Molecular Study to Detection Diarrheagenic E. Coli in Infant Children

Marwa Nassir AL-Lamy[1]*, Khairi Jameel Al-Ruaby[2]
[1,.2]Department of Biology, College of Science, Waist University, Iraq.
E-male: Marwanassir51@gmail.com

Abstract
Diarrheagenic Escherichia coli are a major cause of diarrhea diseases in both developing and developed countries. Furthermore, in developing countries, Diarrheagenic E. coli are responsible for a high morbidity of diarrheal cases and mortality in children under 5 years of age. A total of 177 diarrheal samples were collected from children under five years Range from 5 days to 2 years suffering with Acute watery diarrhea who attended to Al–Kut Hospital for Gyneclogy and obstetrics and pediatrics during the period from 15 October 2021 to 15 April 2022. All isolates were identified according to their morphological characteristics, biochemical tests and confirmed by VITEK 2 system. Results were showed that 85 /177 (48.02%) isolates were identified as Escherichia coli, while 54(30.5 %) isolates belong to other bacteria, by PCR Diarrheagenic E. coli were detected in 41.1 % (35 /85). The distribution of 35 DEC pathotype isolates were: EPEC was found in 40% (14/35), EAEC in 37.14% (13/35), ETEC in 11.42% (4/35), EIEC in 11.42% (4/35) and 0% (0/35) in STEC. The Distribution of the Diarrheagenic E. coli strains was in males 13 (37%) and females 22 (62%), the rate of infection in females was greater than males, statistically this difference was significant($P<0.05$), while the ages groups were distributed as 5D-12M (65%) and (34%) at 13-24 month. The Diarrheagenic E. coli was present in formula-fed infants in a significant percentage 19/35 (54.2%). (It is the highest percentage) and in infants dependent on natural feeding (breast-fed), the percentage showed the lowest where it was 5/35(14.2%).
Keywords: Escherichia Coli, Diarrhea, PCR, Breast feeding

1. Introduction

Diarrheal disease is the leading infectious cause of childhood morbidity and mortality, most commonly occurring in sub-Saharan Africa and Asia. Although many studies worldwide have reported Rotavirus to be the primary cause of acute diarrhea in children, the role of bacteria in causing diarrhea appeared to differ depending on the geographical area (1). Diarrheagenic Escherichia coli (DEC) is the leading cause of bacterial pediatric diarrhea in developing regions and has been suggested to frequently occur in young children. Results from a study in Iraq revealed DEC to be the most common bacterial pathogen among children younger than 5 years of age (2). On the basis of specific virulence properties, DEC can be classified into 6 major categories: enteropathogenic E. coli (EPEC), enteroaggregative E. coli (EAEC), enterotoxigenic E. coli (ETEC), enteroinvasive E. coli (EIEC), Shiga toxin-producing E. coli (STEC), and diffusely adherent E. coli (DAEC) (3). Enteropathogenic E. coli is associated with the development of a characteristic intestinal histopathological lesion, known as attaching and effacing (A/E), the adherence on cells mediated by intimin this intimin protein coded by eae gene which generates a condition of watery diarrhea, it subdivided to typical and atypical according to presence or absence of bundle-forming pilus (4).
Enteroaggregative E. coli, the main mechanism of induce infection started with attachment on intestinal mucosa by aggregative adherence fimbriae and it's under the control of agg gene (5), in addition there is other secreted proteins that play important role in pathogenicity (6). Enteroinvasive E. coli is a unique from other strains because it is intracellular pathogens can multiplicate inside colon cells and induce bacillary dysentery,this invasion mediated by presence of virulence plasmid (Pinv) (4).
Enterotoxigenic E. coli is the major cause of children and traveler's diarrhea acquired by tourists visiting developing nations. The main mechanism that induces pathogenicity mediated through colonization the bacteria in intestine by specific colonization factors, as well as secretion of heat-stable and heat-labile enterotoxins (6). Shiga toxin-producing E. coli is important food borne pathogen, presence virulence factors like stx genes, locus of enterocyte effacement and high molecular weight plasmid that coding for a hemolysin, all contribute to induce bloody diarrhea (7).

