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RESEARCH ARTICLE

PREVALENCE AND CHARACTERIZATION OF WATERBORNE MULTI DRUG RESISTANT *E. COLI* O157:H7

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Keywords

- Water-borne disease
- *E. coli*
- MUG
- Shiga toxin
- Multi drug resistance

ABSTRACT

The water samples were collected from pre-identified spots. To isolate *E. coli* from the water samples a number of nutrient media with selective substrates were used. MacConkey agar plates are used to differentiate by fermenting lactose. Lactose fermenting strains which grew as red or pink. MUG-EC broth was used for in the *E. coli* strains. Lastly, confirmation of selected suspected *E. coli* 0157:H7 isolates were taken to test for indole production test and methyl red red. Four sampling sites S1, S2, S5 & S6 were found positive for the presence of *E. coli* 0157:H7. Isolated virulent bacteria were further characterized based on the presence of stx2 gene that codes for shiga like toxins. The product targeting Stx2 of 485 bp was found in all suspected *E. coli* isolates from the sampling site S1, S2, S3 & S6. This four *E. coli* strains possessed virulence marker Stx–2 gene as tested by PCR. Phylogenetic relationship among isolated bacteria was determined by the sequence analysis of 16S rDNA gene. The specific oligomers target 16S RNA sequence of *E. coli* and 100% sequence similarity was found with the reference sequence after BLAST analysis of most conserved region of 16S r DNA gene. Antimicrobial susceptibility testing revealed the multi drug resistant pattern of isolates. All *E. coli* isolates were resistant to ampicillin, 6 isolates were resistant to erythromycin, 4 isolates were resistant to tetracyclin, and 2 isolates were resistant to kanamycin

INTRODUCTION

Water is essential to sustain life. Microbial water quality may vary rapidly and over a wide range. The occurrence of waterborne pathogens in short-term peaks may increase disease risks considerably and may also trigger outbreaks of waterborne diseases. A third of world populations suffer from diseases derived from contaminated water. Every year 13 million people die from waterborne diseases (World Health Organization). World Bank estimates that more than 30 million life years are lost annually due to water related diseases in India (Brandnson, 1995) valued at a per capita income of Rs. 12,000 this amounts to an annual loss of Rs. 36,000 billion (about US $ 850 billion).

Most of the pathogens in drinking water are generally of faecal origin and can be found in human and animal waste, therefore used as “indicators” of microbial contamination in water. Faecal waste can be a source of pathogenic microorganisms like bacteria, viruses, protozoa, and helminthes.

Coliforms are present in large numbers among the intestinal flora of humans and other warm-blooded animals and thus found in faecal wastes. As a consequence, Coliforms are detected in higher concentrations than other pathogenic bacteria; therefore coliforms are used as an index of the potential presence of entero-pathogens in water environment. The use of Coliforms group, and more specifically *Escherichia coli,* as an indicator of microbiological quality of water has been adapted by regulatory agencies worldwide.

Escherichia coli are an important member of enterobacteriacae which contains gram negative bacilli. *E.coli* is normal inhabitant of animal and human intestinal tract. There are 700 serotypes have been identified on the basis of somatic (O), capsular (K) and flagellar (H) antigen. These serotypes are causal agent of wide range of clinical manifestations, including mild illness, vomiting, sudden onset of diarrhea, hemolyticuremic syndrome and death (Su and Brandt, 1995). Among all serotypes, *E. coli* O157:H7 is an increasing public health concern because of incidence of food poisoning outbreaks. Since the initial identification of *E. coli* O157:H7 as the etiological agent of Hemolytic Uremic Syndrome (HUS) and hemorrhagic colitis (HC), this pathogen has risen as a major concern of food safety in many countries(Johnson et al., 1996) . The infective dose of *E. coli* O157:H7 in humans is very low (Jones, 1999). *E. coli* O157:H7 that produces a toxin called the shiga toxin (O'Brien and Holmes 1987). This toxin causes premature destruction of the Red Blood Cells which then clogs the body filtering systems in the kidneys causing HUS. This is turn causes strokes due to small clogs of blood which lodge in capillaries in the brain.

