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Prevalence of Multidrug Resistance and Virulence Genes in *Escherichia coli* Isolates from Hindon River Water, Western Uttar Pradesh, India

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ABSTRACT:
The prevalence of multidrug resistance (MDR) and virulence genes in *E. coli* of river water pose serious public health risks. Addition of Pollutants such as domestic and wild animal faeces, septic trench malfunctions, stormwater drainage, municipal and industrial waste contribute to water quality degradation. The contaminated river water currently serves as a prominent reservoir for diverse virulence genes in *E. coli* that exhibit antibiotic resistance. Therefore, it is essential to determine the prevalence of virulence genes and multidrug resistance in *E. coli*. The present study involved the isolation and characterization of *E. coli*, as well as assessing the prevalence of virulence genes and multidrug resistance in *E. coli.* The total *E.coli* isolates (n = 151) of the Hindon river water were investigated for their virulence genes of Shiga toxin-producing *E. coli* (EHEC), enterotoxin-producing *E. coli* (ETEC), enteropathogenic *E.* coli (EPEC) and susceptibility to (n =11) antimicrobial agents of six different classes of antibiotics. The virulence gene *stx1* in the EHEC was found to be highest 35.4% in the month of December and *stx2* gene was not detected. Whereas, virulence genes *LT1* and *ST1* for ETEC was maximum in the month of April and found to be 29% and 27% respectively. Similarly, virulence genes (*eaeA*, and *bfp)* in EPEC isolates were also highest in the month of April and found as 87% and 84% respectively. The MDR profile for EHEC, ETEC and EPEC showed more than 70% resistance to tetracycline and Ampicillin. The prevalence of multi-drug-resistant *E.coli* strains of EHEC, ETEC and EPEC in the water of Hindon river water poses a significant health risk of waterborne outbreaks.

Introduction

The Hindon river, a crucial tributary of the venerated Yamuna river, is used as the primary water source in Western Uttar Pradesh, India, since decades. The Hindon river originates from the Upper Shivalik region of India and it covers a distance of about 400 km before joining the Yamuna river on the outskirts of Delhi, and it flows through four major cities such as; Saharanpur, Muzaffarnagar, Meerut, and Ghaziabad. Additionally, the river Krishni and Kali are the two main tributaries that joins the Hindon river. Unfortunately, the unregulated discharge of domestic sewage, municipal byproducts, industrial byproducts, and agricultural runoff of toxic chemicals and pesticides has turned the river water unsafe for life. Moreover, the waste products from sugar, pulp, paper, distilleries, tanneries, waste water treatment plants (WWTPs), and sewage treatment plants (STPs) are carelessly discharged into the river without being fully or partially treated (Mishra et al., 2016; Sharma et al., 2021). The pollution level at the confluence of the Krishna and Kali rivers, the tributaries of the Hindon River, has been assessed to be highly polluted (Mishra et al., 2016).

The targeted detection of each pathogenic species is challenging because specific pathogens are present in a very low concentration in the river water (USEPA,2010). The regulatory agencies worldwide recommend *E. coli* as the principal indicator organism in river water. The OECD and WHO guidelines recommend the absence of total coliforms and *E. coli* in 100 ml of potable water. The pathogenic *E. coli* that produces Shiga toxin (*stx1* and *stx2*) spread to peoples

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through contaminated water and via direct contact of infected animals or humans (Bian et al., 2015; Chekabab et al., 2013; Mead & Griffin, 1998). In addition, this infection can potentially lead to hemolytic uremic syndrome, hemorrhagic colitis, and bloody diarrhoea (Kaper et al., 2004; Nataro & Kaper, 1998). Waterborne diarrhoeal disease kills 2 million peoples worldwide each year, primarily children under the age of five. An estimated 663 million peoples in the global population have regular access to improved drinking water sources (WHO, 2015; Gallay et al., 2006). The researches revealed that the *E. coli* O157:H7 can cause sickness or death even at low concentrations (10-100 cells). An extended detection interval for *E. coli* O157:H7 may result in antibiotic use without microbiological testing and enhancing the antibiotic resistance (Brunetti et al., 2021; Di Toma et al., 2023).

In their study Kotloff et al. (2013) reported that enterotoxigenic *E. coli* (ETEC) is a waterborne pathogen that significantly contributes moderate to severe diarrhoea in children, particularly in low and middle income countries. The symptoms of an ETEC infection can vary greatly ranging from mild diarrhoea to a severe sickness that closely resembles to cholera. It also affects adults who visit areas where the disease is prevalent. ETEC, which is one of the leading causes of diarrhoea globally, has been associated with more than 50,000 deaths and 223 million cases per year, as reported in a study on the Global Burden of Disease (Khalil et al., 2018). Upon colonisation of the ETEC in small intestine, the release of heat labile toxin (*LT*) and heat stable toxins (*ST*) occurs. Only a few studies have shown physicochemical and microbiological quality of Hindon river water (Bhutiani et al., 2017; Yadav et al., 2020). The seasonal variation regarding physicochemical and microbiological quality of water has also been recentlly studied (Anita et.al.,2023). Under this investigation it has been found that the river was heavily contaminated with fecal coliforms and unfit to use for any purpose (Anita et.al., 2023).

Moreover, distinct pathogenic strain of *E. coli* known as EPEC is a diarrheagenic pathotype in water. In low-income countries, these pathogens are frequently responsible for watery diarrhoea in infants, but they are rarely cause of diarrhoea in adults (Kaper et al., 2004). The presence of the EPEC, which includes the *eaeA* gene, distinguishes EPEC strain from other strains, which has been previously explained. Furthermore, The EPEC adherence factor (EAF) plasmid distinguishes typical EPEC strains from atypical EPEC strains. The plasmid encodes the bundle-forming pilus via the *bfp* operon. This pilus is considered to be responsible for the initial attachment of strains to intestinal epithelial cells (Kaper et al., 2004).

The overuse and misuse of antibiotics have developed global antibiotic resistance in microorganisms. According to the Centres for Disease Control and Prevention (CDCP), the United States has an annual incidence of over 2.8 million infections caused by antimicrobial drug resistance. Furthermore, over 35,000 people die each year as a result of infections caused by drug-resistant microbes (Reddy et.al.,2022). Antimicrobial drug resistance is estimated to kill 700,000 people worldwide each year. If no further action is taken, it is projected that the number of deaths will surpass 10 million by 2050, resulting in a global economic burden of around one hundred trillion dollars. (Li et al., 2023). Waste products discharged into river water by industrialization, urbanization, hospitals, agriculture, poultry, aquaculture, and animal husbandry are the primary source of selective pressure that leads to the development of antibiotic resistance (Karkman et al., 2016; Zhao et al., 2020). We previously have identified variations in physiological and biochemical characteristics in Hindon river water in western Uttar Pradesh in the year "2021" in the month of April, August, and December (Anita et al., 2023). Regardfully, additional study is necessary to examine the virulent types of *E. coli*, including EHEC, ETEC, and EPEC, and their antibiotic resistance levels in the Hindon river water. Therefore, the objective of the present study was to determine the occurrence of multidrug resistance *E.coli* harbouring significant virulence genes of EHEC, ETEC and EPEC strains in river water samples collected during various seasons from the multiple sites over the year in the months April, August, and December 2021 from Hindon river water, running in western Uttar Pradesh, India.

