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BACTERIOLOGICAL AND MOLECULAR CHARACTERIZATION OF DIARRHEAGENIC *ESCHERICHIA COLI* PATHOTYPES FROM CHILDREN IN DUHOK CITY, IRAQ

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ABSTRACT:
Background: A variety of diarrheagenic *E coli* (DEC) are responsible for causing different types of diarrhea in children especially in developing courtiers.
Objectives: This study was primarily aimed to isolate and bacteriological characterizing of *E coli* from diarrheic infant stool and to investigate their antibiotic resistance patterns and then using molecular identification of DEC pathotypes for better discrimination.
Methods: Total of 400 fresh stools specimens were collected from children with diarrhea in Heevi Hospital in Duhok city, Iraq. The samples were cultured on selective media such as (MacConkey and MacConkey sorbitol agar). Colonies were identified through biochemical reaction and VITEK 2 system and then antibiotic susceptibility profiles were determined.
Results: A total of 349(87.2%) samples were yielded positive for growth of *E coli.* Out of these, 50 phenotypically-identified *E coli* were then subjected to PCR assay targeting certain virulence factors (*alt, eae, sxt1* and *sxt2*) for discrimination of pathotyes. 13/50(26%) Enterotoxigenix *E. coli* (ETEC) was detected, 5/50(10%) Enterohemorrhagic *E coli* EHEC was detected, while no Enteropathogenic *E coli* (EPEC) was detected. All pathotypes were more frequent in samples from male children aged between 2-3 years that were artificial feeding pattern. Moreover, all pathotypes expressed high resistant to ampicillin, cephalosporin and tetracycline while little resistance to imipenem was observed. **Conclusion:** The study concludes presence of diarrheagenic *E coli* pathotypes in our community causing diarrhea in children and emphasize on using of PCR assay for best discrimination.

KEYWORDS: Diarrheagenic *Escherichia coli,* Stool, MacConkey-Sorbitol agar, Virulence factors, PCR.

1. INTRODUCTION

A variety of recognized microorganisms can be associated with diarrhea in children, including: bacteria and parasites. Several studies have stated that, among bacterial pathogens associated with diarrhea in developing countries, the diarrheagenic *Escherichia coli* (DEC) strains are the most bacterial pathogens worldwide (Canizalez *et al*, 2016; Spano *et al*., 2017). In fact, the frequencies of these DEC differ with geographic region and according to the socioeconomic/sanitary conditions achieved (Black *et al.,* 2010).
Escherichia coli are, in fact, an important member of the normal human intestinal microflora, but some of their strains having developed abilities to cause diseases in the human host: such as in gastrointestinal, urinary, and nerves system (Canizalez *et al*, 2016). It has been observed that, *E. coli* is one of the common causative agents of diarrheal over the world (Nguyen *et al.*, 2005). However, based on specific virulence factors, and phenotypic traits, there are 6 pathotypes of diarrheagenic strains of *E. coli* including: enteropathogenic *E. coli* (EPEC), enterotoxigenic *E. coli* (ETEC), Vero toxin-producing/Shiga toxin-producing *E. coli* (VTEC/STEC) which include its well-known subgroup enterohaemorrhagic *E. coli* (EHEC), enteroinvasive *E. coli* (EIEC), enteroaggregative *E. coli* (EAEC), and diffusely adherent *E. coli* (DAEC) (Kaper *et al.,* 2004).
In order to identify diarrheagenic *E. coli*, bacteriological characterization are necessary to be performed routinely in all diagnostic laboratories, including: stool cultures in order to isolate *E. coli,* and biochemical test. MacConkey-sorbitol agar is selective and differential medium for presumptive isolation of enterohaemorrhagic *E. coli* (EHEC) pathotype that fails to ferment sorbitol sugar and gave colorless colonies. Some of the suspected *E. coli* was subjected to automated system susceptibility testing, i.e. bacterial identification system (well-known as VITEC 2 system). However, unfortunately, bacteriological examination and characterization methods are not enough. In other words, these methods cannot be performed alone, since pathogenic *E. coli* cannot be distinguished from the non-pathogenic *E coli* which, in turns, can be found normally in human feces (Spano *et al*., 2017). Therefore, the discrimination of diarrheagenic *E coli* strains requires molecular techniques based on DNA target screening for isolates harboring virulence genes.
Polymerase Chain Reaction (PCR) is a major develop in molecular diagnostics of pathogenic microorganisms including *E coli*, which has a great sensitivity and high specificity in detection of target templates over classical bacteriological methods (Fujioka *et al*., 2013). No molecular methods applied for detection and discrimination of diarrheagenic *E coli* pathotypes