Although significant research has been done in the field of waterborne pathogens infection detection in water at low doses needs better detection strategies. Development of molecular techniques better understanding of mechanisms of disease and molecular microbial ecology will ultimately provide solutions for both detection and control of waterborne pathogens.

Additionally, antimicrobial resistance among enteric bacteria is an increasing global public health concern. The widespread administration of antimicrobials promotes the selection of antimicrobial resistant strains, which complicates the treatment of bacterial infections. Furthermore, antimicrobial resistance also has negative effects on animal health and the environment as per the One Health concept which highlights the interconnection of human, animal, and environmental health. Livestock in particular, are often considered as sources of antimicrobial resistance in industrialized countries, where antimicrobials are commonly used to improve productivity (Van Boeckel et al., 2015). Also, more information is needed on antimicrobial resistance patterns in developing countries like India, where medical drugs are often misused, and creating ideal conditions for the development of resistant strains. To reduce this knowledge gap, this study was carried out to assess and characterize the contamination of *E. coli* O157:H7 in water sources of Satna, Madhya Pradesh, India, and determine the antimicrobial susceptibility pattern of the isolates of *E. coli* O157:H7.

MATERIALS AND METHODS

Sample Collection

Water samples from different locations were collected in order to isolate *E. coli* 0157:H7. These sample points were selected, based on their position relative to potential sources of contamination or being representative of a specific catchment area. The position of the sample points are summarized in table1:

Table 1: Sample details

S.No.	Location of Sampling	Sample Code
1.	Nagod Drinking water.	S1
2.	Railway Platform Drinking water taps	S2
3.	Drinking water at New basti	S3

4.	AKS University Tap Water	S4
5.	AKS University Sewage Water	S5
6.	Maihar railway platform drinking water	S6
7.	Utaili drinking water	S7
8.	Magardaha river water	S8
9.	Premvihar colony drinking water	S9
10.	Maihar drinking water	S10

Isolation of *E. coli* variants

All media components were weighed by the digital weighing machine & were then dissolved in distilled water. This mixture was then heated for 2-3 minutes over Hot Plate. The mixture was kept for Autoclaving for 20 minutes. The media was then poured into petridishes.

MacConkey agar plates were incubated at 37ºC for 18-36 hrs. After the colonies grew, their size, shape and colour pattern were noted from MacConkey agar plates. Colonies were then picked and streaked onto fresh MacConkey plates and incubated at 37ºC for 24-36 hrs.

From MacConkey agar plates, the pink colonies were picked and streaked onto Sorbitol MacConkey agar for selection of colorless colonies. All these isolates were streaked onto the MUG-EC media and incubated at 37ºC for 24-36 hrs. These plates were then, exposed to UV light at 365 nm to detect blue colour fluorescence.

The colonies which showed all the positive results were then converted into slants using Nutrient Agar Media & sent for DNA Sequencing.

Molecular Characterization

Genomic DNA isolation

LB broth was prepared & autoclaved for 20 minutes. The bacterial culture was then inoculated and kept overnight in the Shaking Incubator at 37ºC. Then culture was taken out for protocol. The culture was then transferred into Eppendorf tubes. Culture tubes were the centrifuged at 14000 rpm for 10 minutes. After centrifugation, the supernatant was discarded & the Pellet was retrieved. 500 µl of 10% SDS solution was then added into the pellet for the degradation of the cell wall. This culture was then incubated at 65°C for 30 minutes in a Water Bath. Supernatant was again discarded & the Pellet was retrieved after incubation in the Water Bath. 500 µl of Saturated Phenol was mixed in the above retrieved pellet. Centrifugation was again done at 12000 rpm for 5 minutes. The Aqueous phase was then transferred into a new tube. The Supernatant was again discarded & 500 µl of Chloroform: Isoamyl Alcohol in the ratio of 24:1 was added. Centrifugation was again done at 12000 rpm for 5 minutes. The Upper Layer was then transferred into new tubes. 1000 µl of Chilled Ethanol was added. This mixture was then incubated at -20°C in a Deep Freezer for 30-60 minutes. After incubation, white threads were visualized. Centrifugation was again done at 12000 rpm for 10 minutes. Supernatant was again discarded & the Pellet was retrieved. The culture was then dried for 30 minutes at room temperature. 50-100 µl of Nuclease Free water was added.

