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(RESEARCH ARTICLE)

A study of enteropathogenic *E .coli* (EPEC) and the contributing risk factors in diarrheal patients under five

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Abstract

Diarrhea can be defined as "passing of a liquid stool more than three times within 24 hours", it is a major cause of infantile deaths in underdeveloped world in young children<5 years. According to WHO report a child dies every minute due to diarrhea. In this study 1465 stool samples of acute watery diarrhea were analyzed. Demographic data analysis showed male to female ratio of 55.2%, 44.8%. All lactose fermenting bacterial colonies were processed for the identification of bacterial pathogenic *E. coli* and verified biochemically, were also serotyped by commercially available strain specific antiserum. All genotypes of importance were confirmed by advanced molecular techniques like PCR and RT-PCR. *E. coli* (uidA), EPEC virulent genes eae and bfpA and ETEC virulent gene It and st, EIEC virulence gene inv and stx1, EHEC virulent gene stx2, EAEC virulent gene east-1and east were investigated. Among *E. coli,* isolates, EPEC *(*48.6%) was found to be most dominant diarrheal pathogen followed by ETEC (24.3 %), EAEC (21.6 %), EIEC (5.4%) and EHEC (0.0%). Use of advanced molecular techniques like PCR is necessary to detect pathogenic *E. coli* is highlighted from clinical samples.

Keywords: Body Mass Index; SES Socioeconomic status; HRG High Risk Group; AMP Ampicillin; AK Amikacin; SXT Trimethoprim-sulphamethoxazole

1. Introduction

Diarrhea can be defined as "passing of a *liquid* stool more than three times within 24 hours". It is consistency rather than frequency which is more important and major cause of infantile deaths in underdeveloped world in young children<5 years [1]. In Pakistan five million children are born every year, 10% of these die before reaching their first birthday and 14% die before the age of five years which accounts to nearly 70,000 - 150,000 children under the age of five years annually i.e. nearly 2000 children die every day [2, 3]. More than one third of the total hospital admissions are due to diarrheal disorder and ultimately leading to 5-6 episodes of infection per child annually leading towards malnutrition and stunted growth [4]. The role of hand hygiene, food handlers, quality of water, vender food and its safety data is missing and information regarding the storage and transport of food materials, fast food items sold in local market as important contributing factors needs to be determined [5]. Serious health consequences due to multiple episodes of avoidable diarrhea are not highlighted due to lack of availability of reliable data.

In Pakistan high turnover of non-pathogenic *Escherichia coli* strains are reported in intestinal flora of infants [5, 6]. It is reported that if borderline between extra intestinal virulence and intestinal fitness is diminished as compromised competitiveness may promote intestinal colonization of extra intestinal infection causing *E. coli* [7]. Although

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pathogenic *E. coli* is major cause of diarrhea among young children in Pakistan India, Bangladesh, Angola, Thailand and Italy, Nigeria & Zaire [7, 8].

Diarrheogenic *E. coli* cannot be distinguished from non-pathogenic *E. coli* commonly found in human feces, on the basis of culture and biochemical criteria. Moreover, serotyping with O and H antigens is of limited use, as there is not always a correlation between pathogenicity and expressed antigens. Different classes of diarrhea causing *E. coli* are characterized based on the presence of genes that codes for specific virulence factors detected by PCR technique that are not present in non-pathogenic strains. So far, no PCR techniques are available in routine that can assist the complete screening of the virulence genes characteristic of all diarrheogenic *E. coli* [9].That is why the study was designed to highlight the importance of modern technique in diagnosis of virulent strains to minimize unnecessary use of antibiotics and in return emergence of multidrug resistant strains.

2. Material and methods

2.1. Recruitment of Study Subjects

Study subjects were identified and recruited from outdoor and Emergency ward of Public and private Hospitals in Karachi Pakistan presenting with acute watery diarrhea. The individuals meeting the criteria and gave consent to participate were included in the study.

2.2. Study design

We designed this study to determine the pathogenic *E. coli* in children reporting to the diarrheal unit of local government hospitals catering to the needs of urban and rural low socioeconomic areas of Karachi and other cities of Sindh province. This study also aimed to conduct a detailed survey of sanitary, phytosanitary and environmental conditions as contributing risk factors for high incidence of diarrhea.

2.3 Study duration

This study was carried out during Jan 2015 to June 2018 with the recruitment of a total of 1465 patients.

