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STUDIES ON SUSCEPTIBILITY AND RESISTANCE PATTERNS OF VARIOUS *E. COLI* ISOLATED FROM DIFFERENT WATER SAMPLES AGAINST CLINICALLY SIGNIFICANT ANTIBIOTICS

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Abstract: *Escherichia coli* is an emerging pathogen of the greatest concern as it is the leading cause of various severe infections of stomach, urinary tract, ear, wound etc. in humans. Increasing rates of antimicrobial resistance among *E. coli* is another furthermost fret worldwide. This problem is more traumatic when water bodies are getting contaminated with faecal pollution and inappropriate use of antibiotics that led to emergence of multi-drug resistant strains of this normal microbiota of human intestine. The current study dealt with the isolation of around twenty *Escherichia coli* strains from water samples collected from different prominent hilly locations of Uttarakhand region of India. These bacteria were evaluated for their resistance and susceptibility patterns against seven commonly prescribed clinically-significant antibiotics viz. Azithromycin, Cefixime, Ciprofloxacin, Doxycycline, Gentamycin, Streptomycin and Tetracycline. *E. coli* strains were shown to have variable susceptibilities during the study. Doxycycline was found to be most inert antibiotic as it was not effective against any of *E. coli* isolates. Around 85% isolates were shown to be sensitive against Cefixime making this antibiotic more promising. More evident finding of the study was that none of antibiotic used in the study was 100% effective. Cefixime was found to be the most effective antimicrobials against *E. coli* isolates followed by Streptomycin, Tetracycline, Gentamycin, Azithromycin and Ciprofloxacin. Multiple antimicrobial resistances (MAR) index for all the isolates were calculated on the basis of resistance patterns. It has been observed that only 10% strains showed the MAR Index less than 0.1 while rest of 90% isolates were having MAR index between 0.2 to 0.9 showing the multi-drug resistance.

Keywords: Antibiotic resistance, *Escherichia coli,* Susceptibility, Agar well diffusion assay, MAR index.

INTRODUCTION

Escherichia coli (commonly abbreviated *E. coli*) is a Gram-negative, rod-shaped bacterium that is commonly found in the lower intestine of warm-blooded organisms (endotherms). It is also found in water, soil and vegetations. The genus belongs in a group of bacteria informally known as "coliforms" and is a member of the *Enterobacteriaceae* family (the enterics) of the *Gammaproteobacteria*. This family is largest among all other bacterial families and includes many of other familiar pathogens, such as *Salmonella, Escherichia coli, Yersinia pestis, Klebsiella* and *Shigella*. *E. coli* and other related enteric bacteria constitute about 0.1% of gut flora. Although most *E. coli* strains are harmless, some serotypes can cause serious food poisoning in humans, and are occasionally responsible for product recalls due to food contamination. The harmless strains are part of the normal flora of the gut, and can benefit their hosts by producing vitamin K_2, and by preventing the establishment of pathogenic bacteria within the intestine. *E. coli* cells are able to survive outside the body for a limited amount of time, which makes them ideal indicator organisms to test environmental samples for fecal contamination. The major route through which pathogenic strains of the bacterium can cause disease is faecal-oral, therefore, *E. coli* becomes the most common cause of food and water-borne human diarrhea worldwide and in developing countries. It is the leading pathogen causing urinary tract infections[8,14,34] and is among the most common pathogens causing blood stream infections[4], and other complications like diarrhea[9,15] in humans.

Considering the facts about the increased water pollution, faecal contamination, increased transportation between countries, lack of knowledge about using antibiotics, it has been greatly notified that several microorganisms are emerged as resistant to antibiotics and are very difficult to be killed by existing antimicrobials. Therefore, aim of the bacteriological tests it is not only to discover the pathogenic germs, but in the same time to establish if this microorganisms could be destroyed by some chemicals or antibiotics. The broadly uses of antibiotics constitute a high risk of bacterial development. Antibiotics are substances produced by living organisms, which are able to kill or inhibit the growth of microorganisms. Antibiotic usage is possibly the most important factor that promotes

the emergence, selection and dissemination of antibiotic-resistant microorganisms in both veterinary and human medicine. The increase in the number of resistant and multi-resistant strains of bacteria is a major concern of health officials worldwide, particularly with the decline in the number of new antibiotics available for treatment.