Materials and methods

Study area and sampling: The Hindon river that flows through the upper Shivalik region of the lower Himalayas. (Latitudes 28° 4' to 35° 5'N and longitudes 77° 8' to 77° 4'E) was chosen for the current study. The river maintains a sluggish current throughout the year except during the monsoon season. Under this investigation different sites of Hindon river from distinct locations were selected, which includes: (Site 1#: Star papermill Saharanpur; Site 2#: Muzzafarnagar merger of river Kali near Malira village; Site 3#: Meerut Baghpat Road Daluheda; Site 4#: Noida- Greater Noida extension; Site5#: Safipur village Greater Noida; Site 6#: Kulesra village, Greater Noida; Site 7#: Momnathal village, Greater Noida, Confluence of the river in the river Yamuna) (Fig.1). For the collection of the samples, sites of various human activities, including washing, cooking, bathing, littering, agricultural activities, animal rearing and the discharge of industrial waste into the river were identified. The activities mentioned above were considered while collecting samples for April, August, and December 2021 (Fig.1). The water samples were collected at a depth of 10-15 centimetres, approx. 5-6 metres from the river bank in 1L amber glass reagent bottles. The collected samples were kept at 4°C and

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analysed for faecal coliforms within 24 hours (Rice et al., 2012; APHA, 1998).

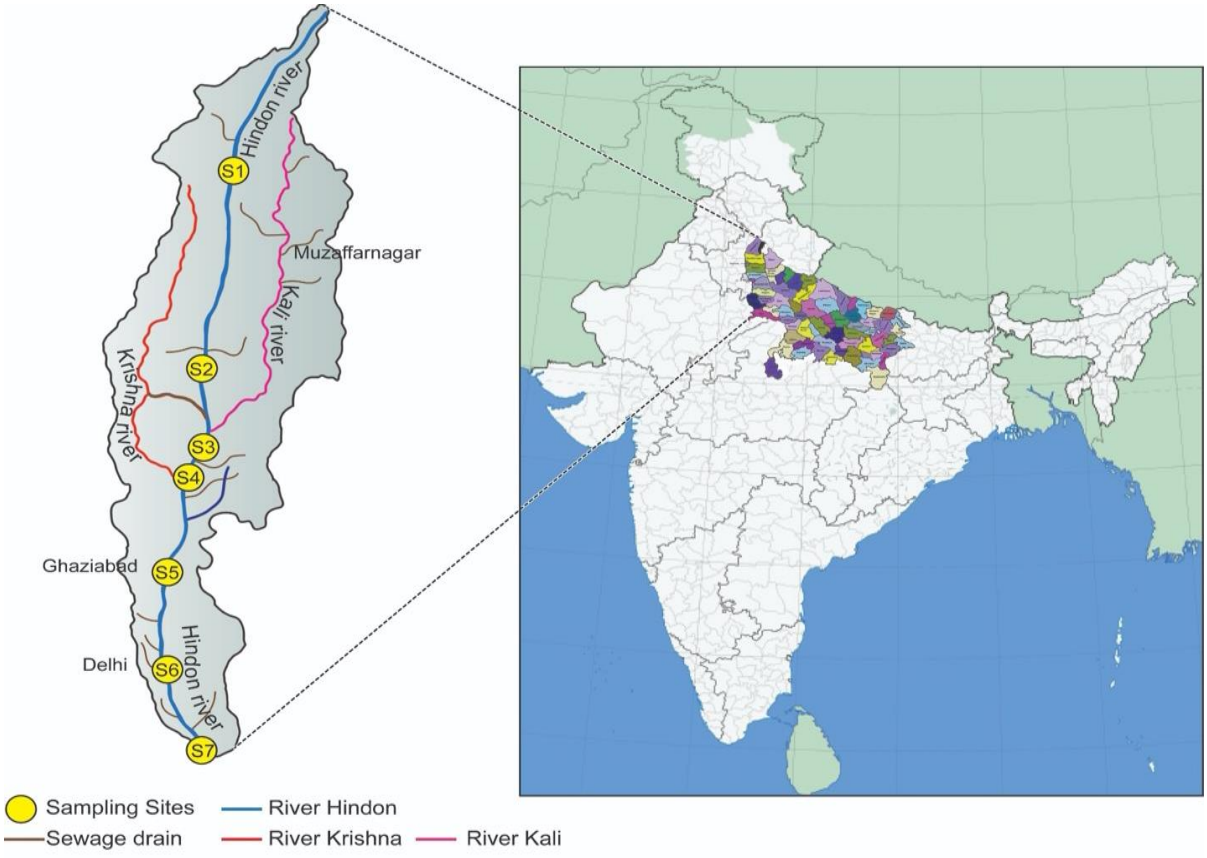

Fig.1. Various sampling locations along the Hindon river water, running in different districts of Western Uttar Pradesh, India

Isolation and identification of *E. coli strains*

Distinct *E. coli* isolates were isolated from the Hindon river and confirmed as earlier described by Ram et al. (2007). For different isolates of *E.coli*, 100 millilitres of water were collected from each sampling site (from the left, middle, and right sides) of the river Hindon was mixed. Thereafter, one millilitre of river water from each sampling site was mixed with 99 millilitres of sterile distilled water. These mixed samples were filtered in duplicate using a cellulose nitrate membrane filter with a pore size of 0.45 micrometres. The membrane filters were removed aseptically with sterile forceps, and each filter was cut into four pieces. These pieces were placed in 25 mL Erlenmeyer flasks containing 10 mL of MacConkey broth. The flasks were incubated at 37 (1 ±°C) for 24 hours at 220 rpm, with agitation provided by a rotary shaker (CLIMO SHAKER ISF1-X, Kuhner, Switzerland) set to two hundred and twenty revolutions per minute. Then, a single loopful of culture was evenly spread on Levine EMB (Eosin methylene blue) agar plates, which were then placed in an incubator and kept at 37 (1±°C) overnight. From each site, 20 blue-black colonies with green metallic sheen growing on EMB agar plates were randomly chosen for further investigation. These isolates were tested for ONPG, lysine utilization, ornithine utilization, urease, phenylalanine deamination, Vogus-Proskeur reaction, methyl red, indole, PYR, ß-glucuronidase, ß-galactosidase, ß-xylosidase, esculin hydrolysis, sucrose, sorbitol, trehalose, glucose, cellobiose and MUG-EC test. A pure strain of *E. coli* (ATCC-25922) was used as a positive control for biochemical characterization. All confirmed *E. coli* isolates were kept at -80 °C in Luria Bertani (LB) broth supplemented with 50% (v/v) glycerol for further studies.

Extraction of genomic DNA

DNA from different *E. coli* isolates was extracted using the Phenol-Chloroform method as described by Sambrook and Russell (2002). The obtained genomic DNA was subsequently analysed using electrophoresis on a 1% agarose gel. The purity and concentration of the

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genomic DNA obtained were determined through 260/280 nm absorbance measures using the NanoDrop spectrophotometer 2000 (NanoDrop 2000/2000c. *Spectrophotometer V1.0*. ThermoScientific).

Detection of virulent genes through PCR

All presumptive positive *E. coli* were isolated from Hindon river water and identified using traditional PCR. The *16S rRNA* and *uidA* gene of each isolate were amplified for the genetic characterization of the isolates (Osinska et al. 2023). Screening of *E. coli* isolates having virulence genes (*stx1, stx2, LT1, ST1, eaeA and bfp*) specific to enterohemorrhagic *E. coli*, enterotoxigenic *E. coli* and enteropathogenic *E. coli* was done using specific primers (Table 1).