Bacterial species identification using PCR and sequencing method Universal eubacterial primers FD1 5`CCGAATTCGTCGACAACAGAGTTTGATCCTGGCTCAG3` and RD1 5`CCCGGGATCCAAGCTTAAGGAGGTGATCCAGCC3` (Weisberg *et al.*, 1991) were used for amplification of 1492 bp region of the 16S rRNA gene on PTC 100 (M.J. Research, USA) thermal cycler. A 50 µl reaction mixture included 5-10 ng of bacterial DNA as template, 1 µl of each primer, 1U of *Taq* DNA polymerase (Banglore Genei, India) and 100 µg dNTPs. The reaction conditions were: initial denaturation of 7 min at 94^0C followed by 29 cycles of denaturation of 1 min at 94^0C, extension of 1 min at 72^0C and annealing temperatures 54^0C for 7 cycles; 53^0C and 52^0C for one cycle each, 51^0C for 20 cycles and a

final extension of 10 min at 72^0C. Amplified gene was visualized in 0.8% agarose after electrophoresis.

16s rRNA gene sequencing

Partial 16S rRNA gene sequencing was performed in the same reaction mixture and following the same amplification conditions as described in full 16S rDNA. The only difference was in primers. In the partial gene amplification, primers f1 and r1 were used. The PCR products were analyzed on 1.2 % agarose gel in TAE buffer, run at 50 V for 2 h. Gels were stained with ethedium bromide and visualized as described above. The amplicons were purified with Banglore Genei, PCR purification kit and quantified spectrophotometrically at 260 nm compare with calf thymus DNA. The cleaned partial 16S rDNA amplicon was sequenced with DNA sequencing system.

Sequence analysis of 16S rDNA sequences using bioinformatics tools

The PCR products were purified and sequenced. Theses all sequences were subjected for phylogenetic analysis. The homology of partial sequences were compared with the sequences from the DNA databases and similar sequences showing above 95% were retrieved by nucleotide BLAST (basic local alignment search tool) program at NCBI BLAST server (www.ncbi.nlm.nih.gov/BLAST). Multiple sequence alignment of retrieved sequences was done by EBI ClustalW server (www.ebi.ac.uk.clustal/index.html). Phylogenetic tree constructed by using *genebee* server (www.genebee.msu.ru/services/phtree-reduced.html). Phylogenetic tree obtained with bootstrap values in cluster algorithm, phylip format and topological algorithm

Amplification of Stx 2 gene

PCR oligomers were designed targeting an amplification product of 485 bp in the *Stx*2 gene. The primers used were forward primer SII-1 5'- CAGGCGCGTTTTGACCATCTT-3' and reverse primer SII-2 5'-CTCCCCACTCTGACACCATCCTCT-3' (Osek, 2001).

Antimicrobial susceptibility testing

Antimicrobial resistance tests were performed by standard disc diffusion technique (Wayne, 2012). The selection criteria of antimicrobials depended on the frequency of use of antimicrobials. Resistance testing discs included 10 antimicrobial agents (HiMedia, India) as listed in Table 1. The isolates were considered resistant if the diameter of the inhibition zone was less than or equal to the resistance. Each isolated bacterial colony from pure fresh culture was transferred into a test tube of 5 ml tryptone soya broth and incubated at 37°C for 6 hours. The turbidity of the culture broth was adjusted using sterile saline solution, or more isolated colonies were added to obtain turbidity that is usually comparable with that of 0.5 McFarland standards (approximately 3×10^8 CFU ml^{-1}). Mueller-Hinton agar (HiMedia, India) plates were prepared according to the manufacturer. A sterile cotton swab was immersed into the suspension and rotated against the side of the tube to remove the excess fluid and then swabbed in three directions uniformly on the surface of Mueller-Hinton agar plates. After the plates dried, antimicrobial disks were placed on the inoculated plates using sterile forceps. The antimicrobial disks were gently pressed onto the agar to ensure firm contact with the agar surface, and incubated at 37°C for 24 hours. Following this, the diameter of inhibition zone formed around each disk was measured using a black surface, reflected light and transparent ruler by laying it over the plates.