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in screened diarrheal stools sample in children in Duhok city, Kurdistan region, Iraq. In order to detect these categories of *E. coli* strains, it is necessary to perform several PCRs with different primers specific for these genes. Therefore, in this work we used a rapid and reliable diagnosis of human diarrheagenic *E. coli* pathotypes by PCRs with different primer pairs targeting virulence genes.

2. PATIENTS AND METHODS

2.1 Clinical specimens

This study was conducted in Heevi Hospital, Duhok city, Kurdistan region, in northern of Iraq. The period that has been devoted was carried out from May 2018 to April 2019. In this cross-sectional study, a total of 400 diarrheic stool samples were collected from children aged (1-5) years (238 male and 162 female) that suffered from diarrhea. As well as, nutritional status of children whether breast (285) or bottle (115) feeding was recorded in this study. The study excluded those children who had used antibiotic drugs before admission.

2.2 Ethical consideration

Study proposal and informed consent was approved by ethics committee of the college of Medicine, Duhok University and Duhok Public Health Directorate, Kurdistan Region, Iraq. From all patients, formal consent was taken from their parents/guardians before collecting the samples.

2.3 Sample collection and processing

Fresh stool samples were collected from each child and put in an appropriate place, clean, wide-mouth container. The macroscopic examination of fresh stool was performed such as consistency (formed, soft, watery, liquid, and mucoid), colour (brown, yellow, green, black, and red), blood (not seen, few, moderate, present, and abundant), mucus (not seen, few, moderate, present, and abundant).
A loopful from fresh watery and mucoid stools samples were streaked on Blood agar, MacConkey agar, while, bloody stools samples were streaked on MacConkey-sorbitol agar. Both sets of media were incubated for 18-24 hours at 37°C. Three lactose-fermenting purified colonies and a corresponding representative non-lactose fermenting colonies from MacConkey agar and MacConkey-sorbitol agar were picked, respectively for phenotypic identities using a classical biochemical tests (Engelkirk & Duben-Engelkirk, 2008) and then subjected to VITEK 2 automated system for the identification of bacterial species (Versalovic *et al.*, 2011). Confirmed *E. coli* isolates were stored at - 20° C in Trypticase soy broth supplemented with 20% glycerol for further procedures (Cheesbrough, 2006).

2.4 Antimicrobial susceptibility test

All *E coli* isolates were tested for antibiotic susceptibility assay according to Kirby-Bauer disc diffusion technique (Bauer *et al.*, 1966) as per the recommendation of Clinical and Laboratory Standards Institute (CLSI) (Weinstein *et al.,* 2017). Antibiotics panels have been used as a guide to testing the sensitivity of bacteria to the 13 antibiotics (Oxoid) including the following: cefotaxime (30µg), amikacin(10 µg), ceftriaxone(10 µg), cefixime(5 µg), tetracycline (30 µg), gentamicin(10 µg), ciprofloxacin(10 µg), imipenem(10 µg), amoxicillin(20 µg),, chloramphenicol(10 µg), amoxicillin/ clavulanic acid(20/10 µg), trimethoprim/ sulphamethoxazole(10/50 µg) and ampicillin(20 µg). Briefly, an overnight suspension broth culture that matched to 0.5 MacFarland Standard were uniformly inoculated by a cotton swab on Muller-Hinton agar then incubated at 37 ^{0}C for 24 hours. Plates were then read by measurement of the zones of growth inhibition (millimetre) around each of the antibiotic disks. The zone diameters of drugs were interpreted based on Clinical and Laboratory Standards Institute criteria (noted as CLSI) (Weinstein *et al.*, 2007).