Table 1. PCR primers utilized to amplify housekeeping and virulent genes specific for EHEC, ETEC and EPEC isolates.

Name	Primer sequence (5'-3')	Nt.	Annealing Temp.	Product Size	Reference
Common genes for all *E.coli*					
16sRNAF	TCCTACGGGAGGCAGCAGT	19	61	467bp	Ahmed et.al.,2015
16sRNAR	GGACTACCAGGGTATCTAATCCTGTT	26	63.2		
UidF	CAGTCTGGATCGCGAAAACTG	21	59.8	143bp	Jinneman et.al,2003; Godambe et. al.,2017
UidR	ACCAGACGTTGCCCACATAATT	21	58.4		
Enterohemorrhagic *E. coli* (EHEC)					
*Stx1*F	GTGGCATTAATACTGAATTGTCATCA	26	58.5	109bp	Lopez-saucedo et.al., 2003; Jinneman et.al,2003;Critzer et.al.2008
*Stx1*R	GCGTAATCCCACGGACTCTTC	21	61.8		
*Stx2*F	GATGTTTATGGCGGTTTTATTTGC	24	57.6		Jinneman et.al,2003
*Stx2*R	CGGGCACTGATATATGTGTAA	21	55.9	255bp	
Entero toxigenic *E.coli* (ETEC)					
*LT1*F	GGCAGGCAAAAGAGAAATGG	20	57.3	210bp	Patel et.al.,2011
*LT1*R	TTGGTCTCGGTCAGATATGTG	20	57.9		
*ST1*F*	CAACTGAATCACTTGACTCTTC	22	56.5	145bp	Ahmed et.al.,2007
*ST1*R	GCACAGGCAGGATTACAAC	19	56.7		
Enteropathogenic *E.coli* (EPEC)					
*eaeA*F	GACCCGGCACAAGCATAAGC	20	61.4	384bp	Gunzburg et al. 1995; Gomi et al.,2015; Paton & Paton 1998
*eaeA*R	CCACCTGCAGCAACAAGAGG	20	61.4		
*Bfp*F	AATGGTGCTTGCGCTTGCTGC	21	55	324 bp	Paton & Paton (1998)
*bfp*R	GCCGCTTTATCCAACCTGGTA	21			

To conduct PCR, 50-µL polymerase chain reaction assay mixture containing 5 µL 10X PCR buffer, 3 µL 25 mM magnesium chloride, 1 µL 10 mM dNTP (deoxyribonucleotide triphosphate), 2 µL genomic DNA (25–50 ng) or 5 µL total DNA, 1 µL Taq polymerase (1 unit/µL), and 1 µL of each primer (10 pmol) was prepared. PCR amplifications were carried out for 40 cycles for each gene, the initial denaturation was at 94°C for 4 min, and a final extension was carried out at 72°C for 7 min (Thermal cycler; BR-PCR-2000, BR Biochem, INDIA). The PCR-amplified products were resolved on 1.5% agarose gels containing ethidium bromide (0.5 µg/mL) at 3.5 V/cm, and were visualized and recorded using a (VILBER BIOPRINT, gel documentation system, France). The positive controls used were: *E. coli* ITRC-18 [Indian Institute of Toxicology Research (IITR) Lucknow, India] for *stx1*, *stx2*; *E. coli* MTCC-723 (Microbial Type Culture Collection, IMTECH, Chandigarh, India) for *LT1* and *ST1* genes. On the other hand, *E. coli* ATCC-43887 was utilized as a (+) control for the *eaeA and bfp* genes.

Determination of Susceptibility to Antimicrobials

The isolates positive for virulence genes specific to EHEC, ETEC and EPEC from each site were tested for susceptibility to 11 distinct antimicrobials of six different classes including: aminoglycosides: amikacin, Ak (10mcg), gentamicin, G (10 mcg), streptomycin, S (10 mcg); ß-lactams: amoxycillin, Ac (10 mcg); cephalosporins: cefixime Cfm (10 mcg), ceftazidime ca (30 mcg); fluoroquinolones: ciprofloxacin Cf (5 mcg), norfloxacin Nx (10 mcg); phenicol: chloramphenicol, C (10 mcg); tetracycline: tetracycline, T (30 mcg). These tests were performed as previously described (Bauer et al. 1966; Ram et al. 2007) using disc-diffusion method and antimicrobial impregnated paper discs (Hi-Media Ltd., Mumbai, India) according to protocols of Clinical and Laboratory Standards Institute (CLSI, 2018). Data for susceptibility to antimicrobials tested for each bacterial isolate was recorded as resistant, intermediate, and sensitive, based on Clinical and Laboratory Standards Institute break points (CLSI, 2018). For an antibiotic sensitivity test, both *E. coli* ATCC 35218 and *E. coli* MTCC443 were utilized as positive and negative controls, respectively. The antimicrobial sensitivity and virulence genes were analysed in triplicate (n=3) for each *E. coli* isolate.

Results

Journal of Chemical Health Risks
www.jchr.org
JCHR (2023) 13(6), 3586-3599| ISSN:2251-6727

Seasonal distribution of virulent genes in E.coli isolates

The virulence of potential EHEC was determined by the presence of virulence gene markers *stx1* and *stx2*. The level of *stx1* in the month of April, August and December was found as 29.5%, 25.4% and 35.4% respectively. But, virulence gene for *stx2* was not detected in any of the season. Further, virulence gene makers in the ETEC were *LT1* and *ST1*, and their level in the month of April was found as 29% and 27% respectively. However, the level fluctuated in the month of August and was found as 17% and 19% respectively. Interestingly, in the month of December the value seemed to increase and was found as 22% and 25% respectively. Moreover, the virulence gene markers in the EPEC were *eaeA*, and *bfp*, and their level in the month of April was found as 87% and 84% respectively. However, the level fluctuated in the month of August and was found as 65% and 60% respectively. In, the month of December the value increased and found as 75%and 80% respectively.

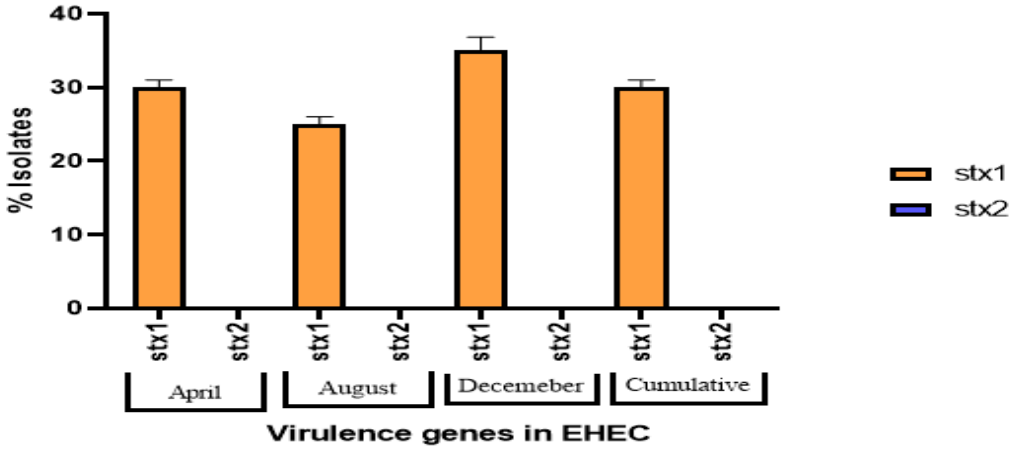

Fig:2. The virulence of potential EHEC strains and level of *stx1* and *stx2* genes during in the month of April, August and December of year 2021.