RESULTS AND DISCUSSION

Isolation of E. coli

The water samples were collected from pre-identified spots of Satna City. To check the prevalence of *E. coli* O157:H7 in water samples, a number of nutrient media with selective substrates were used. MacConkey agar plates are used to differentiate by fermenting lactose. Lactose fermenting strains which grew as red or pink surrounded by a zone of acid precipitated bile. The red color is due to production of acid from lactose, absorption of neutral red and subsequent change of the dye when the pH medium declines to pH 6.8 or lesser value. The selective isolates from MacConkey agar plates were streaked out on the plates of the Cefixime-Tellurite-SMAC. It is a selective medium for *E.coli* 0157:H7 strains. It is a recommended medium for isolation of pathogenic *E.coli* 0157:H7 which does not ferment lactose but ferments sorbitol and produced colorless colonies. Cefixime inhibits the growth of other organisms and tellurite enhances the growth of *E. coli* 0157:H7.

Confirmation of E. coli O157:H7

MUG-EC broth was used for in the *E.coli* strains. Isolates were grown at 44.5ºC for 18 hrs on MUG-EC broth. MUG (4-methylumbelliyferyl- β-D-glucuronide) was hydrolyzed by the ß -glucuronidase yielding 4-methyl umbelliferone, which shows blue fluorescence on being irradiated with long wavelength UV light (366nm) . But interesting point is that *E.coli* 0157:H7 lacks the enzyme, β -glucuronidase, it does not form the 4-methyl umbelliferone and therefore does not exhibit the fluorescence. This medium permits the rapid detection of

E.coli 0157:H7.

Lastly, confirmation of selected suspected *E. coli* 0157:H7 isolates were taken to test for indole production test and methyl red red. Tryptone water was used for indole production test and buffered Glucose broth was used for Methyl red test. Methyl red test, involved addition of methyl red in to grown culture after 5days incubation at 30ºC for production of pink colour as a positive result. After addition of the methyl red to culture of *E.coli* strains, which were grown in buffered glucose broth, red color is produced due to high acidity production during dextrose fermentation. The indole production was determined using Kovac's reagent (p-dimethylamino benzaldehyde), which gives the formation of pink colored ring in grown culture after 24-48 hrs incubation at 30ºC. The organisms thus characterized were tentatively identified as suspected *E. coli* 0157:H7 (Thompson et al., 1990).

Table 2: Biochemical Characterization of isolated bacterial strains

S No.	Name of Test	Sample Name									
		S1	S2	S3	S4	S5	S6	S7	S8	S9	S10
1	Indole Test	+	+	+	-	+	+	-	+	+	+
2	Methyl Red Test	+	-	+	+	+	-	+	+	+	-
3	Vogus Proseukur Test	-	-	+	+	+	+	+	-	+	+
4	Citrate Utilization Test	+	+	-	-	-	-	-	+	-	-

PCR analysis for virulence gene in suspect E. coli 0157:H7

Four sampling sites S1, S2, S5 & S6 were found positive for the presence of *E. coli* 0157:H7. Isolated virulent bacteria were further characterized based on the presence of stx2 gene that codes for shiga like toxins. The conditions used for amplification of each of the isolated DNA were adopted as described by Ji- Yeon Kim et al. (2005). The product targeting Stx2 of 485 bp was found in all suspected *E. coli* isolates from the sampling site S1, S2, S3 & S6. This four *E. coli* strains possessed virulence marker Stx–2 gene as tested by PCR. As demonstrated in the present study, strains had Stx-2 gene that are characteristic for *E. coli* isolates. Therefore these isolates can be considered potentially virulent bacteria for humans. These isolates were recovered from different sites but possessed identical virulence marker.

Chen and Griffiths (1998) recorded similar results by using the similar sets of primers. Stx1 gene was detected in 3%, Stx2 gene in 20% and both the genes in 76 % isolates of human clinical cases (Stephen et al., 1989). Further, selected virulent strain of *E.coli* 0157:H7 was characterized based on the 16S rDNA conserved regions sequencing.