2.3. Inclusion Criteria

Children suffering from acute watery diarrhea between the age group of 4 weeks to < 5 years.

2.4. Exclusion Criteria

Newborn < 4 weeks and children > 5 years. Sick children with other morbid conditions like heart disease, chronic diarrheal patients. Children with bloody diarrhea.

2.5. Informed consent

Meeting with every individual patient or attendant was schedule to sign informed consent, which was read aloud to the eligible participants to explain the study objectives.

2.6. Administration of Questionnaire

Face to face interview was conducted with parent or attendant of eligible candidates to ask questions for demographic data.

2.7. Debriefing and Referrals

Following interview during debriefing session satisfactory answers to any question asked by parents or attendants were given. Patients were informed about the mode of transmission, prevention and contributing risk factors of diarrheal infection.

2.8. Sample collection

Stool samples of diarrheal patients who reported with acute watery diarrhea at private and public hospitals Were collected transported and processed for the presence of bacterial pathogen in IIDRL lab of University of Karachi especially for entero pathogenic *E. coli*. Which were identified by culture and serology techniques. Genotypes of importance were confirmed by advanced molecular techniques like PCR

2.9. Bacterial Pathogens Cultures

All clinical isolates which were lactose fermenter, indole positive and confirmed by strain specific anti-sera were collected and processed and analyzed for determination of antibiotic susceptibility screening by Kirby Bauer disk diffusion method against commonly prescribed antibiotics against all identified isolated strains of EPEC from patients stool samples [7]

2.10. Molecular characterization of pathogenic *E. coli* isolates by PCR

Pathogenic *E. coli* cannot be distinguished from non-pathogenic *E. coli* by culture and biochemical characteristics only. So the characterization of the genes that code for virulence factors is only possible by the molecular identification. In Pakistan the molecular typing of Pathogens from diarrheal stool samples for *E. coli* genotypes is not done routinely in diagnostic settings and we never knew that which type of pathogenic strains are prevalent in our country. That is why all *E. coli* strains were characterized by PCR method using specific primers for genes: *E. coli*; uidA, st / It for ETEC; eae/bfpA for EPEC; stx1/inv for EIEC; stx2 for EHEC by PCR method and amplified products were visualized by gel electrophoreses [8].

2.10.1. DNA extraction

DNA extraction following boiling method was performed. In this method a single colony from each verified isolate was inoculated into 10 mL of tryptic soy broth and was incubated at 37 °C. "100" bacterial pellets after centrifugation were added into "400 µl" distilled water and boiled at 90 °C for 10 minutes. The mixture than was centrifuged at a high speed of 5000 rpm for five minutes. The supernatants contained DNA template stored for PCR. 4. 1% agarose gel electrophoresis was done to obtain product of interest against appropriate set of primers [9]. PCR Procedure Six pairs of primers listed in Table 1 were used for molecular characterization of pathogenic *E. coli* strains.

2.10.2. Steps in PCR procedure

This procedure consists of following steps:

"25 µl" of reaction mixture contained "3 µl" of template DNA, "1 µl" $MgCl_2$, "0.17 µl" of each primer,

1 unit of Taq DNA polymerase, "0.5 µl" dNTP Mix, "2 µl" 10× PCR buffer and "17 µl" dd H_2O.

The reaction mixtures in the initial denaturation stage were heated at 96°C for five minutes and were amplified for 30 cycles using a gradient master cycler.

Each cycle was comprised of denaturation at 94 °C for 30 seconds, annealing at 55 °C and 53 °C (for ST, LT and eae genes) for 30 seconds, and extension at 72 °C for one minute. The final extension was performed at 72 °C for seven minutes. PCR products were analyzed by electrophoresis (100 V for 1 hour) on 1% gel agarose and stained with DNA green viewer [10].