2.5 DNA Extraction

For the molecular characterization and PCR assay, 50 isolates of *E coli* were choice according to their cultural characteristics on MacConkey agar and MacConkey -Sorbitol- agar. Out of these 35 isolates were taken from MacConkey agar and another 15 were from MacConkey -Sorbitol- agar.
The extraction of DNA was performed from the sweep of a few colonies of bacteria that were grown on blood- Agar plates utilizing boiling method (Liu, 2011).The extraction of crude genomic DNA was performed by heating samples for 20 minutes at 120 ^{0}C and high-speed centrifugation(3500 rpm for 20 minutes) for 15 minutes. Supernatants were then used as DNA template for PCR. Determination of DNA concentration and purity of each isolate was determined using Nano-drop spectrometer (Cambridge, England) instrument.

2.6 PCR Amplification protocol

Monoplex PCR for categorization of *E. coli* into ETEC, EHEC and EPEC was performed using; published primers (Fujioka *et al*., 2013) as in Table (1) for identification of virulence genes *elt* and *eae,* respectively. On the other hand, multiplex PCR was applied for assign of EHEC using specific primers for detection of *stx1* and *stx2,* as seen in Table (1).

Table 1. Specific genes, primers sequences and expected products for PCR assays used for various virulence genes among diarrheagenic *E coli.*

Pathotype	Specific gene	Primers (5′→3′)	Product size(bp)
EPEC	*eae*	F:CCCGAATTCGGCACAAGCATAAGC R:CCGGATCCGTCTCGCCAGTATTCG	881
ETEC	*elt*	F:AACGTTCCGGAGGTCTTATG R:CAACCTTGTGGTGCATGATG	511
EHEC	*stx 1*	F:AGTTAATGTGGTGGCGAAGG R:CACCAGACAATGTAACCGC	347
	stx 2	F:TTCGGTATCCTATTCCCGG R:CGTCATCGTATACACAGGAG	589

EPEC = enteropathogenic *E. coli*; ETEC = enterotoxigenic *E. coli*;
EHEC = enterohemorrhagic *E. coli*. Note that: F = forward; R = reverse

The master mix of PCR reaction was performed in final reaction of a total volume of 25 µL .Each reaction contained 2 µL of primer(10 pmol µl-1) mixture (1 µL for each forward and reverse primer), 1 µL of DNA template (25ng/ ml) ,10 µL of Master Mix and 12 µL of Nuclease free water. PCR amplification was performed for the following thermal and cycling condition (Toma et al., 2003) : initial denaturation at 95°C for 3 min, followed by 30 cycles of denaturation at 94°C for 1 min, annealing at 56°C for 1 min, and extension at 72°C for 1 min; and final extension at 72°C for 5 min. The PCR products were then separated in1.5% agarose gel to detect specific amplified product by comparing with standard molecular weight marker.

2.7 Statistical Analysis:

Statistical analysis was carried out using Stata 16 program. The significant difference between variables was determined using Fisher and Chi square test (Sokal and Rohlf, 2013). P value < 0.05 was considered a significant.

3. RESULTS

Out of 400 fresh stool samples that cultivated on MacConkey and Sorbitol- MacConkey agar, 349 (87.2%) isolates were yielded positive growth that distributed between 298 isolates with pink lactose fermentative colonies on MacConkey agar and other 51 isolates with non-sorbitol fermentative colonies on Sorbitol- MacConkey agar that was presumptively identified as a pathotype Enterohemorrhagic *E. coli*. Watery, mucoid and soft stool samples largely yielded positive growth of *E coli* on MacConkey agar, while majority of bloody stool samples were appeared non sorbitol fermenter colonies on Sorbitol- MacConkey agar. All recovered isolates from both media were further subjected to Vitek 2 system for definitive confirmation and identification that was 91.3% similarity of characteristics of *E coli*.

Table (2), show percentages of overall *E coli* isolated from diarrheic stool samples from children aged between 1-5 years, the percentage of infections was slightly higher in male (87.4%) gender than female (87.0%) but statistically was not significant (p<0.71). Furthermore, the highest infection rates were found among children aged between 2-3 years (95.3%) and the lowest rate were seen in age between 4-5 years of age and statistically was not significant (p<0.162). According to feeding status, the highest recovery of *E coli* rate was seen with artificial feeding (89.5%) and also no significant difference was found between them(p<0.7).

Generally, the highest resistance rate of antibiotics was noticed against amoxicillin (100%), followed by ampicillin (98.6%), amoxicillin/ clavulanic acid (97.2%), tetracycline (98.6%), while moderate resistance was recorded against chloramphenicol and ciprofloxacin. The lowest resistance was with imipenem with 10%, and 6.7% for both isolates from MacConkey agar and MacConkey -Sorbitol agar, respectively (Table 3).