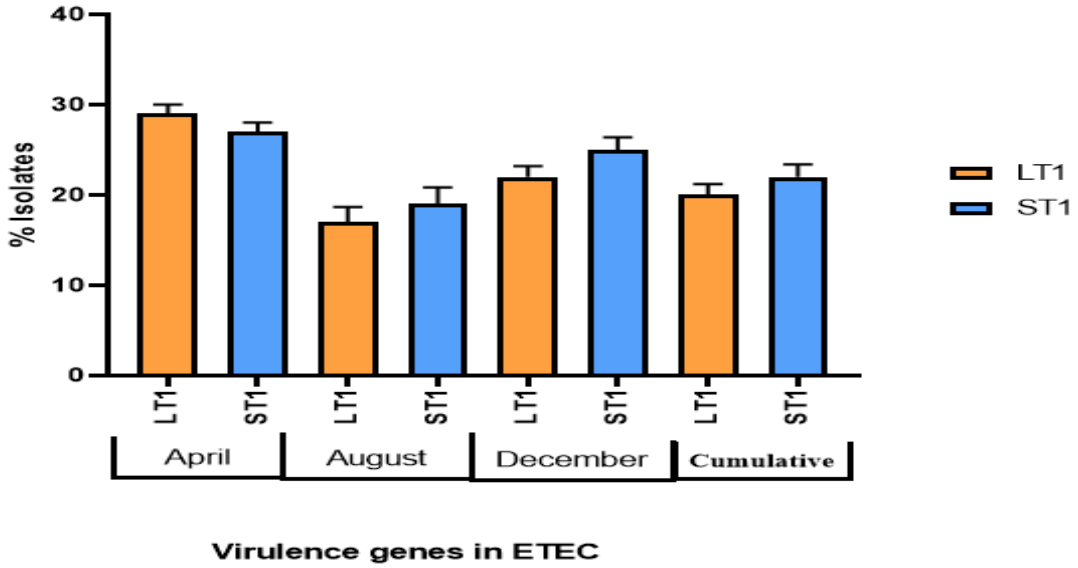

Fig:3. The virulence of potential ETEC strains and level of *LT1* and *ST1* genes during April, August and December of year 2021.

Journal of Chemical Health Risks
www.jchr.org
JCHR (2023) 13(6), 3586-3599| ISSN:2251-6727

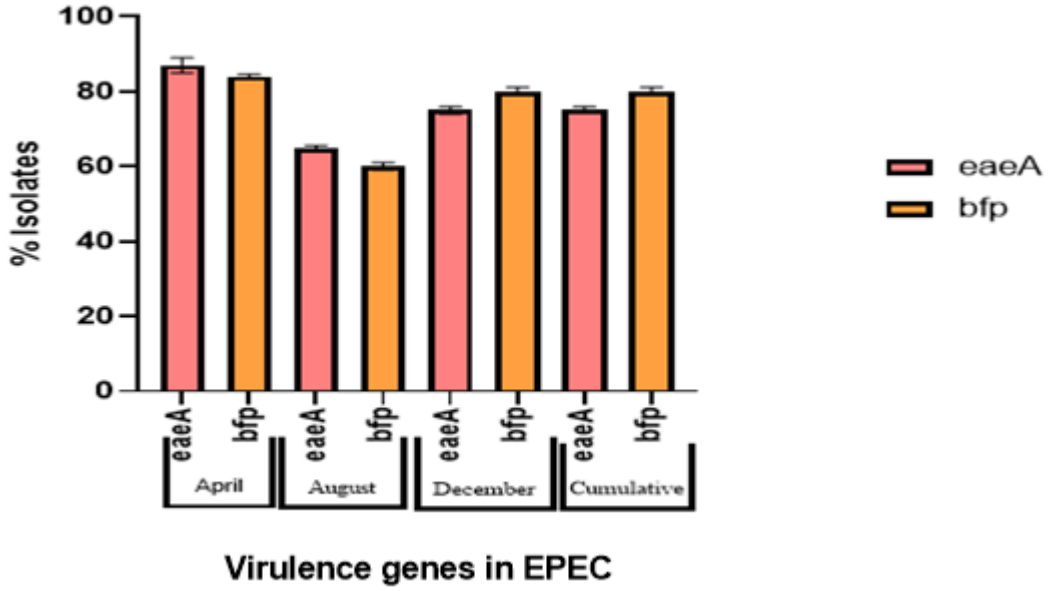

Fig:4. The virulence of potential EPEC strains and level of *eaeA* and *bfp* gene during April, august and December of year 2021.

Antibiotic profiling of EHEC strains

The antibiotic profiling of different EHEC were studied and it was observed that in the month of April the antibiotics amikacin (47.32%), gentamicin (34.32%), streptomycin (57.8%), amoxycillin (53%), ampicillin (75%), cefixime (22%), cetrazidime(28.4%), ciprofloxacin (7.14%), norfloxacin (28.5%), chloramphenicol (9.25%), and tetracycline (78%). The decreasing order of antibiotic susceptibility can be presented as tetracycline > ampicillin > streptomycin > amoxycyllin > amikacin > gentamicin > norfloxacin > cetrazidime > cefixime > chloramphenicol > ciprofloxacin in the month of April 2021.While this level in the month of august were fluctuated and found to be as: amikacin (9.32%)> gentamicin (5.23%)> streptomycin (17.8%)> amoxycillin (23%)> ampicillin (60%)> cefixime (23%)> cetrazidime (1%)> ciprofloxacin (0%)> norfloxacin (11.25%)> chloramphenicol (11.25%)> tetracycline (65%). Further, the decreasing order of antibiotic resistance can be presented as tetracycline < ampicillin < amoxycillin = cefixime < streptomycin < norfloxacin = chloramphenicol < amikacin < gentamycin. < cetrazidime and no resistance to ciprofloxacin. However, the levels increased in month of December and were found to be amikacin (40.3%), gentamicin (17.7%), streptomycin (55.8%), amoxycillin (35%), ampicillin (79%), cefixime (17.7%), cetrazidime (58.25%), ciprofloxacin (17.7%), norfloxacin (17.7%), chloramphenicol (6.2%), and tetracycline (84%). The decreasing order for the month of December 2021 was tetracycline > ampicillin > cetrazidime > streptomycin > amikacin > amoxycillin > cefixime = norfloxacin = ciprofloxacin = gentamicin > chloramphenicol (Figure 5).