3. Results

Demographic data analysis results showed that out of 1465 samples 44.8% were female and 55.2% were males, who presented with the complain of acute watery diarrhea [11]. It was also noted that predominant age groups suffering from diarrhea were twelve months old (65.3%), followed by12-14 months (20.1%) and > 36 months was (6%). According to BMI recorded results, we found that (10%) children were overweight and (5%) were obese. Using the number of family members living per square area indicated that 73% patients had 7-8 family members on average and (73.3%) study population had poor personal hygiene standards at the time of interview. Clinical sign and symptoms of fever, vomiting and diarrheal episodes showed that patients present with combination of clinical indications with history of fever (22.4%), vomiting (48.07%). We recorded that 70% of patients reported 1-2 episodes of vomiting per 24 hours, whereas 62.3% has 3-4 episodes of persistent diarrhea. According to records majority of sick children (90.5%) reported that they did not receive compulsory routine childhood vaccination. Among *E. coli,* isolates, EPEC *(*48.6%) was found to be most dominant diarrheal pathogen followed by ETEC (24.3 %), EAEC (21.6 %), EIEC (5.4%) and EHEC (0.0%). In addition to *E. coli.* Antibiotic susceptibility showed high level of resistance among diarrheal isolates. Most isolates were resistant to Cotrimaxazole (Sxt), Ciprofloxacin (CIP), Amoxicillin (AML), Imipenum (IMP), Amikacin (AK), Augmentin (AMC), Tazocin and piperacillin (TZP), Cefxime (CXM), Cefotaxim (CTX) and Chloramphenicol (C). Most probable reason for this high level of resistance (60% -75%) is misuse or over use under situations where they are not needed like their use in Protozoal and *Rotavirus* infection.

Table 1 Primer list for *E. coli* genotypes

Strain	Target Gene	Amplicon size (bp)	Oligonucleotide sequence	Reference
E. coli	uidA	632	CCAAAAGCCAGACAGAGT GCACAGCACATCAAAGAG	[32]
ETEC	it	322	TCTCTATGTGCACACGGAGC CCATACTGATTGCCGCAAT	[32]
	st	170	TCTTTCCCCTCTTTTAGTCAGTC CCAGCACAGGCAGGATTAC	[32]
EPEC	eae	229	TGATAAGCTGCAGTCGAATCC CTGAACCAGATCGTAACGGC	[32]
	bfpA	450	CACCGTTACCGCAGGTGTGA GTTGCCGCTTCAGCAGGAGT	[32]
EIEC	inv	320	CTGGTAGGTATGGTGAGG CCAGGCCAACAATTATTTCC	[32]
EHEC	stx1	130	GAAGAGTCCGTGGGATTACG AGCGATGCAGCTATTAATAA	[32]
	stx2	510	GGGTACTGTGTGCCTGTTACTGG GCTCTGGATGCATCTCTGGT	[33]
EAEC	east-1	111	CCATCAAACACAGTATATATCCGAG GTCGCGAGTGACGGCTTTGT	[33]
	east	203	ACGATATCCTCATCGCCTGTG CTGCTGGCCTGCCTCTTCCGT	[33]

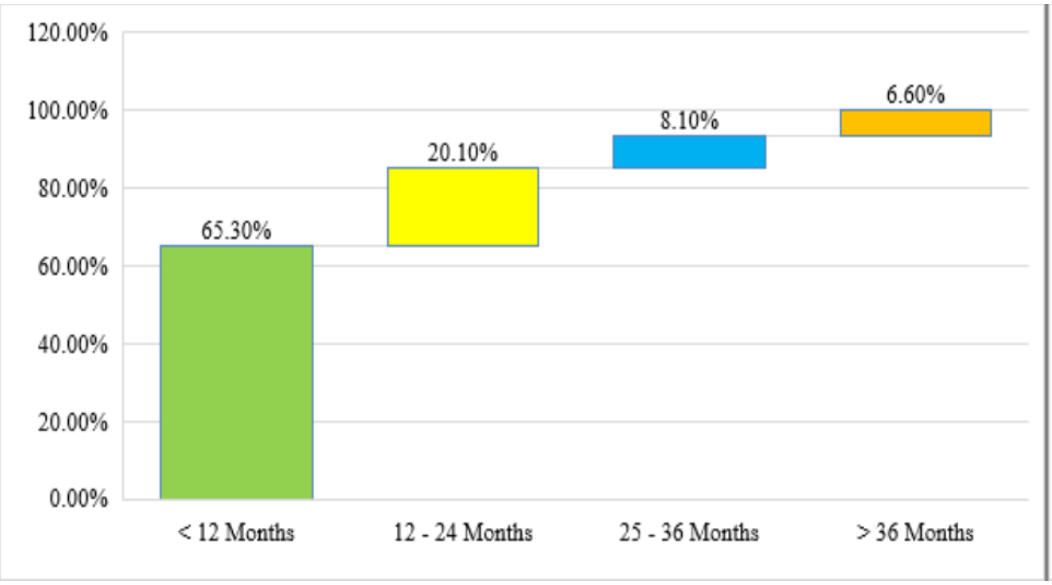

Figure 1 Basic information of studied sample – age group distribution

3.1. Body mass index (BMI)

BMI ranges less than 5th percentile, 10% children were overweight and 5% were obese as indicated in [Table 6 and Fig 3]. Average BMI with Standard deviation was 15.9±4.1.

