ABSTRACT

**Aim:** Multidrug resistance (MDR) is a major public health problem worldwide. The aim of this study was to assess the prevalence, levels of antimicrobial susceptibility and extended-spectrum beta lactamase (ESBL) production by *Escherichia coli* O157: H7.

**Methodology:** A cross sectional study was performed and a total of 116 stool samples were collected from children aged ≤ 5 years presenting diarrhoea from hospital located at district Khairpur.
Khairpur. E. coli O157: H7 was isolated on Sorbitol MacConkey agar and isolates were initially characterized by Gram staining. Subsequently biochemical characteristics except oxidase test (which was performed manually) and drug sensitivity test was performed by using Automated MicroScan® walkaway machine. ESBL production by isolates was determined by disc diffusion method as per Clinical & Laboratory Standards Institute (CLSI) guidelines.

Results: Out of 116 diarrheal samples tested, 16 (13.79%) samples were positive for E. coli O157:H7. High number of E. coli O157: H7 were isolated from children aged up to 1 year followed by 2-3 years aged. Isolates were frequently found in summer season (12 times) followed by winter (3 times) and Autumn (1 time). The isolates were found 100% resistant against Nalidixic acid, Imipenem, Amoxycllin /Clavulanic Acid, Norfloxacin, Chloramphenicol, Vancomycin, Doxycycline, and Ampicillin and 90% resistant to Cifixime. ESBL production was noted in 12 (75%) out of 16 E. coli O157:H7 isolates as confirmed by CLSI protocol.

Conclusion: This study revealed that E. coli O157: H7 should be considered as major pathogenic bacterium causing diarrhea in children less than three years. The prevalence of ESBL producer MDR E. coli O157: H7 is of great concern, which requires monitoring of infection control measures through efficient antimicrobial management to ensure an uncompromised public health.

Keywords: Prevalence; Multidrug resistance; antibiotics; ESBL.

1. INTRODUCTION

Diarrhoeal disease is one of the leading causes of mortality among children of less than five years age [1]. It is accountable for the mortality greater than 1,400 children each day [1]. This disease is particularly prevalent in developing countries where sanitation and hygiene conditions are very poor and safe drinking water is scarce. Bacteria especially Escherichia coli is major contributor in causing enteric infection in children. There are various pathogenic strains of E. coli linked with diarrhoea, known as diarrhoeagenic E. coli. These include Shiga toxin-producing E. coli (STEC) which is also known as Verocytotoxin-producing E. coli (VTEC) or enterohemorrhagic E. coli (EHEC), Enterotoxigenic E. coli (ETEC), Enteropathogenic E. coli (EPEC), Enteroreaggregative E. coli (EAEC), Enteroinvasive E. coli (EIEC) and Diffusely adherent E. coli (DAEC) [2]. EHEC serotype E. coli O157:H7 is accountable for 20% of epidemic diarrhea worldwide [3]. This serotype is responsible for causing hemolytic uremic syndrome (HUS) in humans. HUS causes hemolytic anaemia, thrombocytopenia and renal failure by causing bloody diarrhoea and extreme abdominal pain, which leads to the formation of clots in capillaries. Rehydration, fever, and pain relief are the only medications available to treat this infection [4-6].

Multidrug-resistant E. coli have emerged as a major public health issue around the world [7] and due to the effectiveness of the outer membrane (OM) as a barrier and multidrug efflux pumps, the E. coli O157:H7 strain has much higher intrinsic levels of resistance to different antibiotics. Efflux pumps (EPs) decrease OM permeability, lowering antibiotic absorption and resulting in drug resistance [8]. EPs affects almost all antibiotic groups, including Macrolides, Tetracyclines, and Fluoroquinolones. In addition, the E. coli O157:H7 also produce extended-spectrum beta-lactamas (ESBLs), which render them resistant to many antibiotics and these strains have been widely reported in recent years on a global scale [9]. Indeed, Enterobacteriaceae that produce ESBLs are linked to higher rates of morbidity and mortality, longer hospital stays, and higher healthcare costs [10]. According to previous reports ESBL have been related to the development of antibiotic-resistant bacterial strains and due to the spread of these strains, antimicrobials used to treat patients with pathogen bacteria such as E. coli are likely to become less successful [11]. Since antimicrobial resistance among enteric bacteria is an increasing global public health concern which limits the treatment options for bacterial infections. Furthermore, antimicrobial resistance also has negative effects on human health and the environment, this study was designed to evaluate prevalence of multidrug resistant E. coli O157:H7 causing diarrhoea in children of District Khairpur, Sindh, Pakistan. In addition to this, levels of antimicrobial susceptibility and extended-spectrum beta lactamase (ESBL) production by E. coli O157: H7 was also assessed.
2. MATERIALS AND METHODS