2. Materials and Methods

A total of 177 diarrheal samples were collected from children under five years Range from 5 days to 2 years suffering with Acute watery diarrhea who attended to Al–Kut Hospital for Gyneclogy obstetrics and pediatrics during the period from 15 October 2021 to 15 April 2022. The collected specimens were streaked directly on MacConkey agar and positive colonies (lactose fermented) were purified on EMB agar, incubated at 37°C for 24 hrs. The identification of the isolate included morphological characteristics and biochemical testes which carried out depending on Bergy's Manual of Systematic Bacteriology, 2nd

doi.org/10.31838/hiv22.02.564 Received: 23.07.22, Revised:04.09.22, Accepted: 09.08.22

edition. Epi20E, Vitek 2 system and molecular detection was employed to confirm the identification.

Molecular detection Bacteria genomic DNA Extraction

Genomic DNA was extracted from E. coli by using Geneaid presto mini gDNA Bacteria Kit according to company's instructions.

Genomic DNA investigation

The extracted DNA was examined by using Nanodrop which measure the DNA concentration (ng/µL) and examined the DNA purity by reading the absorbance at (260 /280 nm) as following stages

1- After starting up the Nanodrop software, select the appropriate application (Nucleic acid, DNA.(

2- A dry wipe, was taken and cleaned the measurement sites several times. Then carefully pipette 2µl of free nuclease water on to the surface of the lower measurement pedestals for blank the system.

3- The sampling arm was lowered,and clicking OK to initialize the Nanodrop, then cleaning off the pedestals and 1µl of DNA was added to measurement.

PCR Thermocycler conditions

PCR cycling program parameters used in this reaction for detection of (eaeA, aggR, invE, stx1, elt,) genes were shown in table (1).

Table (1): Thermal cycling program for Virulence genes

Step	Temperature	Time	No. of cycles
Initial denaturation	95°C	5 min.	1
Denaturation	94°C	30 sec	35
Annealing	58 °C	30 sec.	
Extension	72°C	1 min.	
Final extension	72°C	7min.	1
Holding	4°C	∞	1

Gel Electrophoresis of PCR product

The following steps were tested for PCR products of each gene using the agarose gel Electrophoresis method:

1.5% Agarose gel was prepared using 1X TBE and dissolved for 15 minutes in a water bath at 100 ˚C, after which 45˚C was left to cool.

In the agarose gel solution, 5µL of,SafeView Classic stain was then applied. After fixing the comb in the correct position, the agarose gel solution was poured onto the tray and allowed to solidify for 15 minutes at room temperature. then the comb was gently removed from the tray and 10µl of PCR product was applied to each comb well and 5ul (100bp Ladder) in one well. In the electrophoresis chamber, the gel tray was fixed and filled with a 1X TBE buffer. The electrical current was then carried out for 40-45 min at 100 volts.Using ultraviolet trans illuminators, PCR goods have been visualized.

3. Statistical Analysis

The data of the present study was analyzed statistically by statistic package for social science (SPSS) version 27 program using chi-square test (X 2) and two-way ANOVA & Least significant differences (LSD). The level of significance was set to 5%. P<0.05 was considered significant while P>0.05 was considered as non-significant (8).

4. Results

Diarrheagenic E. coli Diagnosed by PCR

The DNA of 85 isolates from diarrheal cases and 30 from control were extracted. Purity and concentration were confirmed with Nanodrop, the results listed in table in appendix 3, the purity of E. coli isolates between ~ (1.8-2) and concentration were between 50-360 mg/µl, the intact DNA bands were confirmed through gel electrophoresis. Diarrheagenic E. coli were detected in 41.1 % (35 /85) among diarrheal children compared with 0% (0/30) among control children. The results of PCR amplification of 35 DEC pathotype isolates were: EPEC was found in 40% (14/35), EAEC in 37.14% (13/35), ETEC in 11.42% (4/35), EIEC in 11.42% (4/35) and 0% (0/35) in STEC. The frequency of virulence gene in the Diarrheagenic E. coli pathotypes shown in table (2).

Table (2) The frequency of genes in Diarrheagenic E. coli pathotypes (35 isolate)

Diarrheagenica *E. coli* Pathotypes	Genes	Frequency of virulence Gene	%
EPEC	EaeA	14	40
EAEC	AggR	13	37.14
ETEC	Elt	4	11.42
EIEC	InvE	4	11.42
STEC	Stx1	0	0
X2	34.64		
P value	0*		
ignificantly difference at P<0.05			

Distribution of the Diarrheagenic *E. coli* strains with age of studied children.