Antibiotic profiling of ETEC strains

The antibiotic profiling of different ETEC were studied and it was found that in the month of April the antibiotics amikacin (51.32%), gentamicin (37.23%), streptomycin (59.80%), amoxycillin (38%), ampicillin (82%), cefixime (18%), cetrazidime (58%), ciprofloxacin (20%), norfloxacin (28.50%), chloramphenicol (8.25%), and tetracycline (92.00%). So, the decreasing order of resistant isolates was tetracycline > ampicillin > streptomycin > cetrazidime > amikacin > amoxycillin > gentamycin > norfloxacin > ciprofloxacin > cefixime > chloramphenicol. While this level in the month of august were fluctuated and found to be amikacin (10.32%), gentamicin (6.23%), streptomycin (18.80%), amoxycillin (25%), ampicillin (60%), cefixime (24%), cetrazidime (1%), ciprofloxacin (0%), norfloxacin (11.25%), chloramphenicol (11.25%), and tetracycline (65%). So, the decreasing level was tetracycline > ampicillin > amoxycillin > cefixime > streptomycin > norfloxacin = chloramphenicol> amikacin> gentamycine> cetrazidime. Further, the isolates were resistant to ciprofloxacin. Interestingly the levels increased in month of December and were found to be amikacin (43.30%), gentamicin (19.70%), streptomycin (40.80%), amoxycillin (38.40%), ampicillin (72.20%), cefixime (18.70%), cetrazidime (60.25%), ciprofloxacin (18.70%), norfloxacin (18.54%), chloramphenicol (6.2%), and tetracycline (70%). The decreasing order of % isolates resistance was ampicillin > tetracycline > cetrazidime > amikacin > streptomycin > amoxycillin >

Journal of Chemical Health Risks
www.jchr.org
JCHR (2023) 13(6), 3586-3599| ISSN:2251-6727

cefixime = gentamycin > ciprofloxacin > norfloxacin > chloramphenicol (Figure 6).

Antibiotic profiling of EPEC Strains

The antibiotic profiling of different EPEC were studied and it was found that in the month of April the antibiotics amikacin (42%), gentamicin (19%), streptomycin (59%), amoxycillin (38%), ampicillin (74%), cefixime (18%), cetrazidime(58%), ciprofloxacin (20%), norfloxacin (20%), chloramphenicol (8%), and tetracycline (95%). So, the decreasing order of antibiotic resistancetetracycline>ampicillin>streptomycin>cetrazidime>amikacin>amoxycillin>ciprofloxacin=norfloxacin>gentamycin>cefixime>chloramphenicol. While this level in the month of august were fluctuated and found to be amikacin (10%), gentamicin (5%), streptomycin (21%), amoxycillin (26%), ampicillin (60%), cefixime (22%), cetrazidime(0%), ciprofloxacin (0%), norfloxacin (10%), chloramphenicol (10%), and tetracycline (74%). The decreasing level of antibiotic Tetracycline > ampicillin > amoxycillin > cefixime > streptomycin > amikacin = chloramphenicol = norfloxacin > gentamycin. Interestingly the levels increased in month of December and were found to be amikacin (41%), gentamicin (18%), streptomycin (59%), amoxycillin (38%), ampicillin (77%), cefixime (17%), cetrazidime (60%), ciprofloxacin (21%), norfloxacin (21%), chloramphenicol (9%), andtetracycline (85%). Tetracycline > ampicillin > cetrazidime > streptomycin > amikacin > amoxycillin > norfloxacin = ciprofloxacin > gentamicine > cefixime > chloramphenicol (Figure 7).

Multi-class drug resistance in EHEC

The prevalence of multi-class drug resistance in EHEC isolates were studied and it was found that in the month of April the class aminoglycosides (45.61%), penicillin (61.29%), cephalosporin (42%), fluoroquinolones (15.84%), phenicol (13.52%) and tetracycline (84.7%). Thus, the decreasing order was tetracycline > penicillin > aminoglycoside > cephalosporines > fluoroquinolones > phenicol (Figure 8). The levels seem to be fluctuated in the month of August and were found to be aminoglycosides (10.6%), penicillin (45%), cephalosporin (12.5%), fluoroquinolones (6.25%), phenicol (11.75%) and tetracycline (75%). Thus, the decreasing order was tetracycline > penicillin > cephalosporine > phenicol > aminoglycoside > fluroquinolones. Interestingly, these levels seem to be increased in the month of December and were found to be class aminoglycosides (37.56%), penicillin (52%), cephalosporin (26.75%), fluoroquinolones (13.64%), phenicol (11.5%) and tetracycline (84%). Therefore, the decreasing order was tetracycline > penicillin > aminoglycoside > cephalosporin > fluroquinolones > phenicol (Figure 8).

Multi-class drug resistance in ETEC

The prevalence of multi-class drug resistance in ETEC isolates were studied and it was found that in the month of April the class aminoglycosides (40%), penicillin (61.29%), cephalosporin (28%), fluoroquinolones (17.84%), phenicol (15.52%) and tetracycline (92. %). Thus the decreasing order was tetracycline > penicillin > aminoglycoside > cephalosporine > fluoroquinolones > phenicol. The levels seem to be fluctuated in the month of August and were found to be aminoglycosides (24.6%), penicillin (45%), cephalosporin (13.5%), fluoroquinolones (6.25%), phenicol (13.25%) and tetracycline (75%). So, the decreasing order was tetracycline > penicillin > aminoglycoside > cephalosporine > phenicol > fluoroquinolones. Interestingly, these levels seem to be increased in the month of December and were found to be class aminoglycosides (35%), penicillin (51%), cephalosporin (44.7%), fluoroquinolones (14%), phenicol (13.5%) and tetracycline (82%). The decreasing pattern thus obtained was tetracycline > penicillin > aminoglycosides > cephalosporine > fluoroquinolone > phenicol (Figure 9).

Multi-class drug resistance in EPEC

Likewise, the prevalence of multi-class drug resistance in EPEC isolates were studied and it was found that in the month of April the class aminoglycosides (42%), penicillin (62%), cephalosporin (43%), fluoroquinolones (18%), phenicol (15%) and tetracycline (95%).Thus, the decreasing levels was tetracycline > penicillin > cephalosporin > aminoglycoside > fluoroquinolones > phenicol. Further, in the month of August it was found to be aminoglycosides (22%), penicillin (45%), cephalosporin (12.5%), fluoroquinolones (10%), phenicol (15%) and tetracycline (74%).Thus, the decreasing order was tetracycline > penicillin > aminoglycoside > phenicol > cephalosporin > fluoroquinolone. Interestingly, these levels seem to be increased in the month of December and were found to be class aminoglycosides (39.56%), penicillin (52%), cephalosporin (27.75%), fluoroquinolones (14%), phenicol (12.5%) and tetracycline (84%).Thus, the decreasing order of resistance was tetracycline > penicillin > aminoglycoside > cephalosporin > fluoroquinolones > phenicol. Therefore, based on the cumulative observations of different months, most of the EHEC, ETEC and EPEC isolates were resistant to more than three different classes of variable drugs, majorly tetracycline, penicillin, aminoglycoside, cephalosporin which confirmed their prevalent multiple drug resistance (MDR) nature not affected by a cluster of antibiotics (Figure 10).