While much effort has been directed toward management and monitoring of antibiotic use and the prevalence of bacterial infections within communities; bacterial resistance to antibiotics in the aquatic environment has received comparatively little attention. The rapid emergence of resistance to antibiotics amongst pathogen generates visions of the "potential post antibiotics era threatening present and future medical advances" the emergence of bacteria resistant to antibiotics is common in areas where antibiotics are used, but antibiotic resistant bacteria are also increasingly occurs in aquatic environment.

Bacterial contamination of surface waters, particularly contamination with faecal-derived bacteria, has long been a water quality issue due to the potential for disease transmission. Because of this and the potential for antibiotic resistance, there is a new level of risk associated with these bacteria. Recent studies have also identified antibiotics in surface waters[2,5,7,17] but the role of these antibiotics in the development, transfer, and maintenance of resistance is largely unknown.

The number of antimicrobial-resistant (AMR) bacteria in the environment increases exponentially with the use of antimicrobials, as a result of increasing selective pressure on bacterial populations[21,26,31] and its spread between different bacterial strains in different habitats has also been demonstrated[27,29,32] *E. coli* has been the foremost indicator of faecal contamination in water quality monitoring for many decades. The highest resistance rates were found in *E. coli* strain of the sewage treatment plant. It has also been demonstrated that the highest resistance rates in the penicillin group were found for Ampicillin, piperacillin and for tetracycline. All *E. coli* strains isolated from river and polluted waters show a high incidence of multiple antibiotic resistances (MAR) phenotype. Many investigators have recognized that wastewater treatment plants are the principal recipients of enteric bacteria with multiple antibiotic resistances[13,28].

The aim of our study was to determine the antimicrobial susceptibility and resistance pattern of *E. coli* from different water samples. The study involves; (a) Collection of water samples from different sources of Uttarakhand, India; (b) Isolation of *E. coli* by Most Probable Number (MPN) technique; (c) Determination of susceptibility and resistance pattern against different antibiotics by agar well diffusion assay; (d) Determination of multiple antibiotic resistance (MAR); and (e) Interpretation of the data generated for the benefit of new antibiotic drug discovery research.

MATERIALS AND METHODS

Collection of water samples: Gamma irradiated, clean and sterilized bottles (200ml capacity) were used for sampling of water from different sources of Uttarakhand, India. For dechlorination sodium thiosulphate was added to the clean, dry sampling bottles before gamma sterilization in an amount to provide an approximate concentration of 100mg/lit in the sample. Aseptic conditions were maintained during the collection of samples. The samples were kept in an ice pack to prevent any changes in the microbial flora of the samples during the transportation. The water samples were transported to the lab in vertical position maintaining the temperature 1-4°C with ice pack enveloped conditions. Samples were analyzed within 6h of collection.

Isolation and Identification of *E. coli*: The estimation of *E. coli* was done by determining the Most Probable number (MPN) technique as per IS1622:1981[12] in test samples. The test procedure included three phases namely presumptive, confirmative and completed phase.

Presumptive Test: For each water sample; 5 tubes of each 10ml, 1ml, and 0.1ml were used. 10ml sample was inoculated in double strength McConkey broth media and rest 1ml and 0.1ml was inoculated in single strength McConkey broth media. All the inoculated tubes were incubated at 37°C for 24-48hrs. Tubes showing presence of growth (turbidity) with or without gas were submitted to confirmatory phase.

Confirmative Test : All presumptive tube showing positive results were gently shaken and using micropipette and sterile tips culture were added to BGBL (Brilliant Green Bile Lactose Broth) and incubated at 37°C for 24-48hrs. Formation of gas within the period of 48hrs was taken for completed phase. Numbers of positive tubes were recorded.

Completed Test: The cultures from the positive tubes were streaked on EMB (Eosine Methylene Blue) agar and these plates were incubated at 37°C for 24 hrs in inverted position. Green metallic sheen colonies were observed which confirms the presence of *E. coli*. Some of these colonies from individual plates were transferred on non-selective media nutrient agar slants. Further incubation yield the pure culture of *E. coli*.