2.1 Study Design and Study Area

A cross-sectional study was conducted from December 2019 to December 2020 to evaluate prevalence of multidrug resistant E. coli O157:H7 causing diarrhoea in children of District Khairpur, Sindh, Pakistan. Sampling was carried out over a period of 12 months to cover all the four seasons. Children aged ≤ 5 years and presenting severe diarrhoea and admitted to hospital Pir Abdul Qadir Shah Jeelani Institute of Medical Sciences Gambat Khairpur (PAQSJIMS) were included in this research. The patients who had taken antibiotics in last 72 h and critically ill patients were excluded from this study.

2.2 Sample Size and Collection of Fecal Samples

Sample size was determined using the formula reported previously [9]. A total of one hundred sixteen (116) stool samples were collected from both male (58) and female (58) patients equally whose parents gave consent. Rectum stool swab samples were collected and placed in a cairy-blair transport media. Subsequently these were transported to the Microbiology research laboratory of PAQSJIMS at 4°C in polystyrene cool box [12]. A data regarding the age, area, water source in households, feeding stage and antibiotics usage was collected.

2.3 Isolation of E. coli O157:H7

Stool swab samples were inoculated onto Sorbitol containing MacConkey agar plates and incubated aerobically at 37°C for 24 hrs [12]. Subsequently light-yellow colored colonies (a characteristic of E. coli O157:H7 by fermenting sorbitol, a carbohydrate) were sub-cultured on Eosin Methyline Blue (EMB) agar, and then incubated aerobically at 37°C for 24 hrs. The green metallic sheen colonies appearing on EMB were picked and sub-cultured to nutrient agar for obtaining pure cultures [12,13].

2.4 Characterization and Drug Sensitivity Test of E. coli O157:H7

E. coli O157:H7 isolates were initially characterized by Gram staining. Subsequently, biochemical characteristics except oxidase test (which was performed manually by soaking a filter paper piece with 1% tetramethyl-p-phenylene-diamine dihydrochloride and then inoculating culture with sterilized stick on moist area of filter paper) and drug sensitivity test was performed by using Automated MicroScan® walkaway machine [12-14]. Antibiotics tested were Amikacin (AK), Amoxycillin /Clavulanic Acid (AMC), Ampicillin (AMP), Aztreonam (ATM), Cefepime (FEP), Ceftazidime (CAZ), Cefotaxime (CTX), Ceftazidime (CAZ), Chloramphenicol (C), Cefixime (CFM), Ciprofloxacin (CIP), Doxycycline (DO), Imipenem (IMP), Levofloxacin (LEV), Nalidixic acid (NA), Norfloxacin (NOR), Piperclillin/Tazobactam (TZP), Trimethoprim/ Sulfamethoxazole (SXT), Vancomycin (VA). The results for drug sensitivity test were recorded as susceptible, intermediate, and resistant.

2.5 Molecular Identification of E. coli O157:H7 Isolates

16SrRNA gene based molecular identification was performed for confirmation of E. coli O157:H7 isolates. For this samples were sent to Macrogen Company (Korea) for commercial analysis. After obtaining sequencing results data was analyzed using NCBI Blast available free online.

2.6 Extended Spectrum Beta Lactamase (ESBL) Production by Isolates

Since Microscan machine indicated/assumed the ESBL production by majority of isolates, a phenotypic test as per CLSI guidelines was performed and for this test both cefotaxime (CTX, 30 μg) and ceftazidime (CAZ, 30 μg) disks alone and in combination with clavulanate (CA, 30μg) (Oxoid) were placed on Mueller Hinton Agar plates containing E. coli O157:H7 bacterial lawn. The test was interpreted positive when the growth-inhibitory zone around either the CTX with CA or CAZ disk with CA was ≥ 5 mm [15].

2.7 Statistical Analysis

Where required the samples were processed in triplicates and results were declared in average ± standard deviation. The Chi square test was performed to detect the significant difference in male and female by using XLSTAT365-Freemium.
3. RESULTS

3.1 Prevalence of *E. coli* O157:H7

Out of 116 diarrheal samples tested, 16 (13.79%) samples were positive for *E. coli* O157:H7 as confirmed by cultural characteristics, microscopic characteristics, Biochemical characteristics (Table 1, Fig. 1) and 16 SrRNA sequencing (Fig. 2).