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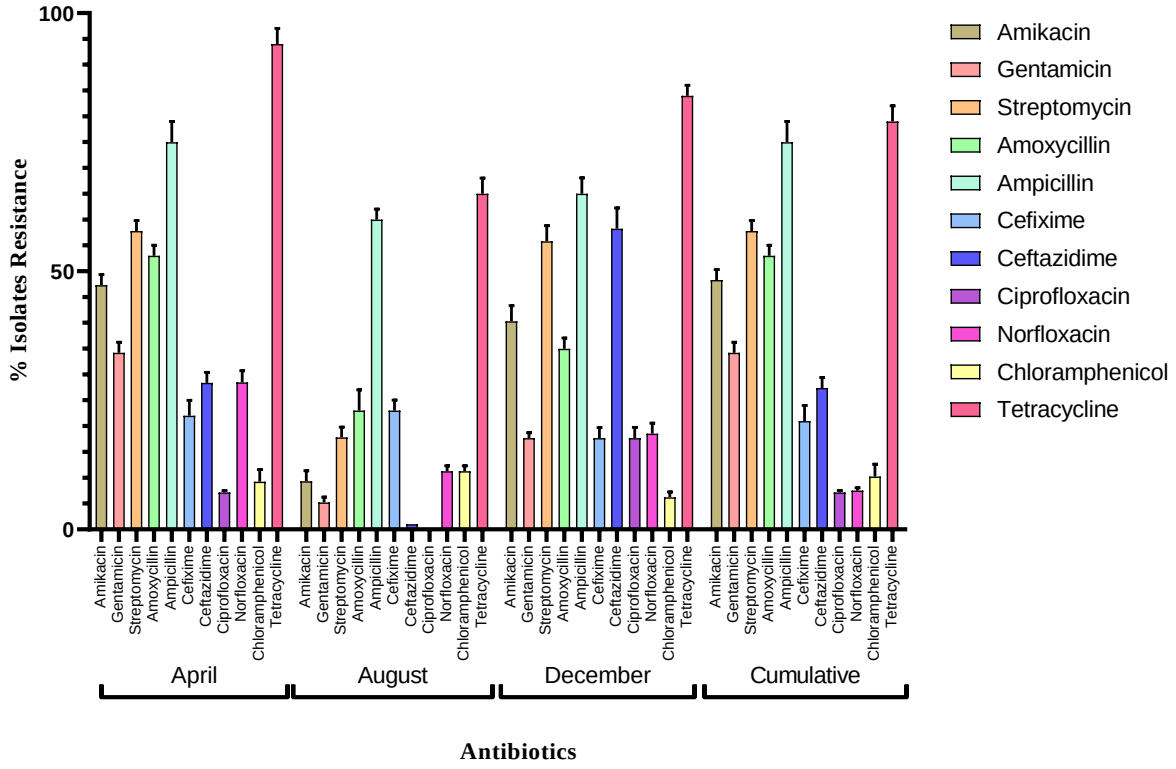

Fig: 5; The antibiotic susceptibility profile of different EHEC strains identified during the month of April, August and December of the year 2021.

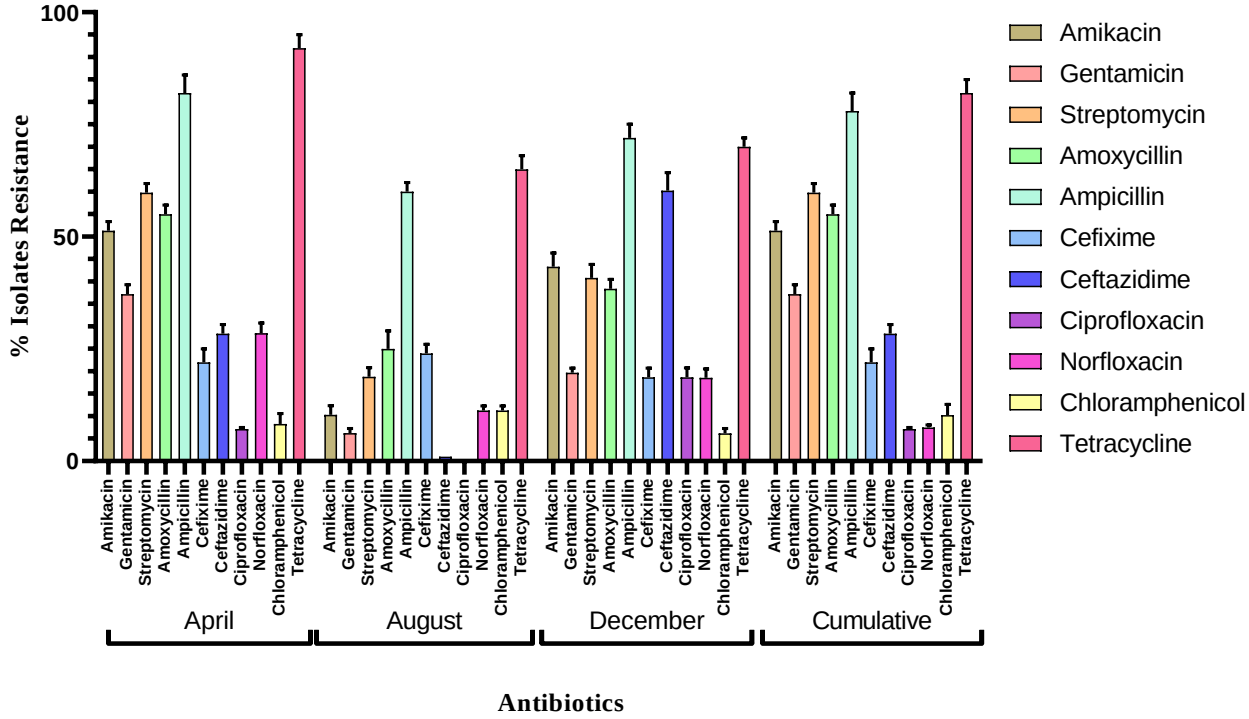

Fig:6; The antibiotic susceptibility profile of different ETEC strains identified during the month of April, August, and December of the year 2021.

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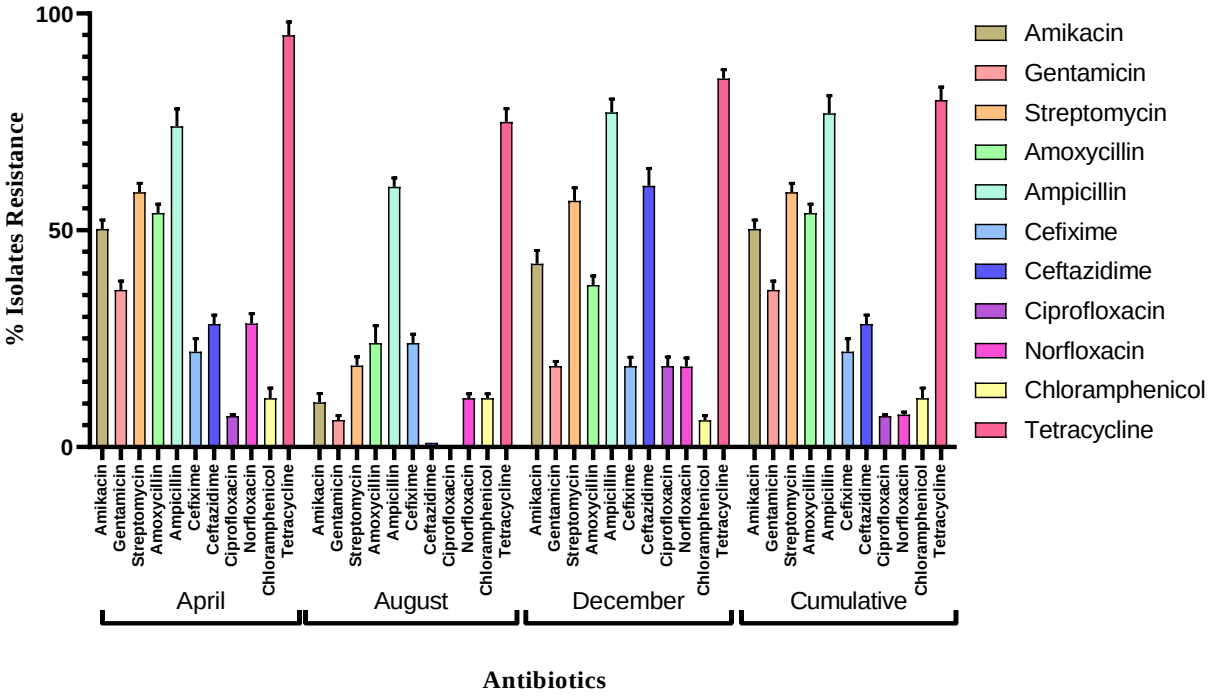

Fig:7; The antibiotic susceptibility profile of different EPEC strains during the month of April, August and December of the year 2021.