Identification of *E. coli* by Biochemical Test: These isolates were further confirmed by Gram's staining and HiMedia IMViC biochemical kit for *E. coli* as per IS: 5887

part 1-1976 Reaffirmed 2000[11]. Following biochemical test like Motility, Indole production, Citrate utilization, Glucorinidase, Nitrate reduction, ONPG, Lysine utilization, Lactose, Glucose, Sucrose, Sorbitol were used for confirmation of *E. coli*.

Antibiotics and their solutions: Seven antibiotics like - Azithromycin (Azithromycin tablet IP 1g, Mfd by: Zest pharma), Cefixime (Cefixime tablet IP 400mg, Mfd by: Zest pharma), Ciprofloxacin (Ciprofloxacin tablet-IP 500mg, Mfd by: Cipla Limited), Doxycycline (Doxycycline hyolate IP 100mg, Mfd by: Jagsonpal Pharmaceutical limited), Gentamycin (Gentamycin 40mg, Mfd by: Abbott healthcare Pvt Ltd), Streptomycin (Streptomycin IP, Mfd by: Vital healthcare Pvt-Ltd) and Tetracycline (Tetracycline hydrochloride capsules IP 250mg, Mfd by: Aventis pharma limited) were used to check the susceptibility and resistance pattern of *E. coli*. All these antibiotics were obtained from local pharmacy store and they were used in 10µg/ml concentration against *E. coli* isolates.

Inoculum Preparation: All *E. coli* isolates were sub cultured on non selective nutrient agar slants. The bacterial cultures were incubated overnight at 37°C. 0.5 McFarland density of bacterial isolates was adjusted using normal saline (0.85% NaCl) using densitometer to get bacterial population of 1.0 x 10^8 cfu/ml.

Agar Well Diffusion Assay (Zone of Inhibition Evaluation): Antibiotic susceptibility and resistance were evaluated by agar well diffusion assay[6]. 100µl of each of the adjusted cultures were mixed into separate 100 ml of sterile, molten, cool MHA, mixed well and poured into sterile petri plates. These were allowed to solidify and then individual plates were marked for each individual *E. coli* isolates. Each plate was punched to make wells of 6 mm diameter with the help of sterile cork borer at different sites of the plates. 100µl of respective antibiotic solutions were pipette into the well in assay plates. Plates were incubated overnight at 37°C. Following incubation, petri-plates were observed for the inhibition zones, diameters of which were measured by using Vernier Calipers.

RESULTS AND DISCUSSION

In the present study, water samples were collected from the hilly regions of Uttarakhand, India. These samples were analyzed for the presence of *E. coli* which is a prominent member of family Enterobacteriaceae and also is an indicator microorganism of faecal contamination of water bodies. During the study, a total of twenty *E. coli* were isolated from different locations of sampling locations. These isolates were biochemically characterized for the confirmation of *E. coli* strains. Isolated *E. coli* strains were then evaluated for their antimicrobial susceptibility patterns against seven commonly prescribed clinically significant antibiotics by using agar well diffusion assay. Table.1 demonstrated the antibiotic resistance patterns in terms of average zones of diameter considering 4 plates for each *E. coli* isolates against each of seven antibiotics of 10µg/ml concentration. Percentage of resistant *E. coli* out of total isolates (i.e. zone of inhibition <12mm and percentage of *E. coli* showing zero mm (0mm) zone of inhibition were also calculated and shown in Table.2.

Table. 1: Antibiotic resistance patterns of *E. coli* isolates

E. coli Isolates	Zone of inhibition* against Antibiotics						
	Azithromycin	Cefixime	Ciprofloxacin	Doxycycline	Gentamycin	Streptomycin	Tetracycline
S1	16	38	0	0	17	14	16
S2	0	40	0	0	15	16	0
S3	10	19	13	0	0	18	16
S4	14	36	0	0	18	0	18
S5	18	19	0	0	16	17	16
S6	19	40	20	0	18	18	0
S7	0	42	0	0	0	0	17
S8	0	16	0	0	15	17	16
S9	0	39	30	0	17	15	15
S10	0	0	0	0	0	18	0
S11	15	39	31	0	19	0	16
S12	0	36	0	0	0	18	17
S13	16	20	0	0	17	19	19
S14	0	38	0	0	19	19	0
S15	14	35	0	0	0	16	18
S16	14	39	0	0	0	16	0
S17	0	0	0	0	16	0	18
S18	16	37	31	0	16	17	17
S19	17	37	25	0	18	18	14
S20	17	0	0	0	0	0	0