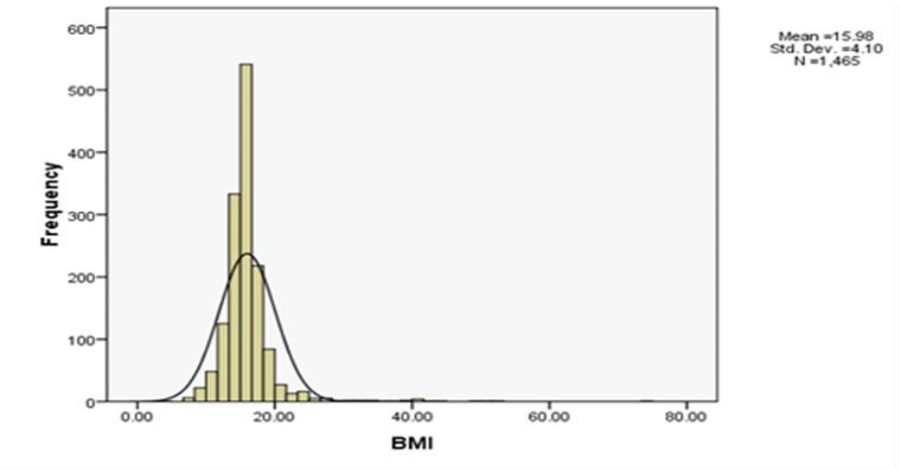

Figure 2 Body mass index (BMI)