Phylogenetic analysis of isolated E. coli strains

The phylogenetic relationship among isolated bacteria was determined by the sequence analysis of 16S rDNA gene. The specific oligomers target 16S RNA sequence of *E. coli* and 100% sequence similarity was found with the reference sequence after BLAST analysis of most conserved region of 16S r DNA gene. The given sample-1 has shown 100% sequence similarity with *Escherichia coli* isolates (fig. 4.1).

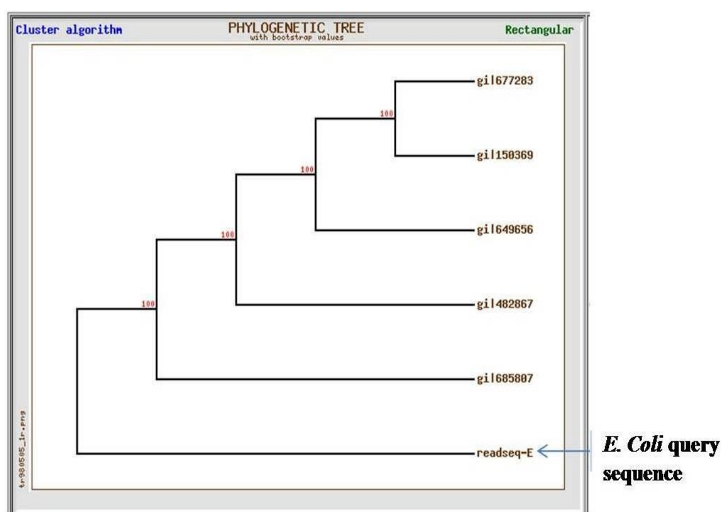

Fig. 4.1: Phylogenetic tree presenting the E. coli query sequence grouping with the reference sequences of E. coli isolates retrieved from the database.

Multi-drug resistance pattern

Antimicrobial susceptibility testing (photo plate 4.6) results showed that of the 7 isolates, all were resistant to ampicillin, 6 were resistant to erythromycin, 4 were resistant to tetracyclin, and 2 were resistant to kanamycin. None of the isolates were resistant to cefotaxime, ceftriaxone, cefuroxime sodium, chloramphenicol, ciprofloxacin, gentamicin, nalidixic acid and norfloxacin. All isolates were resistant to at least two antimicrobials (table 3 & 4).

Table 3: Antimicrobials used and interpretation of resistance

Antimicrobial disc	Code	Conc.(µg)	Diameter of zone of inhibition(mm)		
			Resistant	Intermediate	Susceptible
Ampicillin	AMP	10	13	14-16	17
Amoxycillin - Clavulanic acid	AMC	20/10	13	14-17	18
Cefotaxyme	CTX	30	22	23-35	26
Chloramphenicol	C	30	14	13-17	18
Ciprofloxasin	SIP	5	15	16-20	21
Erythromycin	E	15	13	14-20	23
gentamycin	CN	10	12	13-14	15
kanamycin	K	30	13	14-17	18
Nalidixic acid	NA	30	13	14-18	19
nitrofurantoin	F	50	14	15-16	17
norfloxacin	NOR	10	12	13-16	17
streptomycin	S	10	11	12-14	15

Tetracyclin	TE	30	11	12-14	15

Table 4: Antimicrobial resistance patterns of E. coli O157:H7 isolates.

E. coli O157:H7 isolates	Resistant pattern
S1	AMP, E
S2	AMP, E
S3	AMP, TE, E
S4	AMC, K
S6	AMC, AMP,TE, E, K
S7	AMP, TE, E
S8	AMP, TE, E

CONCLUSIONS

In our research work, we have found presence of multi drug resistant *E. coli* 0157:H7 in samples such as, Birla factory drinking water, railway platform drinking water, Municipal drinking water at New *basti*, and AKS University sewage water as well as in water supply of Municipal Corporation of Satna which is being used by the community of potable water. The results obtained here in this work are promising and could be useful tool tracing *E. coli* infection and for epidemiological studies for outbreak studies as this pathogen has arisen as a major concern. We here strongly recommended the municipal corporation to treat the water in proper way that can prevent or disinfect the water before supply to the community otherwise may cause the huge health hazards in the human population.

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