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PREVALENCE OF RESISTANCE TO CLINICALLY SIGNIFICANT ANTIBIOTICS AGAINST *Escherichia coli* ISOLATED FROM HOLY RIVER GANGA, INDIA

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ABSTRACT:

Ganga is a divine water body which has acquired a status of mother goddess in Indian culture civilization. Ganga water is considered as sacred water and Hindu rituals from birth to death cannot be consecrated without Ganga water. The microbiological quality of holy river Ganga, life sustaining surface water resource for large population of northern India is adversely affected due to rapid industrialization and population growth. The current investigation surveyed on total number of 10 water samples collected from river Ganga flowing in different cities of India. The samples were analyzed for Most Probable Number (MPN) of coliforms and found to be in the range of 940 organisms to 33000 organisms per 100ml. Similarly, MPN Fecal coliform was done at an incubation of higher temperature, and resulted from lower of 109 organisms to a higher at 2800 organisms per 100ml of samples analyzed. The Ganga water samples also showed the presence of Escherichia coli and further its antibiotic susceptibility pattern was performed by Kirby Bauer Agar well diffusion method. Various clinical significant antibiotics were used namely Streptomycin, Kanamycin, Meropenem, Norfloxacin, Ciprofloxacin, Ampicillin, Sulbactum and Tazobactum. All isolated *Escherichia coli* strains were found resistant to Tazobactum. The isolated E.coli from upstream water of Varanasi has shown resistance towards four out of eight antibiotics used. *Escherichia coli* present in all the samples have shown susceptibility towards Meropenem and Ciprofloxacin.

Keywords: Ganga water, microbiological quality, Most Probable Number (MPN) of Coliform, MPN Faecal coliform, *Escherichia coli,* Antibiotic susceptibility and resistance

INTRODUCTION

The 2,525km long river Ganga rises in the western Himalayas in the Indian state of Uttarakhand, and flows to the south and east through the genetic plain of north India into Bangladesh, where it empties into the Bay of Bengal. This holy river flows through five states in the country namely Uttarakhand, Uttar Pradesh, Bihar, Jharkhand and West Bengal. It originates at coordinate 30°59'N, 78°55'E which is known as Gangotri. It accounts for 26.3% of the total geographic area of India [1].Ganga is considered as life itself as it

provides water for cooking, bathing, irrigation crops and for sustaining livelihoods in many parts of the country. It is also known as India's greatest pilgrimage site. Its banks such as Rishikesh, Haridwar, Varanasi, Allahabad and Kolkata are visited by millions of people every day from different corners of the world to quench their thirst for knowledge and liberation. Rivers of India plays an important role in the survival of Indian people. But with the rapid increase inhuman population, urbanization and economic activities a lot of pressure is created on river water resources which has become a serious issue and requires a lot of attention. People living in nearby areas are directly dependent on these water resources for their day to day activities such as drinking, bathing, laundry and domestic function, [2].

The main reason for the contamination of aquatic environment and increasing biological oxygen demand is the industrial, urban and agricultural wastes that enter the water bodies [3, 4]. The sacred rituals which are performed in these rivers during festive season also contribute a lot to the pollution of the water bodies. The microorganisms which are introduced into these water bodies due to increase in organic and contaminated decomposed material, utilizes the great amount of decomposed material and also great amount of dissolved oxygen [5,6].This situation is leading to reduced oxygen content in the water which disrupts the aquatic life. Thus, posing a serious threat to water resources and nature too [7].

Ganga Action Plan was launched by Shri Rajiv Gandhi, then Prime Minister of India on 14th January, 1986. The main objective of this action plan was to improve water quality by proper treatment of Domestic Sewage, Industrial Chemicals and Toxic Waste directly entering into the river. It focused on introducing new technologies of sewage treatment like up-flow anaerobic sludge blankets (UASB) and controlling pollution from agricultural runoff, human defecation and throwing of unburned and half burned into the river. The ultimate objective of the GAP is to have an approach of integrated river basin management considering the various dynamic interactions between abiotic and biotic eco-system. The Government of India proposed to extend this model with suitable modification to the national level through a National River Action Plan (NRAP) [8].

In the budget tabled in Parliament on 10 July 2014, the then Union Finance Minister Arun Jaitley, announced an integrated Ganga development project titled 'Namami Gange' and allocated Rs. 2,037 crore for the same. As a part of the program, government of India ordered the shutdown of 48 industrial units around Ganga to avoid effluents being released into the river.