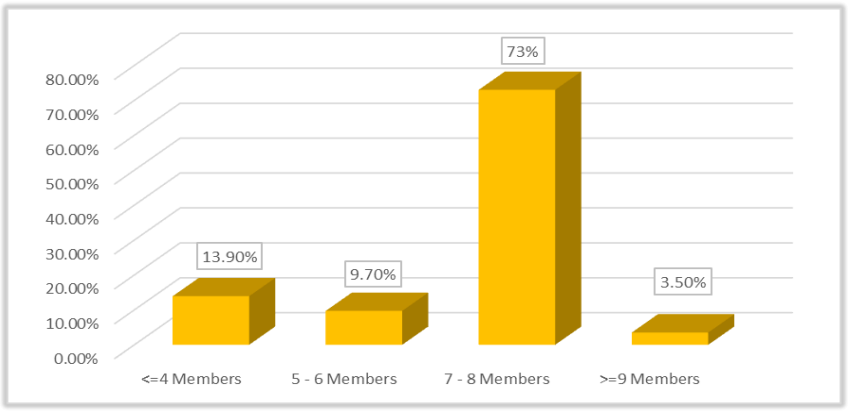

Figure 3 Number of family members

Figure 4 Personnel hygiene status

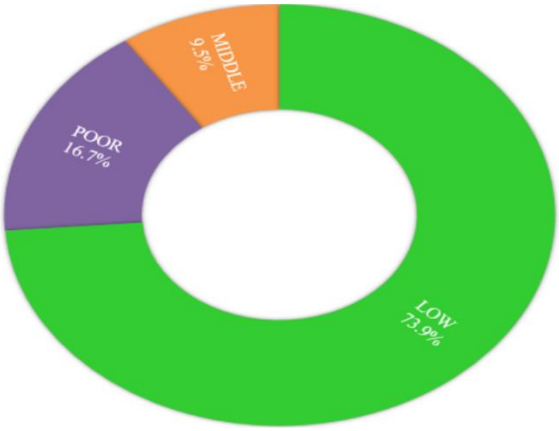

Figure 5 Socioeconomic status

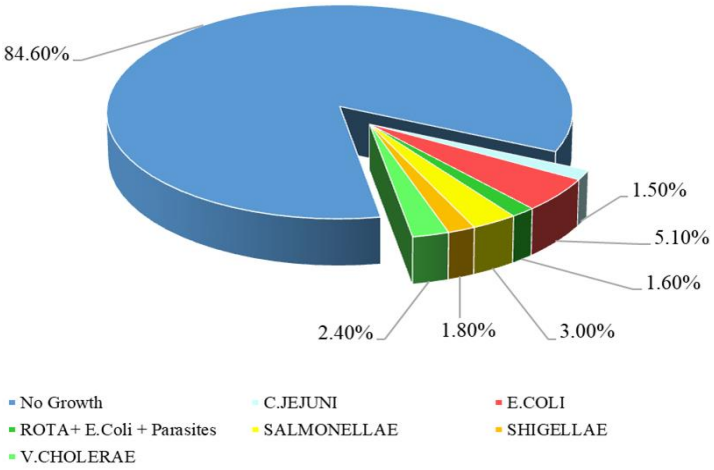

Figure 6 % Age distribution of bacterial pathogens

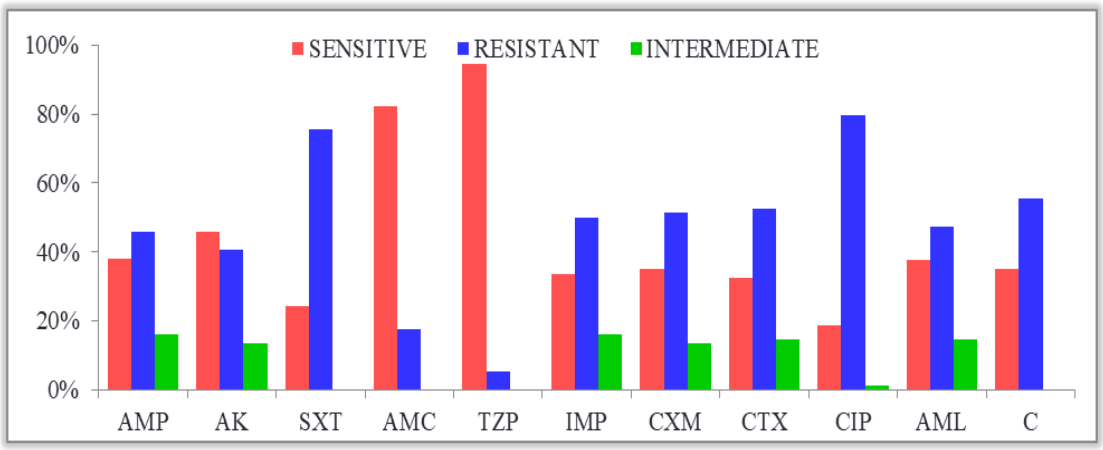

Figure 7 Antibiotic sensitivity patterns for *E. coli*

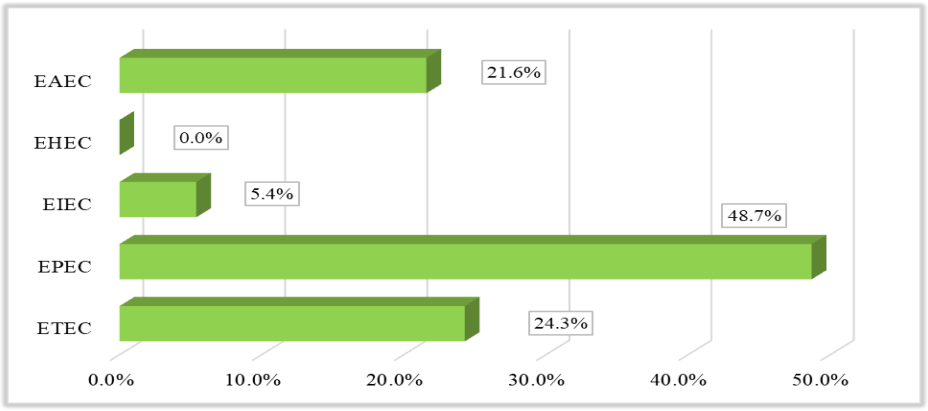

Figure 8 % Age of genotypes of pathogenic *E. coli*

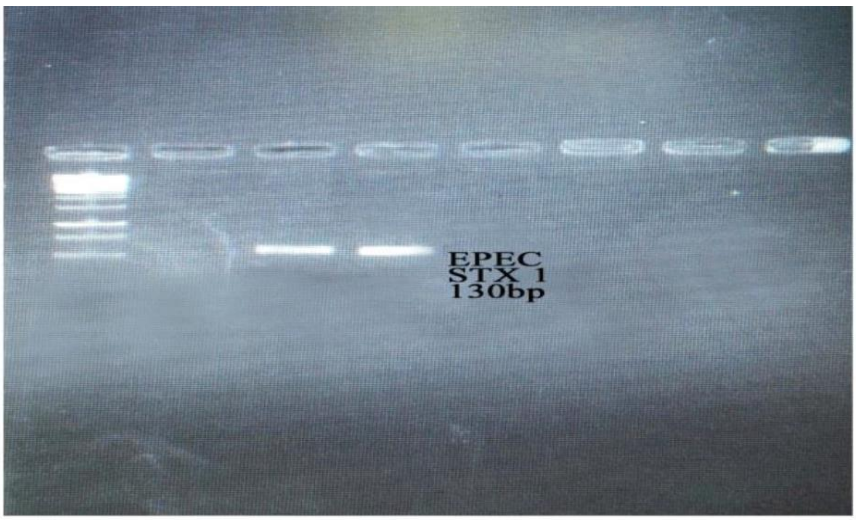

Figure 9 EPEC genotype 130 bp expression of STX 1 genes

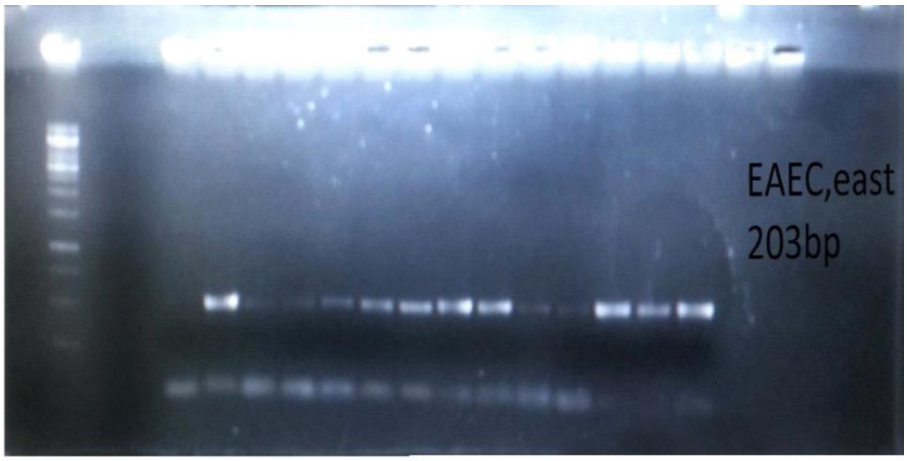

Figure 10 EAEC Genotype 203 bp expression of east genes

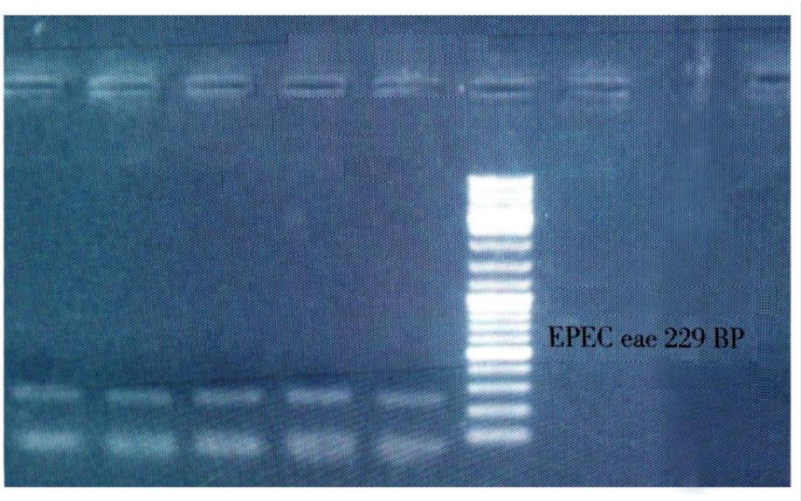

Figure 11 EPEC Genotype 229 bp expression of eae genes

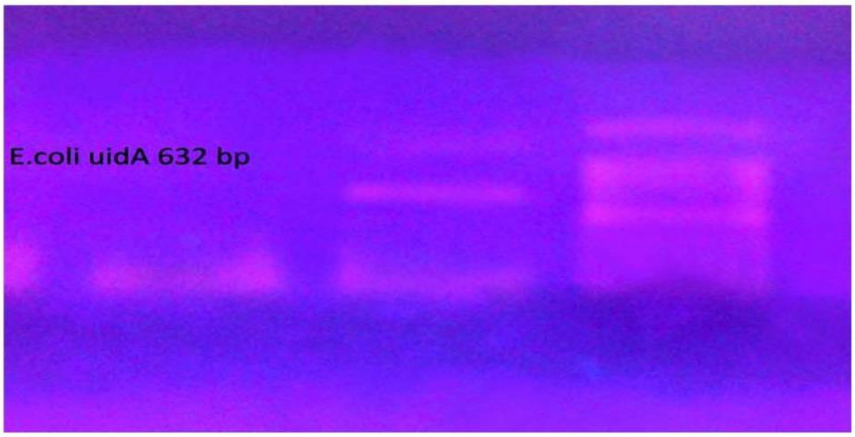

Figure 12 *E. coli* uidA 632 bp expression of genes

4. Discussion

Diarrheal diseases kill more than 700 thousand children annually although it is preventable manageable disease. Major causes of infantile deaths in children < five years of age. In a WHO 2015 report, about 1.7 billion cases of diarrhea were registered accounting 9% of all deaths among children in early childhood [12]. They are still resulting in an extra burden on health related budget of different countries. In 1996, almost 2.5 million people died due to diarrheal illnesses. 1.87 million diarrheal deaths contribute annually to nearly 19% of the 10 million reported cases worldwide [13]. In Pakistan more than 150,000 young children die every year due to multiple episodes of diarrhea depriving children from essential nutrients leading towards malnutrition and stunted growth [14]. Most of the people in Karachi live below poverty line and