloxacine	ofx	5	19	23	76.7	6	20.0	1	3.3
Amikacin	AK	10	16	1	3.3	11	36.7	18	60.0
Amoxicillin	AX	25	0	28	93.3	2	6.7	0	0.0
gentamycine	GN	10	14	2	6.7	18	60.0	10	33.3
Tobramycine	Tab	10	15	5	16.7	17	56.7	8	26.7
Cefotaxime	CTX	30	22	3	10.0	7	23.3	20	66.7