The presence of coliform is the indicator for the quality and safety for human consumption. *E.coli* and other groups of coliform may be present where there has been fecal contamination originating from warm blooded animals. Most strains of *E. coli* are nontoxic, but some can cause serious illness in humans. The presence of *E.coli* in drinking water represents a health concern because they are usually associated with sewage or animal wastes. Infection symptoms and signs include bloody diarrhea, stomach cramps, vomiting and occasionally, fever and other water borne diseases are caused due to the fecal contamination, excreted by human which passes into the sewage treatment plant, without effective treatment they are then passed onto water bodies. Fecal coliform (thermo-tolerant coliform) and fecal streptococci (intestinal Enterococci) are most widely used as indicator bacteria [9].

The main objective of this experiment is to detect, isolate and identify *E.coli* from holy river Ganga and study the antibiotic resistance pattern of the isolated *E.coli* using the following methodology:

1. Collection of water sample from different sites of River Ganga
2. Most Probable Number technique (MPN) for presence of coliforms and Fecal coliform
3. Detection and Identification of *E.coli* using biochemical tests.
4. Determination of susceptibility pattern of isolated *E. coli* against clinically significant antibiotics.

The presence of *E.coli* in drinking water represents a health concern because they are usually associated with sewage or animal wastes. *E.coli* is now tested for on a regular basis in water microbiological analysis. Cells are able to survive outside the body for a limited amount of time which make them potential indicator organism to test environment samples for fecal contamination.

The crucial public health concerns have been the presence of antibiotic resistance bacteria associated with the river [10]. With the increased use of commercially available antibiotics, there is a significant increase in the number of antibiotic resistant bacteria in the aquatic environment [11]. The commercially available antibiotics used now a days, are one of the reason, the bacterial species are attaining resistance to the antibiotics via various mechanisms developed by themselves [12]. It is also assumed that bacterial resistance to antimicrobial agents is increasing worldwide because of latest genes instead of by mutation [13]. A bacterium carrying several resistance genes is called Multi drug resistance (MDR) or a superbug or super bacterium.

Materials and Methods

1. Collection of water samples

Gamma irradiated, clean and sterilized bottles (200ml capacity) were used for sampling of water. For Dechlorination sodium thio sulphate 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 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 6 h of collection.

Table 1: List of Location for water collection:

Samples	Location
S1	D/S Haridwar
S2	U/S Rishikesh
S3	U/S Allahabad
S4	D/S Allahabad
S5	U/S Varanasi
S6	D/S Varanasi
S7	U/S Kanpur
S8	D/S Kanpur
S9	Patna
S10	Howrah (Kolkata)
D/S= Downstream of River Ganga U/S = Upstream of River Ganga	

2. Microbiological analysis

The estimation of coliform and *E.coli* was done by determining the Most Probable number (MPN) technique as per IS1622:1981 [14] in test samples. The test procedure included three phases namely presumptive, confirmative and completed phase.

i. Presumptive phase (to check the presence of coliform)

The water sample was homogenized by shaking it thoroughly and the sample taken out with pipette. 10 ml sample was introduced into five tube each of 10 ml double strength MacConkey broth containing inverted Durham's tube, 1 ml sample into five tube each of 10 ml single strength MacConkey broth containing inverted Durham's tube and 0.1 ml sample into five tube each of 10 ml single strength MacConkey broth containing inverted

Durham's tube. All the above tubes were incubated at 37 C for 48 hrs. All the tubes were observed for acid formation (yellow in Colour) and gas production (bubble formation in Durham's tube). In case of acid and gas observed, these tubes would be taken as positive tubes and would be subjected to confirmed test. In case of no acid and gas, the test shall be discontinued and results are reported as less than 2 organism/ 100 ml by MPN table.

ii. Confirmative phase (to confirm the observation of presumptive test for coliform)

Loopful from each individual positive tube is inoculated into 10 ml Brilliant Green Bile Lactose (BGBL) broth and incubated at 37°C for 48 hrs. The tubes are observed for gas (bubble formation in Durham's tube). If bubble is observed, the set of positive tube are recorded out of the total 18 tubes as confirmed test and proceed for the complete test. If no bubble observed, the test is discontinued and results are reported as less than 2 organism/ 100 ml by MPN table. The tubes are discarded.

iii. **Completion phase** (for further confirmation of coliform)

All the positive tubes are streaked onto MacConkey agar plates and incubated at 37°C for 24 hrs. If pink colonies are observed, from each plate, typical or atypical colonies are picked and (i) inoculated into 10 ml Lactose broth containing inverted Durham's tubes and incubated at 37°C for 24-48 hrs and observed for gas production, (ii) streaked onto nutrient agar slant and incubated at 37°C for 24 hrs for gram staining. If non spore forming rods are formed and if gas is produces in lactose broth the test is considered completed (gram negative) and the presence of coliform organism is confirmed. Positive set of tubes are recorded in confirmed test and no. of organism/100 ml are calculated from the MPN table and results are reported as no. of organism /100 ml.