3.2 Demographic Analysis

Out of 116 samples tested (Males: 58, Females 58), 9 (56.25%) *E. coli* O157: H7 strains were isolated from males and 7 (43.75%) from females. (Table 2). The Chi square test did not show significant difference in gender groups (P=0.06).

3.3 Seasonal Distribution of *E. coli* O157:H7

*E. coli* O157: H7 (Total isolates=16) were isolated more frequently in summer season (12 times) followed by winter (3 times) and Autumn (1 time).

3.4 Antibiotic Resistance Pattern

All the bacterial isolates (N=16) were resistant to most of the antibiotics tested in this study. Phenotypic resistant profile for isolates is shown in Fig. 3.

3.5 ESBL Production

ESBL production was noted in 12 (75%) out of 16 *E. coli* O157:H7 isolates as confirmed by CLSI protocol (Fig. 4).

4. DISCUSSION

Diarrhoeal illness is one of the most important health related problem globally. More than 2 million of mortality happens from diarrhoea among children of below five years age especially in developed countries [16]. *E. coli* is most frequent contributor in causing diarrhea in children. The result of this study revealed high prevalence of *E. coli* O157:H7 in the sample studied, corresponding to children <5 years age. These results are almost similar to some studies [17-19] and higher compared to developed countries [20,21].

Table 1. Cultural, microscopic and biochemical characteristics for confirmation of *E. coli* O157:H7

| Characteristics                                | Result                                      |
|------------------------------------------------|---------------------------------------------|
| 1. Cultural characteristics                   |                                             |
| Colonial characteristics on EMB agar           | Green metallic sheen colonies              |
| Colonial characteristics on Sorbitol-         | Colorless colonies (Indicative of Sorbitol  |
| MacConkey agar                                 | negative)                                  |
| 2. Microscopy                                  | Gram staining                              |
| Gram Negative, rods                           |                                             |
| Motility                                      | Positive                                   |
| 3. Biochemical tests                           |                                             |
| Catalase                                      | Positive                                   |
| Oxidase                                       | Negative                                   |
| Indole                                        | Positive                                   |
| Methyl red                                    | Positive                                   |
| Voges Proskauer                               | Negative                                   |
| Citrate                                       | Negative                                   |
| Urease                                        | Negative                                   |
| Nitrate reduction                             | Positive                                   |
| 3.1. Sugar fermentation tests                 |                                             |
| Glucose                                       | Positive                                   |
| Maltose                                       | Positive                                   |
| Lactose                                       | Positive                                   |
| Sorbitol                                      | Negative                                   |
Fig. 1. Cultural and microscopic characteristics of *E. coli* O157:H7: A) Green metallic sheen on EMB agar; B) Colorless colonies on Sorbitol-MacConkey agar; C) Gram negative rods

Fig. 2. Phylogenetic tree showing resembling of isolated strain with *E. coli* O157:H7

Fig. 3. Antibiotic susceptibility pattern of isolated *E. coli* O157:H7 strains
Table 2. Age and gender wise distribution of E. coli O157: H7 isolates

| Age group (Years) | Male | Female | Total | (%) Prevalence |
|-------------------|------|--------|-------|---------------|
| 0-1               | 06   | 04     | 10    | 8.62%         |
| 2-3               | 03   | 03     | 06    | 5.17%         |
| 4-5               | 0    | 0      | 0     | 0             |
| Total             | 09   | 07     | 16    | 13.79%        |

Fig. 4. ESBL confirmatory test: A) CTX and CAZ alone with no zone of inhibition; B) CTX + CA and CAZ + CA with zone of inhibition > 5 mm which confirms the ESBL production

In this study, when age groups were compared, it was noted that children with age < 1 year were more susceptible in getting infection with E. coli O157: H7. Increased age decreased the chances of getting infection. The low incidence among elder children must be linked with developed immune system with the increase of age [21]. Previous studies have also reported E. coli O157:H7 as the major pathogen causing childhood diarrhoea below two years age in developed countries [22,23]. Although the difference in gender group was observed but it was not significant. This could be because there is no difference in social contact of children and growing children do not have much hormonal difference that could have effect in causing enteric disease.

Season wise distribution of E. coli O157:H7 in this study revealed that the infection caused by this organism was more disseminating in summer followed by winter season. A similar pattern has been noted in previous studies [24,25], representing that E. coli O157:H7 was associated with ecological factors including heat and moisture.

In this research 100% resistant among E. coli O157: H7 was observed against NA, IMP, AMC, NOR, C, VA, DO, AMP and 90% resistant to CFM.