majority of cases are reported from these areas. This study was designed by keeping this hard fact in mind that in Pakistan, a child dies after every minute due to diarrhea and no comprehensive study regarding the spectrum of pathogens involved is available and role of contributing risk factors in the spread of disease are not identified [16,17]. This sort of epidemiological surveillance studies can give better understanding of infection causing etiological agents affecting child health and could contribute to more effective approaches towards saving children's lives by knowing contributing risk factors as an exact cause of avoidable diarrheal episodes [18, 19]. In Pakistan, unfortunately more than 20 million children suffer from multiple episodes of diarrhea in a year while more than one billion diarrheal episodes occur every year among children younger than 5 years of age in socioeconomically developing countries causing 2 to 2.5 million deaths[20,21,22]. Majority of the patients (73.3%) when examined in the presence of medical experts had poor personnel hygiene status in this study and had 7– 8 family members

Escherichia coli strains are major causes of human infectious diseases, partly because of the wide variety of virulence mechanisms and pathotypes and new pathotypes continue to be described [23]. Diarrheogenic strains of *E. coli* possess distinct virulence factors which have been classified into six classes, namely Entero-pathogenic, *E. coli* (EPEC), Entro-toxogenic *E. coli* (EIEC), Entero-hemorrhagic *E. coli* (EHEC), Entero-aggregative *E. coli* (EAEC) [24].