Table 2. Percentages of *E coli* isolated from total examined diarrheic stool samples among genders, age groups and feeding status

Variables	Prevalence of *E coli* infections by culture			
	Positive No. (%)	Negative No. (%)	Total	*P Value
Gender				0.710
Male	208 (87.4)	30 (12.6)	238	
female	141(87.0)	21 (13)	162	
Age				
>1 year	163(85.8)	27(14.2)	190	
1-2 years	87 (86.1)	14(13.9)	101	0.162
2-3 years	41 (95.3)	2(4.7)	43	
3-4 years	35(94.6)	2(5.4)	37	
4-5 years	23(79.3)	6(20.7)	29	
Feeding				
Breast	94(81.7)	21 (18.3)	115	0.7
Bottle	255(89.5)	30 (10.5)	285	

*The data was statistically analysed using Chi-Square test (or Fisher exact test). *P values* <0.05 were considered as a significant.

Table 3. Patterns of antibiotic-susceptibility test of all phenotypically *E coli* isolates grown on MacConkey and Sorbitol- MacConkey agar.

Antibiotics	Resistance Rates (MacConkey agar) %	Resistance Rates (MacConkey-Sorbitol agar) %
Trimethoprim/ Sulphamethoxazole (SXT)	77.1	86.7
Imipenem (IPM)	10	6.7
Ampicillin (AM)	98.6	96.7
Amoxicillin (AX)	100	100
Chloramphenicol (C)	51.4	33.3
Cefotaxime (CTX)	85.7	86.7
Tetracycline (TE)	98.6	100
Ciprofloxacin (CIP)	42.9	33.3
Ceftriaxone (CRO)	87.1	90
Gentamicin (CN)	82.9	70
Amikacin (AK)	85.7	93.3
Amoxicillin/clavulanic acid (AMC)	97.1	96.7
Cefixime (CFM)	92.9	90

It was found that out of 35 isolates of lactose-fermenter growth on MacConkey agar that subjected to PCR assay by using specific primer of *elt* and *eae* virulence genes, 13 of them successfully produced amplified products (511bp) with *elt* and no isolate was contained *eae* (Figure 1) and (Figure 2) ,respectively. Five out of fifteen isolates on MacConkey-Sorbitol agar amplified only *stx1* gene and no *stx2* gene as it is shown in Table (4) and in Figure (3).

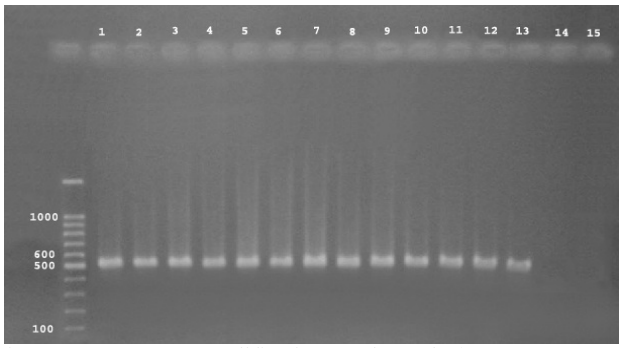

Figure 1. Represent amplification product of *elt* gene on 1.5% agarose gel, ladder 100 bp, lanes (1 to 13) isolates produced amplified products (511 bp); lanes 14 and 15 not produced amplified products.

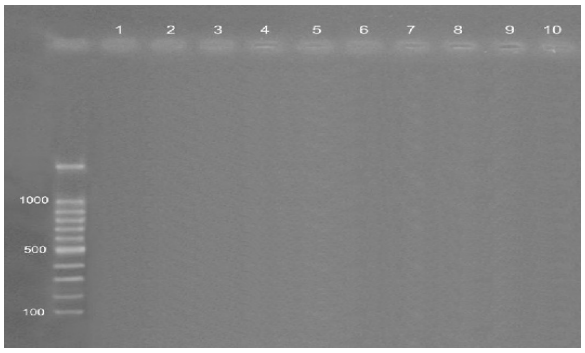

Figure 2. Represent no amplification product of *eae* gene on 1.5% agarose gel, ladder 100 bp, lanes from 1 to 10 not produced amplified products (881 bp**).**