The infants ages are 5 days old to 2 years, this study included Therty five (n =35) DEC infected infant's patients were investigated which included males 13 (37%) and females 22 (62%), the rate of infection in females was greater than males, statistically this difference was significant(P<0.05), while the ages groups were

distributed as 5D-12M (65%) and (34%) at 13-24 month as showed in Table (3).

Table (3): Gender and Age Groups				
Parameters		N (n=85)	%	X^2 / P value
Gender	Male	13	37.14	4.62/0.031*
	Female	22	62.85	
Age groups day/month	5d – 12m	23	65.71	6.91/0.009*
	13 – 24	12	34.28	
* Significant difference (P <0.05)				

Distribution of Diarrheagenic E. coli Strains with Feeding Type of Studied Children

the results that we obtained in our study; it was shown that Diarrheagenic *E. coli* was present in formula-fed infants in a significant percentage 19/35 (54.2%). (It is the highest percentage), While in infants who were fed mixed breast fed, the percentage was lower as 11/35 (31.4%) and in infants dependent on natural feeding (breast-fed), the percentage showed the lowest where it was 5/35(14.2%).

Table (4) Distribution of Diarrheagenic E. coli in feeding type							
Type feeding	DEC frequency	EPEC	EAEC	ETEC	EIEC	X2	P value
Formula-fed	19(54.28)	7(36.84)	10(52.63)	1(5.26)	1(5.26)	17.05	0.001*
Mixed breastfed	11(31.42)	5(45.45)	3(27.27)	1(9.09)	2(18.18)	4.24	0.236
Breast-fed	5(14.28)	3(60)	1(20)	1(20)	0(0)	5.06	0.167
Total	35	15(42.85)	14(40)	3(8.57)	3(8.57)	34.64	0*
X^2		0.911	2.83	1.1	2.03		
P value		0.634	0.242	0.576	0.362		

5. Discussion

In present study, four Diarrheagenic E. coli pathotypes EPEC, EAEC, ETEC, EIEC were responsible for children diarrhea. Among all the Diarrheagenic E. coli pathotypes, Enteropathogenic E. coli (EPEC) were found to be the most common pathotypes for children with 40% (14/35), these results compatible with local study by (9) in Sulaimaniah who showed EPEC as most than other pathotypes (63%), and in contrast with (10) in Baghdad and (11) in Dhiqar because they show it came second after EAEC. The current result was similar to globally studies with (12) in China, (13) in India that also reported a high frequency of EPEC pathotypes associated with pediatric diarrhea.

Enteroaggregative E. coli 37.14% (13/35) isolates came second after Enteropathogenic E. coli as causative agent of diarrhea among Diarrheagenic E. coli pathotypes in our study, that agreed with locally (14) in Wassit city, also Globally with (15). But EAEC considered the major cause of diarrhea between diarrheagenic E. coli pathotypes in local studies by (10,11), also Globally, (16) in India, and (17) in Burkina Faso. AggR gene was appeared in all EAEC isolates detected in our study that mean all of them were typical EAEC,

The infants at First year were more probable to have diarrhea than those who were older because of protection against diarrhea may be conferred via a number of mechanisms for example maternal antibodies against enteric organisms and as well as breastfeeding which after the age of 6month the protection is lost due to giving of additional foods and changing nutritional behaviors (18).

In our study Diarrheagenic E. coli pathotypes were detected in 2 age group and the most cases were occurred among children in first years, this result consistent with local study reported by (19,10) in Baghdad showed high prevalence of DEC in children less than 2 years and reported that most infections were *EPEC* and EAEC.

Our results close agreement with (20) in Vietnam who showed that EPEC and EAEC were more frequently isolated in children less than 1 years, while ETEC and EIEC were more frequently in children more than 1 years, (21) in Nigeria also report high prevalence of Enteropathogenic E. coli in children below 2 years, and in Brazil showed that Enteroaggregative E. coli was found to be more frequently associated with diarrhea in children less than 2 years. (22) in AL-Kut also showed that EPEC isolates were the important cause of acute gastroenteritis particularity in infants under 2 years.