Enterotoxigenic *E. coli* (ETEC) are also known for causing traveler's diarrhea by ingesting contaminated food and water. The major virulence determinants are production of enterotoxins (LT) heat labile, (ST) heat stable as well colonizing factors (CFs) in proximal small intestine. In a study carried out in Bangladesh, approximately 14% of the infants and small children up to twelve months of age were suffering from diarrhea with ETEC [25]. It was reported that in Southern Israel among young children, ETEC is a causative diarrheal agent (12.9%), while in another study from Malaysia, the infection rate among young children with ETEC causing diarrhea is reported to be 61%, which is much higher than in Bangladesh. The difference in prevalence may be due to the better hygiene in Southern Israel [24]. In our study, 24.3% isolates were found to be ETEC. All of these isolates were (LT) toxin producers. None of the samples were positive for both heat labile (LT) and heat stable (ST) toxins production. The frequency of ETEC isolates in our study is in agreement with the neighboring countries, as we have the same living conditions including low personal and public hygiene standards. The toxin production from these ETEC strains differs from those reported in Bangladesh. They had more ST producing strains in contrast to our study as we found more LT producing strains. In developing countries entero pathogenic *E. coli* (EPEC) is considered as leading cause of infantile diarrhea, whereas sporadic diarrhea in developed world [25]. The EPEC (bfpA) gene encodes protein involved in bundle forming Pilus. *E. coli* (eae) genes are major virulent genes for attaching and effacing lesion formation. The strain possessing both genes (bfpA+, eae+) are termed as typical EPEC, having only one gene (eae+, bfpA-) are called atypical EPEC isolates [24].