Detection for *E.coli* (continuation of MPN)

Loopful from each confirmed positive Brilliant Green Bile Lactose (BGBL) broth tubes were inoculated into 10 ml BGBL broth and incubated at 44.5°C for 24 hrs. Gas production is checked. If no gas production observed, the test is discontinued and *E.coli* is reported as negative / 100 ml. If present, loopful from positive BGBL broth was further inoculated to 5 ml Tryptone water medium and incubated at 44.5°C and also, streaked onto EMB agar plates and incubated at 37^0C for 24 hrs. For the isolation of *E.coli*. Few drops of Kovac's reagent were added into the Tryptone water tubes after incubation. Observation of pink colored ring in the tubes and green metallic sheen Colonies on EMB plates is reported as *E. coli* positive / 100 ml. If no pink colored ring observed, *E. coli* is reported as negative / 100 ml.

MPN Fecal coliform:

Inoculate loopful from each Positive tubes from Presumptive phase in 10ml Brilliant Green Bile Lactose (BGBL) broth and incubate at 44^0 C for 24hrs.after 24 hrs observed the gas production in tubes (bubble formation in Durham's tubes).if gas observed in BGBL tubes(Numbers in sets of tubes recorded). These tubes would be taken as Positive tubes and report results as MPN Fecal coliform/100ml.If no gas observed discontinued the test and report results as no growth observed in MPN Fecal coliform/100ml and discard the tubes.

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 [11]. Following biochemical test like Motility, Indole, Citrate utilization, Glucuronidase, Nitrate reduction, ONPG, Lysine utilization, Lactose, Glucose, Sucrose, Sorbitol were used for confirmation of *E.coli*.

After Biochemical confirmation by standard biochemical tests, confirmed *E.coli* isolates were further streaked on NA slants for antibiotic sensitivity test.

3. Antibiotic Sensitivity Test

All isolated bacterial strain culture 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. The working solution of antibiotics was 5mg/ml. Antibiotic susceptibility and resistance were evaluated by agar well diffusion assay. The antibiotic solution was prepared by taking calculated amount of antibiotic drugs and dissolving it in 50ml of solubilizing agent and then sonicating it. Final working solution of 0.5mg/ml was prepared from stock solution in a volumetric flask. 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 individual plates for each individual isolates which were allowed to solidify. Each plate was punched to make wells of 6 mm diameter with the help of sterile cork borer at four marked sites of the plates. 100 µl of respective antibiotic solutions (0.5mg/ml) were pipette out into the well in assay plates. Plates were incubated overnight at 37^{o}C. Following incubation, petri-plates were observed for the inhibition zones, diameters of which were measured by using Vernier Caliper.

Results and discussion

In the present study, 10 water samples were collected from the Upstream and Downstream regions of Holy River Ganga, India (Table 1). These samples were analyzed for the Enumeration of Coliform & Fecal coliform by Most Probable Number Techniques by using Multiple Tube Dilution Method and presence of *E.coli* which is a prominent member of family Enterobacteriaceae and also is an indicator microorganism of fecal contamination of water bodies. During the study, all the collected samples have shown the presence of coliform in the range of 940 organisms to 33000 organisms per 100ml and the presence of fecal coliform in the range of 110 organisms to 2800 organisms per 100ml. It was found that the contamination in upstream water samples was less as compared to downstream water samples (Table 2).we can conclude that the coliform was found to be the maximum in the samples collected from Kanpur (downstream) which is 33000 organisms per 100ml and the minimum contamination was found in the samples collected from Rishikesh (upstream) which is 940 organisms per 100ml.

Table 2: Results of MPN Coliform and MPN Fecal coliform per 100ml in water sample collected from river Ganga

Samples	Location	MPN Coliform per 100ml (organisms)	MPN Faecal Coliform per 100ml (organisms)	*Escherichia coli*
S1	D/S Haridwar	$7x10^3$	$9x10^2$	Present
S2	U/S Rishikesh	$9.4x10^2$	$1.7x10^2$	Present
S3	U/S Allahabad	$2.8x10^3$	$1.1x10^3$	Present
S4	D/S Allahabad	$1.4x10^4$	$1.6x10^3$	Present
S5	U/S Varanasi	$5.4x10^3$	$1.1x10^2$	Present
S6	D/S Varanasi	$7x10^3$	$9x10^2$	Present
S7	U/S Kanpur	$2.2x10^4$	$1.4x10^3$	Present
S8	D/S Kanpur	$3.3x10^4$	$2.8x10^3$	Present
S9	Patna	$2.8x10^4$	$9.4x10^2$	Present
S10	Howrah (Kolkata)	$1.4x10^4$	$1.1x10^3$	Present