*Zone of inhibition in mm. Diameter including well diameter of 6.0mm

Data revealed that all *E. coli* isolates shows the variable sensitivities against the different antibiotics used in the study. Susceptibility patterns of these isolates against antibiotics have been shown in Fig.1. The most vulnerable antibiotic was found to be Doxycycline against which all *E. coli* isolates shows the resistance i.e. 100% resistance. Ciprofloxacin was found another in danger antibiotic as a total of fourteen isolates (i.e. 70%) were found to have resistance against this antibiotic. Cefixime was found to be more promising as only 15% isolates were found to be resistant against this antibiotic, while all other isolates were shown to have very high susceptibilities. It has been observed that Gentamycin, Streptomycin, Tetracycline and Azithromycin were moderate antibiotics as *E. coli* isolates found resistant were between 25 to 45% (Table.2). It has also been observed that two isolates viz. S18 and S19 were highly susceptible against every antibiotics used for study except Doxycycline. Other organisms found to be fragile were S1, S5 and S13 as these three isolates were sensitive against all the antibiotics except Ciprofloxacin and Doxycycline. Other isolates were found to have variable sensitivities against the antibiotics used in the study.

Figure. 1: Susceptibility patterns of *E. coli* isolates against antibiotics

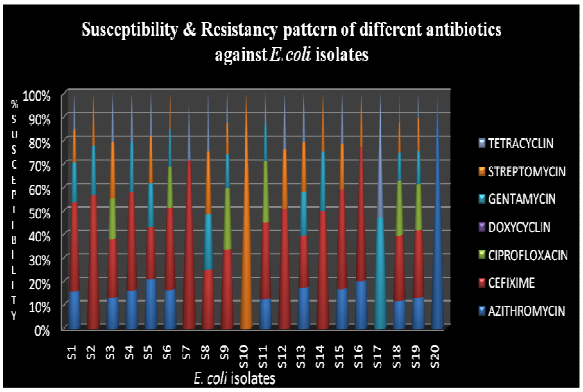

Table. 2: Percentage of pathogens resistant to antibiotics

Name of Antibiotics	% Resistant *E. coli*	% of *E. coli* with 0 mm Zone
AZITHROMYCIN	45.00 (9)	40.00 (8)
CEFIXIME	15.00 (3)	15.00 (3)
CIPROFLOXACIN	70.00 (14)	70.00 (14)
DOXYCYCLINE	100.00 (20)	100.00 (20)
GENTAMYCIN	35.00 (7)	35.00 (7)
STREPTOMYCIN	25.00 (5)	25.00 (5)
TETRACYCLINE	30.00 (6)	30.00 (6)

The fast increase in the appearance of the multi-drug resistant *E. coli* is a major problems faced by medicine and science today. Antimicrobial resistance in *E. coli* has increased worldwide and its susceptibility patterns show substantial geographic variation as well as differences in population and environment[33]. A lot of study has previously been done in this area to evaluate the faecal contamination of water bodies and isolation of resistant microorganisms from different environment and clinical samples because the presence of antibiotic resistant bacteria in natural habitat can pose severe public health risk. Resistant bacteria have been isolated from a variety of sources, including domestic sewage, drinking water, rivers, and lakes and it may be due to the selection resulting from metals present in the particular environment. The levels of antimicrobial agent resistance have been reported from 72% to 100% and 87% for faecal and non faecal coliforms, respectively. In a study, a total of 113 enteric bacteria were isolated from the Malathauze River. The antibiotic resistance pattern of these isolates showed that 94.7% were multi-resistant. The result suggests that the environmental, industrial and human activities impact on the level of antibiotic resistance in the environment. It is thus become important to determine the antibiotic resistance pattern of isolated microbes as it is the part of microbial monitoring process of the water. In an effort, twenty rainwater samples were collected from four geographical zones of endo states of Nigeria which fall under a rain zone of 1200mm annually. Among the rain water samples collected from each zone, endo state south had the highest *E. coli* occurrence. Also the *E. coli* isolates from this zone were found less susceptible and resistance in most cases to the antibiotics. These results are in concordance with our study. Our study revealed that susceptibility rate was high (i.e. 85%) in case of Cefixime. The susceptibility rate was 75% against Streptomycin and 30% in case of Ciprofloxacin among the twenty *E.coli* isolates. In a similar study, high sensitivity to Ciprofloxacin and Gentamycin has been recorded[3,35]. In this study, the overall resistance of *E. coli* to antimicrobials was high and results are consistent with the findings of previous studies[10,15,22,23]. Similarly, high level of resistance in *E. coli* was reported to Tetracycline from a study conducted in Ethiopia[1]. In our study high level of resistance was reported to Doxycycline as all isolates were found to be resistant against this antibiotic. In this study, Cefixime was found to be the most effective antimicrobials against *E. coli* isolates followed by Streptomycin, Tetracycline, Gentamycin, Azithromycin and Ciprofloxacin. Furthermore, a high rate of multi-drug resistance was recorded, which is consistent with the reports of studies done elsewhere[10,19].