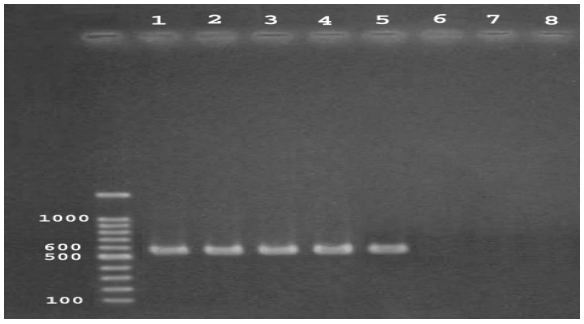

Figure 3. Represent amplification product of *stx1* and *stx2* genes on 1.5% agarose gel, ladder 100 bp, 5 lanes (1 to 5) isolates produced amplified products (589 bp) with *stx1*; and no amplified products produced with *stx2*.

Table 4. PCR assay targeting various virulence genes of three pathotypes of diarrheagenic *E coli*

Results	35 sample on MacConkey agar		15 sample on MaConkey-Sorbitol agar
	ETEC *elt* amplified gene	EPEC *eae* amplified gene	EHEC *stx1* and *stx3* amplified genes
+ ve	13 (37%)	0 (0%)	5(33%)
- ve	22(63%)	35(100%)	10(67%)

Furthermore, Table (6) shows the results of antibiotic susceptibility test for those *E coli* pathotypes that were identified by PCR. The highest resistance rate of Enterotoxigenic *E. coli* (100%) was still found for amoxicillin, ampicillin, cefotaxime and tetracycline and the lowest rate of resistance to imipenem (15%). For the Enterohaemorrhagic pathotypes, the highest rate of resistance (100%) was seen for amoxicillin, sulfa-methoaxazoal, tetracycline and amikacin. Meanwhile, no resistance rate was seen with imipenem in any of the patients (0%) (Table 5).

Table 5. Antibiotic susceptibility patterns of molecularly-identified pathotypes of diarrheagenic *E. coli*

Antibiotics	ETEC (13 isolate) (%)	EHEC (5 isolates) (%)
Trimethoprim/ Sulphamethoxazole (SXT)	12 (92)	5 (100)
Imipenem (IPM)	2 (15)	0 (0)
Ampicillin (AM)	13 (100)	4 (80)
Amoxicillin (AX)	13 (100)	5 (100)
Chloramphenicol (C)	7 (53)	3 (60)
Cefotaxime (CTX)	13 (100)	3 (60)
Tetracycline (TE)	13 (100)	5 (100)
Ciprofloxacin (CIP)	9 (69)	2 (40)
Ceftriaxone (CRO)	13 (100)	4 (80)
Gentamicin (CN)	9 (69)	3 (60)
Amikacin (AK)	10 (76)	5 (100)
Amoxicillin/Clavulanic acid (AMC)	13 (100)	4 (80)
Cefixime (CFM)	12 (92)	4 (80)

4. DISCUSSION

Diarrheagenic *Escherichia coli* pathotypes are commonly involved in diarrheal disease in children in our setting due to factors of contamination. To our knowledge, no study has been performed in line of molecular identification of causes of diarrhea, especially diarrheagenic *Escherichia coli* pathotypes among children equal or less than five years of age in Duhok City, Kurdistan region, Iraq. Therefore, the current study was implanted phonotypical and molecular methods to identify these pathotypes among children equal or less than five years of age in studies region In this study, overall 349(87.2%) of diarrheic stool samples were showed positive culture characteristics of *E coli* on both MacConkey and MacConkey-sorbitol- agar. In the present study, *E coli* were more frequently isolated in male than female and the highest infection rates were also found in age between 2-3 years old. In terms of feeding, the highest *E coli* infection rate was recorded in artificial feeding than breast feeding.

Furthermore, the isolates of *E coli* were showed high resistant to old antibiotics such as ampicillin, cephalosporin and tetracycline but lowest resistance was seen to imipenem. Similar findings have been reported in Zakho city, Kurdistan Region, Iraq, by Badry *et al.* (2014) where *E coli* is the most prevalent enteric pathogen which was (58.43%). Similar findings were also reported in other studies (McAuliffe *et al.,* 2013; Rashedul, 2011). In another study conducted in Libya, it has found diarrheagenic *E col* was the most common detected bacterial pathogens (Rahouma *et al.,* 2011). These high rates of recovery of *E coli* found in the current study may be due to low education of parents, low-grade of health hygiene, poor toilet training, lack of exclusive breastfeeding, artificial feeding, source of water supply, overcrowding and climatic conditions (Hellard *et al.,* 2000).