ESBLs production have been noted in Enterobacteriaceae since last few decades. Infections caused by such bacteria often limit the treatment options and thus cause therapeutic failures. Thus, CLSI has suggested that a phenotypic confirmatory test for ESBL development be performed to accurately detect ESBL production. In this study this test was performed to detect the ESBL production by E. coli O157: H7 and results revealed the presence of ESBL producer (75%) bacterial isolates. These results support the studies conducted in other parts of world [26,27]. The prevalence of multidrug resistance among bacteria and production of ESBL production is alarming. Physicians in Pakistan should pay attention to these results since E. coli is still one of the most common pathogens associated with infectious diarrhea in children.

5. CONCLUSION

The high prevalence of E. coli O157: H7 in this study is worrisome, and it emphasizes the fact that this organism is significant cause of infectious diarrhea in children less than three
years in Khairpur region. In addition, high resistance among isolates to commonly used antibiotics for treating diarrhea is alarming which requires routine laboratory identification of pathogen(s) and antibiotic susceptible testing. Active surveillance is also required in devising strategies to counter multidrug resistance in diarrheal cases of this region of Pakistan.

DISCLAIMER

The products used for this research are commonly and predominantly use products in our area of research and country. There is absolutely no conflict of interest between the authors and producers of the products because we do not intend to use these products as an avenue for any litigation but for the advancement of knowledge. Also, the research was not funded by the producing company rather it was funded by personal efforts of the authors.

CONSENT

A written consent from patient attendants has been collected and preserved by the authors.

ETHICAL APPROVAL

Ethical approval from medical superintendent of PAQSJIMS and Research Ethics Committee of our Institute was obtained.

ACKNOWLEDGEMENT

The authors acknowledge Institute of Microbiology, Shah Abdul Latif University, Khairpur, Sindh Pakistan for providing laboratory facilities to perform experimental work.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

REFERENCES

1. WHO. Progress on drinking water, sanitation and hygiene: 2017 update and SDG baselines; 2017.
2. Gould LH, Kline J, Monahan C, Vierk K. Outbreaks of disease associated with food imported into the United States. Emerg Infect Dis. 2017;23(3):525-8.
3. Holmes KK, Bertozzi S, Bloom BR, Jha P, Gelband H, DeMaria LM, Horton S. Major infectious diseases: key messages from disease control priorities. 2017;1-27.
4. Wong CS, Jelacic S, Habeeb RL, Watkins SL, Tarr PI. The risk of the hemolytic–uremic syndrome after antibiotic treatment of Escherichia coli O157: H7 infections. New Eng J Med. 2000;342(26):1930-6.
5. Kreft H, Jetz W. Global patterns and determinants of vascular plant diversity. Proceedings of the National Academy of Sciences. 2007;104(14):5925-30.
6. Blount ZD. The natural history of model organisms: The unexhausted potential of E. coli. Elife. 2015;4:e05826.
7. Dobiasova H, Dolejska M, Jamborova I, Brhelova E, Blazkova L, Papousek I, et al. Extended spectrum beta-lactamase and fluoroquinolone resistance genes and plasmids among Escherichia isolates from zoo animals, Czech Republic. FEMS Microbiol Ecol. 2013;85:604-11.
8. Amaral L, Martins A, Spengler G, Molnar J. Efflux pumps of Gram-negative bacteria: What they do, how they do it, with what and how to deal with them. Front Pharmacol. 2014;4:168.
9. Holstein A, Grillon A, Yzon L, Morange V, Baty G, Lartigue MF, Mereghetti L, Goudeau A, Lanotte P. Prevalence of extended-spectrum beta-lactamases of the CTX-M type producing Escherichia coli and Klebsiella pneumoniae in Bretonneau hospitals (CHRU Tours). Pathologie-Biologie. 2009;58(1):67-9.
10. Dembélé R, Konaté A, Traoré O, Kaboré WA, Soulama I, Kagambéga E, et al. Extended spectrum beta-lactamase and fluoroquinolone resistance genes among Escherichia coli and Salmonella isolates from children with diarrhea, Burkina Faso. BMC pediatrics. 2020;20(1):1-9.
11. Picозzi SC, Casellato S, Rossini M, Paola G, Tejada M, Costa E, Carmignani L. Extended-spectrum beta-lactamase-positive Escherichia coli causing complicated upper urinary tract infection: Urologist should act in time. Urology Ann. 2014;6(2):107.
12. Irwin PL, Nguyen LHT, Paoli GC, Chen CY. Evidence for a bimodal distribution of Escherichia coli doubling times below a threshold initial cell concentration. BMC Microbiol. 2010;10(1):207.
13. Paresh K, Virpari, JB, Nayak HC, Thaker MN. Brahmbhatt. Isolation of pathogenic Escherichia coli from stool samples of
diarrheal patients with history of raw milk consumption. Vet Worr. 2013;6(9):659-663.