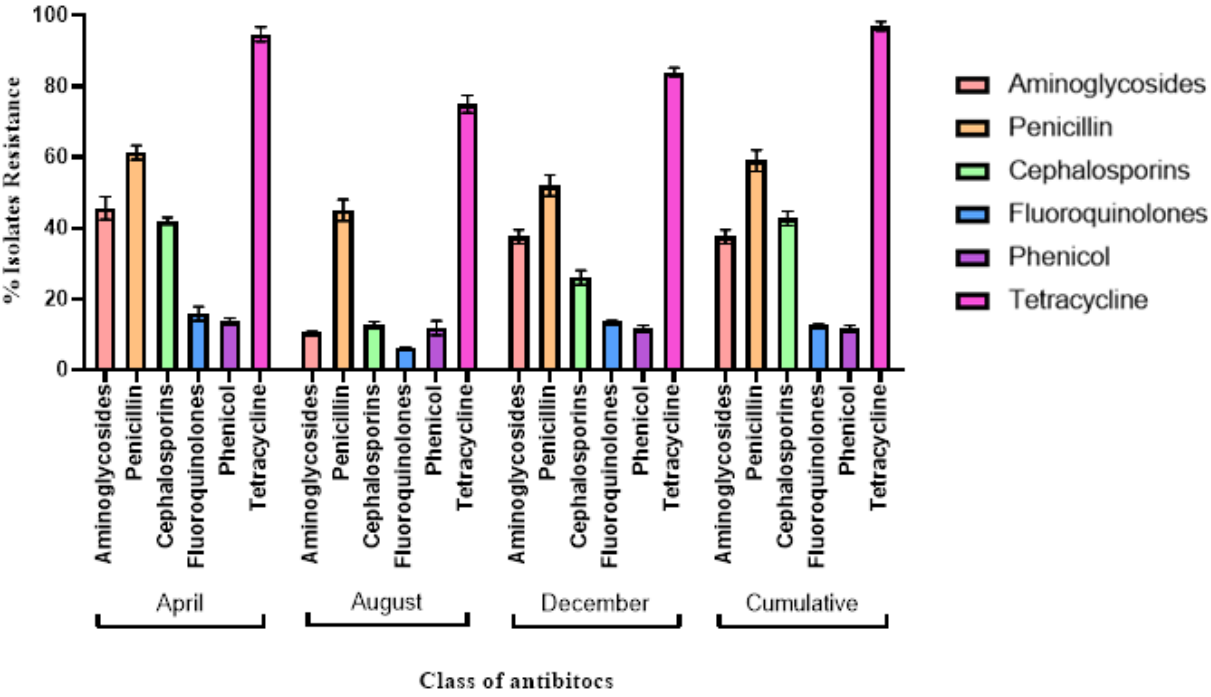

Fig:8; The prevalence of multi-class drug resistance in EHEC isolates identified from the water samples collected during the month of April, August and December of the year 2021.

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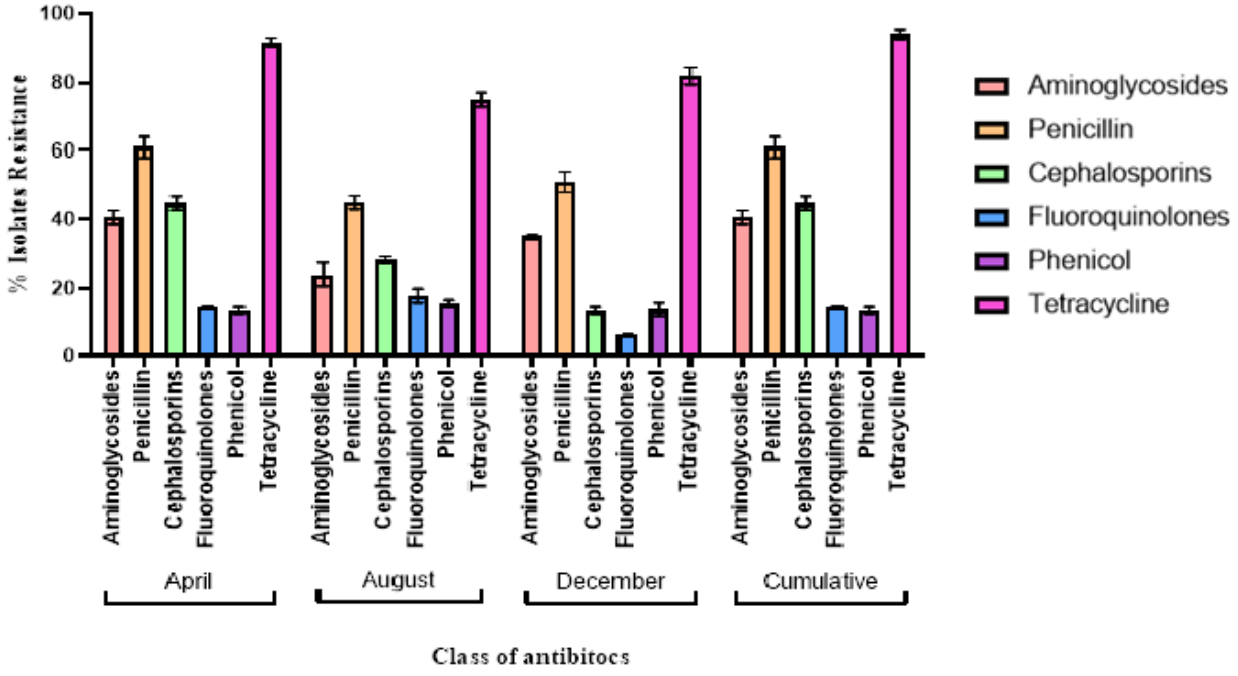

Fig:9; The prevalence of multi-class drug resistance in ETEC isolates identified from the water samples collected during the month of April, August and December of the year 2021.