Table. 3: MAR Index of *E. coli* Isolates

***E. coli* Isolates**	**S1**	**S2**	**S3**	**S4**	**S5**	**S6**	**S7**	**S8**	**S9**	**S10**
***MAR Value**	0.3	0.6	0.3	0.4	0.3	0.3	0.7	0.4	0.3	0.9
***E. coli* Isolates**	**S11**	**S12**	**S13**	**S14**	**S15**	**S16**	**S17**	**S18**	**S19**	**S20**
***MAR Value**	0.3	0.6	0.3	0.6	0.4	0.6	0.7	0.1	0.1	0.9

*MAR refers the Multiple Antibiotic Resistance

The Multiple Antibiotic Resistance (MAR) index of profile was performed to evaluate the heath risk of the environments. Multiple antibiotic resistance index (MAR) (number of antibiotics to which test isolate displayed resistance divided by total number of antibiotic to which the test organism has been evaluated for sensitivity) for each test isolate was calculated as recommended by Krumperman[18]. An isolate with a value of MAR > 0.2 is an indicator of the area with a high risk of contamination (e.g. animal farms, increased human population) where the antibiotics are frequently used. MARs of different *E. coli* strains isolated in the current study are represented in Table.3. In the current study, only two strains were shown to have MAR value <0.2. Rest eighteen strains were having the MAR up to 0.9 showing very high degree of resistance. This study reveals the applicability of the multiple antibiotic resistance (MAR) with the aim to identify the origin of the faecal pollution, offering information about the source of water pollution, which is a very useful tool for water management. The number of antibiotics involved in the multiple drug resistance as obtained in this study, falls within the range obtained by earlier workers[16,20,24,25,30]. The cumulative effectiveness of the antibiotics as obtained in this study is Cefixime > Streptomycin > Tetracycline > Gentamycin > Azithromycin > Ciprofloxacin > Doxycycline.

CONCLUSION

An alarming consequence has been occurred due to widespread emergence of resistance among microorganisms against available antibiotics. This problem is more dangerous if the water bodies from where the common people get their water for drinking purpose are severely contaminated with harmful microbes such as *E. coli* which in turn is resistant to many antibiotics i.e. multi-drug resistant. Although this bacterium is a resident of human intestine, its prevalence in water severely affect the health of community. Thus, this study is highly informative in terms of the evaluation of faecal contamination of water bodies as well as to determine the resistance of *E. coli* against the commonly available clinically significant antibiotics. The results were indicative of very high antimicrobial resistance to Doxycycline and Ciprofloxacin which are. Although, these antibiotics are commonly prescribed antibiotics, increased and uncontrolled use of these antibiotics led to the generation of multi-drug resistant strains. The fourth generation antibiotic i.e. Cefixime is found to have significant efficacy and can be considered appropriate for empirical treatment of *E. coli* infections. Although, the present study involves the investigation only from some locations of Uttarakhand region of India, the study areas can be enhanced for clearer picture of faecal contamination of water bodies and for periodic monitoring of antimicrobial susceptibility in the community. This can led to beneficially assist in the identification of alternate drug to control these multi-drug resistant *E. coli* strains.

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