In a study conducted in Brazil 11.2% - 68.4% strains were reported as EPEC serotypes was the cause of diarrhea. In young children of southern Israel, the prevalence of EPEC strain is reported to be lower (7.3%) [25]. A study conducted in Australia during an outbreak of infantile diarrhea, 59% cases of EPEC were found to be positive. It is reported from developed countries that atypical EPEC strains (eae+ bfpA-) are prevalent as compared to typical EPEC strains (eae+ bfpA+). In a study carried out in Magnolia, they found both typical and atypical strains causing diarrhea. [26].In this study, it was identified that EPEC strains causing diarrhea are in 48.6% cases. Out of these EPEC strains, 32% were atypical EPEC strains (eae, bfpA+) while 64% were (eae+, bfpA) typical EPEC strains. Our results correspond with the results of other countries i.e. the prevalence of atypical EPEC strains is almost same as reported in Australia and Brazil [27].

Another pathotype of *E. coli* i.e. Entero hemorrhagic *E. coli* (EHEC) is involved in causing bloody diarrhea [28]. EHEC is characterized by the production of one or two toxins SLTI and SLT-II coded by genes Stx2. Human infection due to EHEC are primarily associated with the consumption of raw meat. In this study no EHEC was found, most probably due to age restricted study subjects and in our community norm of well cooked food is practiced. 5.4% EIEC strains are found in the diarrheal patients included in this study. Low prevalence might be due to reason that we did not included patients presenting with bloody diarrhea in this study. EIEC in our study population incidence was low which could be attributed to our social norm of not consuming raw meat by the people specially children. We use very well cooked meat. Enteroaggregative Escherichia coli (EAEC) is a pathotype of the *E. coli* which is reported both in developing and developed countries causing infectious diarrhea by its specific aggregative mechanism of adhesion to the intestinal tissue cells worldwide [29]. EAEC strains produce the enterotoxin EAST I, a homologous toxin to the heat-stable (ST) enterotoxigneic *E. coli* with not well understood mechanism [30]. Enteroaggregative *E. coli* (EAEC) strains were reported with the complain of persistent watery diarrhea in children [31] as in India Mexico, Brazil, England, Kenya, Nigeria and Jordan [32] with a prevalence ranging from 11.9% - 19.6%. Our study demonstrated that Enteroaggregative *E. coli* were involved in a moderate proportion of cases of diarrhea included i.e. (21.6%) among children from different parts of Karachi. All were positive for east genotype]. Enteroaggreggative *E. coli* (EAgEC) strains are especially important because of their epidemiological association with persistent diarrhea among infant in developing countries [32].

5. Conclusion

It is reported that in developing countries, on average every child is exposed to three episodes of diarrhea annually, every time each episode deprives them from essential nutrition leading towards malnutrition and stunted growth, which make them prone towards diarrhea and other disease. High level of antibiotic resistant strains reported in this study is a matter of great concern, there is a need of legislation against blind therapy. The overall antibiotic resistance levels against some commonly prescribed drugs was higher due to misusage of antimicrobial agents. Periodic monitoring of the antimicrobial susceptibility pattern is very important in the management of diarrhea in under-five children. Epidemiological factors such as lack of fresh water supply, unhygienic septic tank, low family income, lack of

health information, and low educational level of parents could contribute to the high morbidity of diarrhea in children. These are recorded as contributing risk factors in the transmission of pathogen in infectious diarrheal episodes. Therefore, it is suggested that boiled drinking water and high sanitation standards as well as clean environmental interventions and hand washing be adopted to reduce the high incidence of avoidable diarrheal episodes in young infants and to reduce hospitalization and treatment cost in socioeconomically deprived and marginalized suffering population. All these interventions and proper management by focused attentions incidence of diarrheal patients may reduce emergence of antimicrobial resistance among diarrheal pathogens as well as high morbidity and mortality rate in children less than five years of age.

Compliance with ethical standards

Acknowledgments

This study was conducted with cooperation of IIDRL lab of University of Karachi with the permission of ethical review committee permission.

Disclosure of conflict of interest

It is declare that there is no conflict of interest among authors regarding this research article.

Statement of ethical approval

The present research work was conducted after the approval of ethical review committee of University of Karachi as well from public and private hospitals from where patients were recruited.

Statement of informed consent

Informed consent was taken by patient or by attendant.

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