Shiga toxin-producing E. coli infection commonly associated with bloody diarrhea (10), in this study we didn't targeted just bloody diarrhea and the collected bloody diarrhea were less common than watery diarrhea.Generally, the STEC consider zoonotic disease, the animal and its products play essential role in transmission the infection in community , STEC appears to be more frequent in adults than children (23).

the results that we obtained in our study; it was shown that Diarrheagenic E. coli was present in formula-fed infants in a significant percentage 19/35 (54.2%). (It is the highest percentage), While in infants who were fed mixed breast fed, the percentage was lower as 11/35 (31.4%) and in infants dependent on natural feeding (breast-fed), the percentage showed the lowest where it was 5/35(14.2%). These results are in agreement with the

study (24). Gastro-intestinal infections are less common in breast- fed than in formula-fed infants this seems to be due not only to increased contamination during formula feeding, but also to a protective effect of human milk, recently showed that as little as 5 ml colostrum/kg was sufficient to prevent Enteropathogenic E. coli, gastro-intestinal infections, and more interesting is that EPEC occurred in the faeces of some of the infants without causing any symptoms (25).

The main reason why the number of faecal E. coli and other gram-negative bacilli in breast-fed infants is notably smaller than in formula-fed babies is probably the low buffering capacity and high lactose content of breast milk, other properties of human milk may have a more marginal affect which, however, can become important when virulent bacteria have colonized the newborn infant. Intraluminal agglutination or killing of potential pathogens, prevention of bacterial attachment to the epithelial surface or reduction in number may then play a role (26)

6. Conclusions

Most of pathogenic *E. coli* isolates (EPEC and EAEC) were present in diarrheal stool samples of children less than two years old in Wasit province and most pathogenic *E. coli* isolates were multidrug resistant.

Conflict of Interest: None

Funding: Self

Ethical Clearance: Not required

References

Walker, C. L. F., Rudan, I., Liu, L., Nair, H., Theodoratou, E., Bhutta, Z. A., and Black, R. E. (2020). Global burden of childhood pneumonia and diarrhoea. The Lancet, 381(9875), 1405-1416. doi: 10.1016/S0140-6736(13)60222-6.

Abdel Rahman, T. R. (2020). Detection of *Escherichia coli* O157:H7 Strain by conventional and molecular methods from diarrheal children in Baghdad. Journal of American Science. 12(6):106-109.

Langendorf C, Le Hello S and Moumouni A (2018). Enteric bacterial pathogens in children with diarrhea in Niger: diversity and antimicrobial resistance. PLoS ONE.;10:1–18.

Hazen, T.H., Daugherty, S.C., Shetty, A.C., Nataro, J.P. and Rasko, D.A. (2017). Transcriptional variation of diverse enteropathogenic Escherichia coli isolates under virulence-inducing conditions. MSystems, 2(4), pp. e00024-17.

Blanton, L.V., Wang, L.T., Hofmann, J., DuBow, J., Lafrance, A., Kwak, S., Bowers, L., Levine, M.A., Hale, C.O., Meneely, P.M. and Okeke I.N. (2018). Aggregative Adherence and Intestinal Colonization by *Enteroaggregative Escherichia coli* Are Produced by Interactions among Multiple Surface Factors. mSphere, 3(2), pp. e00078-18.

Kartsev, N.N., Fursova, N.K., Pachkunov, D.M., Bannov, V.A., Eruslanov, B.V., Svetoch, E.A. and Dyatlov, I.A. (2015). Molecular Characterization of Enterotoxin-Producing Escherichia coli Collected in 2011–2012, Russia. *PloS one*, 10(4), p.e0123357.

Soledad-Cadona, J., Bustamante, A.V., González, J. and Mariel-Sanso, A. (2018). Pathogenicity Islands Distribution in Non-O157 Shiga Toxin- Producing *Escherichia coli* (STEC). *Genes*, *9*(2), p.81.

Daniel, W. (2009). Biostatistics: A Foundation for Analysis in the Health Sciences 9th edition. John Wiley and Sons. INC. USA.

Ali, J. A., and Bunyan, I. A. (2021). PATHOGENIC VIRUS AND BACTERIA ASSOCIATED WITH DIARRHEA AMONG CHILDREN IN HILLA PROVINCE, IRAQ

Khalil, Z. K. (2019). 'Isolation and identification of different diarrheagenic (DEC) Escherichia coli pathotypes from children under five years old in Baghdad', Iraqi Journalof Community Medicine, 28(3), pp. 126–132.

Al-Dulaimi, T.H., Aziz, H.W., Al-Marzoqi, A.H., Al-Aziz, S.A. and Mohsin, S.A.A. (2021). Molecular characterization and antibiotic susceptibility of diarrheagenic Escherichia coli from Children. Medical Journal of Babylon, 12(2), pp.541-550.