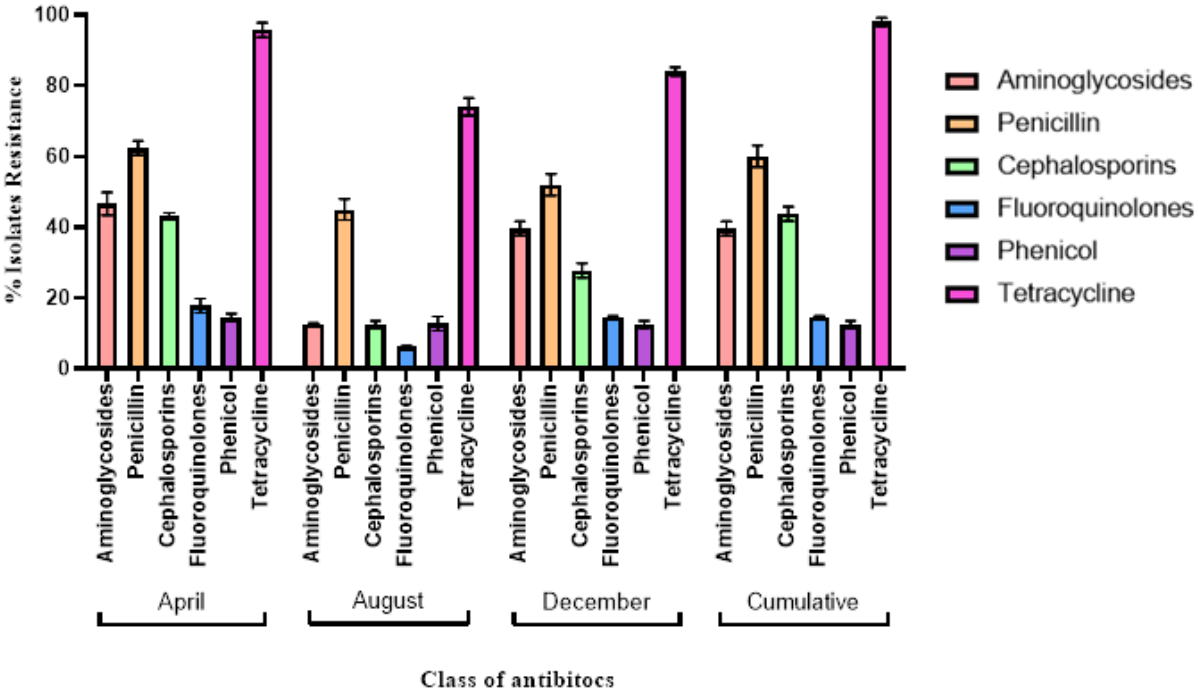

Figure:10; The prevalence of multi drug resistance in EPEC isolates identified from the water samples collected during the month of April, August and December of the year 2021.

Discussion

Rivers are the major source of fresh water globally and in India there are in a crisis and gasping to breathe due to contaminations. The water pollution, abstractions and diversions are nearly killing the rivers. Hindon river is no exception and is dying a slow death. The untreated discharge of STPs, WWTPs, domestic and industrial waste has transformed the river in to a chemical soup. Thus, there is a tremendous deterioration in the quality of river water (Giowanella et al., 2015). In the

Journal of Chemical Health Risks
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JCHR (2023) 13(6), 3586-3599| ISSN:2251-6727

developing countries like India millions of people are dependent on the river water for various household activities, drinking, bathing, agriculture and industrial purpose (Begum et al., 2008). Likewise, the people of western Uttarpradesh are dependendent on the river Hindon despite of the water being a chemical howler (Bhutiani et.al.,2017, Anita et al.,2023). Further, the increasing antibiotic resistant bacteria has increased the chances of severe infection (Burnham,2021). In the current study the virulence genes and their antibiotic reistance was studied in *E.coli* isolates from the different sampling sites of the Hindon river water in the month of April, August and December 2021.This study has used the PCR assay based method for the detection of virulence genes *stx1*, *stx2*, *ST1*, *LT1*, *eaeA* and *bfp* gene.for the detection of EHEC, ETEC and EPEC in the surface water of river Hindon. The PCR assay method is a simple and cost effective method for the detection of virulence genes for viable and non-culturable bacteria. Hence, this assay has high applicability to detect the pathogens in river surface water and prevent the outbreak of diseases associated with the bacteria. This study has shown the presence of EHEC, ETEC and EPEC and the level of antibiotic resistance in the month of April, August and December 2021.Givens et.al., 2023 has shown the presence of ARBs in surface water. *E. coli*, is a predominant species for Horizontal gene transfer of antibiotic resistance genes (Thomson-Nielsen 2005; Browne-Jaque *et al*., 2018). Further, in developing countries lack of proper sanitation and overuse of antibiotics has led to the development of bacteria containing virulence genes (Begum et al., 2008). The presence of virulence genes *stx1* and *stx2* is known for the presence of potential EHEC. The virulence gene *stx2* was not detected in any of the *E.coli* isolates. Further, the presence of virulence gene *LT1* and *ST1* pointed towards the presence of ETEC and the presence of virulence gene *eaeA* and *bfp* are known for the presence of potential EPEC. The EHEC strains are resposible for outbreak of severe and bloody diarrhoea (Nguyen et.al., 2012). The ETEC strains are the major cause of bacterial diarrhoea in the world, the major burden of which is carried by children under 5 years with mortality of 400 million per year in developing countries (Walker et. al., 2007). Humans are the main reservoir of EPEC strain and the principal cause of watery diarrhoea in humans (Torres et.al., 2017; Potgieter et al., 2020). Henceforth, the current study points towards the presence of pathogenic bacteria in the river surface water and the severity of consequences if ingested.

This study has shown that the virulence genes *stx1* in the EHEC in the month of April, August and December was found to be 29.5%, 25.4% and 35.4% respectively. The virulence gene for *stx2* was not detected in any of the season. Studies conducted by Ndlouv et.al., 2015 has shown the occurrence of EHEC in 15% of the isolatesin the Berg river water surface. The shiga toxin genes in EHEC are responsible for haemorrhagic uremic syndrome and haemorrhagic colitis. Further, the virulence genes in the ETEC were *LT1* and *ST1*, and their level in the month of April was highest 29% and 27% respectively. A similar study by Ram et.al., (2007) showed the prevalence of LT1 and ST1 with 66.7% and 33.3% respectively in the river Ganga. Likewise, the virulence genes in the EPEC were *eaeA*, and *bfp*, and their level in the month of April was found to be highest 87% and 84% respectively (Alfinete et al., 2021). Further, the MDR profile for EHEC, ETEC and EPEC showed more than 70% resistance to tetracycline and Ampicillin. Further, several findings have also reported the presence of virulence genes in the river Gomti (Patel et al, 2022; Kumar et.al, 2020). Thus, regular surveillance and monitoring of the river Hindon water at multiple sites is required for public health intervention.

Conclusion

The prevalence of virulence genes in EHEC, ETEC and EPEC strains from the Hindon river water is a matter of great concern. Since, the river water is used by the millions of population in the catchment area of the river for washing, cleaning, bathing, agriculture and other recreational activities. Further, the presence of multidrug resistance in EHEC, ETEC and EPEC in Hindon river water may be due to the discharge of untreated water from STPs, WWTPs, agriculture runoffs, industrial wastes, hospital wastes and carcasses resulting from poor practices. These observations of virulence genes and MDR in pathogenic *E.coli* of Hindon river water surface is alarming for various lifeforms. Further, this research also points to the presence of EHEC, ETEC and EPEC in the surface water and can be used for surveillance of epidemiology. Thus, the prevalence of EHEC, ETEC and EPEC in river Hindon water demands increased surveillance and developing strategies for human health protection.

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Conflicts of Interest

The authors declare no conflict of interest in this work.

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