14. Gould LH, Bopp CH, Stockbne N, Atkinson R, Baberski VB, Carey R, et al. Recommendations for Diagnosis of Shiga Toxin–Producing Escherichia coli Infections by Clinical Laboratories. MMWR-CDC, 58(RR12). 2009;1-14.

15. Clinical and Laboratory Stancolidards Institute. Performance standards for antimicrobial susceptibility testing. Twenty second informational supplement update. CLSI document M100-S2 U. Clinical and Laboratory Standards Institute, Wayne, PA; 2012.

16. Fard AH, Bokaeian M, Qureishi ME. Frequency of Escherichia coli O157: H7 in children with diarrhoea in Zahedan, Islamic Republic of Iran. East Mediterr Health J. 2008;14:1022-27.

17. Yu J, Jing H, Lai S, Xu W, Li M, Wu J, Liu W, et al. Etiology of diarrhea among children under the age five in China: results from a five-year surveillance. J Infect. 2015;71(1):19-27.

18. Tian L, Zhu X, Chen Z, Liu W, Li S, Yu W, et al. Characteristics of bacterial pathogens associated with acute diarrhea in children under 5 years of age: A hospital-based cross-sectional study. BMC Infect Dis. 2016;16:253.

19. Huang F, Deng Y, Qu M, Liu GR, Liu Y, Zhang X, et al. Etiological surveillance and analysis of infectious diarrhea in Beijing in year 2010. Zhonghua yu fang yi xue za zhi. Chin J Prevent. Med. 2011;45(9):820-4.

20. Jafari F, Garcia-Gil LJ, Salzmanzade-Ahrabi S, Shokrzadeh L, Aslani MM, Pourhoseingholi MA, et al. Diagnosis and prevalence of enteropathogenic bacteria in children less than 5 years of age with acute diarrhea in Tehran children's hospitals. J Infect. 2009;58(1):21-7.

21. Moyo SJ, Gro N, Matee MI, Kitundu J, Myrnel M, Mylvaganam H, Maselle SY, Langeland N. Age specific aetiological agents of diarrhoea in hospitalized children aged less than five years in Dar es Salaam, Tanzania. BMC Pediatr. 2011;11:19.

22. Canizalez-Roman A, Flores-Villasenor HM, Gonzalez-Nunez E, Velazquez-Roman J, Vidal JE, Muro-Amador S, Alapizco-Castro G, Diaz-Quinonez JA, Leon-Sicairos N. Surveillance of Diarrheagenic Escherichia coli strains isolated from diarrhea cases from children, adults and elderly at northwest of Mexico. Front Microbiol. 2016;7:1924.

23. Nakhjavani FA, Emaneini M, Hosseini H, Iman-Eini H, Aligholi M, Jabalameli F, Haghi-Ashtiani MT, Taherikalani M, Mirsalehian A. Molecular analysis of typical and atypical enteropathogenic Escherichia coli (EPEC) isolated from children with diarrhea. J Med Microbiol. 2013;62(Pt 2):191-5.

24. Gomes TA, Elias WP, Scaletsky IC, Guth BE, Rodrigues JF, Piazza RM, Ferreira LC, Martinez MB. Diarrheagenic Escherichia coli. Braz J Microbiol. 2016;47(Suppl 1):3-30.

25. Patzi-Vargas S, Zaidi MB, Perez-Martinez I, Leon-Cen M, Michel-Ayala A, Chaussabel D, Estrada-Garcia T. Diarrheagenic Escherichia coli carrying supplementary virulence genes are an important cause of moderate to severe diarrhoeal disease in Mexico. PLoS Negl Trop Dis. 2015;9(3):e0003510.

26. Carattoli A, Garcia-Fernández A, Varesi P, Fortini D, Gerardi S, Penni A, Mancini C, Giordano A. Molecular epidemiology of Escherichia coli producing extended-spectrum beta-lactamases isolated in Rome, Italy. J Clin Microbiol. 2008;46:103-108.

27. Basavaraj MC, Jyothi P, Peerpur BV. The prevalence of ESBL among Enterobacteriaceae in a tertiary care hospital of North Karnataka. India J Clin Diagn Res. 2011;5:470-475.

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Peer-review history: The peer review history for this paper can be accessed here: http://www.sdiarticle4.com/review-history/67894