Zhou, Y., Zhu, X., Hou, H., Lu, Y., Yu, J., Mao, L., Mao, L. and Sun, Z. (2020). Characteristics of diarrheagenic Escherichia coli among children under 5 years of age with acute diarrhea: a hospital-based study. BMC infectious diseases, 18(1), p.6

Thakur, N., Jain, S., Changotra, H., Shrivastava, R., Kumar, Y., Grover, N. and Vashistt, J. (2021). Molecular characterization of diarrheagenic Escherichia coli pathotypes: Association of virulent genes, serogroups, and antibiotic resistance among moderate-to-severe diarrhea patients. Journal of clinical laboratory analysis, p.e22388.

Abdul-hussein, Z. K., Raheema, R. H., and Inssaf, A. I. (2020). Molecular diagnosis of diarrheagenic E. coli infections among the pediatric patients in Wasit Province, Iraq. Journal of Pure and Applied Microbiology, 12(4), 2229–2241.

Broujerdi, S. M., Ardakani, M. R., and Rezatofighi, S. E. (2019). Characterization of diarrheagenic Escherichia coli strains associated with diarrhea in children, Khouzestan, Iran. The Journal of Infection in Developing Countries, 12(08), 649-656.

Rajendran, P., Ajjampur, S.S.R., Chidambaram, D., Chandrabose, G., Thangaraj, B., Sarkar, R., Samuel, P., Rajan, D.P. and Kang, G. (2019). Pathotypes of diarrheagenic Escherichia coli in children attending a tertiary care hospital in South India. Diagnostic microbiology and infectious disease, 68(2), pp.117-122.

Konaté, A., Dembélé, R., Guessennd, N.K., Kouadio, F.K., Kouadio, I.K., Ouattara, M.B., Kaboré, W.A., Kagambèga, A., Cissé, H., Ibrahim H.B. and Bagré, T.S. (2020). a. Epidemiology and antibiotic resistance phenotypes of diarrheagenic Escherichia coli responsible for infantile gastroenteritis in Ouagadougou, Burkina Faso. European Journal of Microbiology and Immunology, 7(3), pp.168-175

Sakhi R. J. (2019). Isolation of Escherichia coli from diarrhea and test their pathogenicity and susceptibility pattern for antibiotic, International Journal of Agricultural science and research, 6 (2), p. 29 – 34.

Hamada, T. A., Abdelghafoor, A. H., and Hussein, A. S. (2018). Evaluation some virulance factor of Eschirichia coli that causes diarrhea in children by using polymerase chain reaction technique in kirkuk city. Medical Journal of Tikrit, 21(1), 308-321.

Nguyen, T. V., Le Van, P., Le Huy, C., Gia, K. N. and Weintraub, A. (2005). Detection and characterization of diarrheagenic Escherichia coli from young children in Hanoi, Vietnam. Journal of clinical microbiology, 43(2), p.755-760.

Achi O. K., Mbajiaka, N. C. (2019). Association of enteropathogenic Escherichia coli with or without diarrhea among children in Aba, Nigeria. International Journal of Microbiology and Application, 2 (2), pp.38-44.

Al-Gharabawee, R. H. R. (2020). The effect of purified bactriocin and ethanolic extract of Plantago lanceolata and Quercus infect Oria on enteropathogenic E. coli. PhD thesis, Baghdad University, College of Vererinary Medicine.

Okeke, I.N., Ojo, O., Lamikanra, A. and Kaper, J.B. (2017). Etiology of acute diarrhea in adults in southwestern Nigeria. Journal of clinical microbiology, 41(10), pp.4525-4530.

Scholar, A. (2020). Prevalence of Escherichia Coli Strains Isolated from Diarrhoiec Stool of Children (0-5) Years Attending Selected Hospitals in Niger State Nigeria. 1Muhammad, IL, 2Olonitola, OS, 2Ameh, JB, 3Olayinka, BO and Suleiman, AL.

Hashem, Z. S., and M Abd El-Baky, R. (2021). In vitro inhibition of uropathogenic Escherichia coli biofilm formation by probiotic Lactobacilli isolated from healthy breast-fed infants. Novel Research in Microbiology Journal, 5(1), 1091-1105.

Yang, I., Corwin, E. J., Brennan, P. A., Jordan, S., Murphy, J. R., and Dunlop, A. (2016). The Infant Microbiome. Nursing Research, 65(1), 76–88.