Generally, *E coli* are the predominant nonpathogenic member of the human intestinal normal flora usually isolated from stool samples (Hellard *et al.,* 2000). This explain the highly rate of presence of *E. coli* was detected in the present study. In fact, the pathogenic strains of *E coli* are well differentiated from the nonpathogenic by using a common molecular test such as PCR using specific primers. For this reason, in current study, 50 isolates of phenotypically-identified *E coli* that subjected to PCR assay by using specific primers targeting virulence genes,

Enterotoxigenix *E coli* (ETEC) was detected in 13 samples (26%), Enterohemorrhagic *E coli* EHEC 5 (10%), while no Enteropathogenic *E coli* (EPEC) detected. Moreover, watery and soft stools samples were mainly contained ETEC while most bloody samples confirmed harboring EHEC. In other parts of Iraq, some PCR-based studies detected 26.3 %, 19.2% and 21.6 % of ETEC in stool samples by (Arif & Salih, 2010; Al-Dulaimi *et al.,* 2015; Khalil, 2015), respectively. Other studies conducted in Bangladesh, Egypt and Iran were recognized ETEC by PCR as major pathotype among children less than 5 years (Nessa *et al.,* 2007; El Metwally *et al.*, 2007; Haghi *et al*, 2014). Low-grade hygienic state of family and artificial feeding may account for this high rate of ETEC.

Enterohemorrhagic *E coli* EHEC was found the second most prevalent detected by PCR test in this study (33%). This finding was higher than two studies performed in Iraq using PCR assay also, one by Al-Dulaimi (2015) in TheQar/Iraq, found (5.27%) (Al-Dulaimi *et al.,* 2015) and other by Khalil, found 7.8% (Khalil, 2015). In Iran, Nigeria and Bangladish, the rates of infection detected by PCR were 1.4 %, 25% and 11.7% (Nessa *et al*, 2007; Sharifi A *et al.,* 2011; Ifeanyi *et al.*, 2015). Whereas, in Sulaimaniah/Iraq, Arif and Salih (2010) were not detected any EHEC pathotype in their study. Khabir (2011), in Bangladesh, there were no EHEC have been detected (Rashedul, 2011). The higher rates of EHEC in our study might be due to taking of contaminated waters and foods including uncooked minced ready meat at fast food restaurants as well as contaminated vegetables with animal feces, in addition to raw milk combined with low infective dose of this pathotype (Sperandio & Pacheco, 2012).

In the current study, none of Enteropathogenic *E coli* pathotype EPEC was detected when we used a specific primer for *eae* gene. Similar result was found in study conducted in Bangladesh, by Rashedul (2011) and in Egypt, by El-Metwally *et al.* (2007), they detected very low rate of EPEC with 3.5%. This present result is in contrast to finding of study performed in Baghdad by Khalil (2015); it has detected the highest infection rate of EPEC (29.4%) among children under 5 years old. The possibility of using more than one primer may yield positive amplification; in addition geographical differences may also effect on the prevalence of EPEC pathotype.

In current study, antibiotic susceptibility patterns for those ETEC and EHEC that molecularly identified were variable. ETEC was still expressed high resistance to commonly used antibiotics except 15% resistance to imipenem, while corresponding phenotypically isolates grown on MacConkey agar expressed 10% resistance. The current findings were accord with two studies conducted in Vietnam and India (Nguyen *et al*., 2005; Sudershan *et al,* 2014). Generally, EHEC pathotype exhibited analogue patterns of antibiotic-susceptibility namely high susceptibility to imipenem. This is in accordance with studies conducted in Bangladesh (Rashedul, 2011) and Germany (Presterl *et al.,* 2003)**.**

5. CONCLUSION

The present study concludes that certain pathotypes of highly antibiotic resistant diarrheagenic *E coli* are circulating in our setting, the study emphasize on employment of molecular tests such as multiplex PCR for discrimination of pathogenic *E coli* from non-pathogenic *E coli* in cases of infants diarrhea.

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