U/S- Upstream and D/S-Downstream

Table 3: Confirmation of MPN Coliform

Samples	Location	MacConkey Agar (pink colonies)	Lactose Broth (gas bubble)
S1	D/S Haridwar	Present	Present
S2	U/S Rishikesh	Present	Present
S3	U/Allahabad	Present	Present
S4	D/S Allahabad	Present	Present
S5	U/S Varanasi	Present	Present
S6	D/S Varanasi	Present	Present
S7	U/S Kanpur	Present	Present
S8	D/S Kanpur	Present	Present
S9	Patna	Present	Present
S10	Howrah (Kolkata)	Present	Present

***D/S-Downstream and U/S-Upstream**

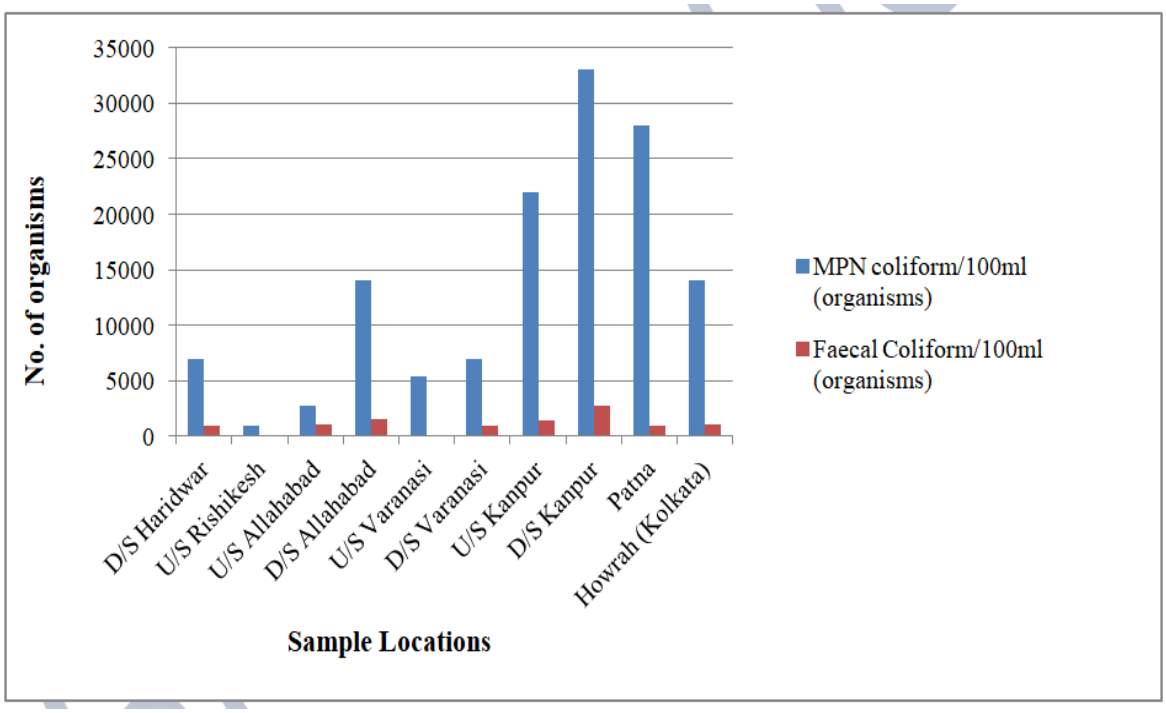

Figure 1: Graphical representation of MPN coliform/100ml and fecal coliform per 100ml of the collected samples

During the study, total ten E.coli were isolated from samples. These isolates were biochemically Identified for the confirmation of *E. coli*. Isolated *E. coli* strains were then evaluated for their antimicrobial susceptibility and Resistance patterns against eight commonly prescribed clinically significant antibiotics by using agar well diffusion assay (Table 4 & Fig 2, Fig 3). Table 5 demonstrated the antibiotic resistance patterns in terms of average zones of diameter considering 4 plates for each *E.coli* isolates against each of eight antibiotics of 5mg/ml concentration.

Table 4: Biochemical test results of *E.coli*

Sr. No.	Test	Sample Code										Positive Control *E.coli* (ATCC 8739)
		S1	S2	S3	S4	S5	S6	S7	S8	S9	S10	
1	Gram Staining	Gram negative rods	Gram negative rods	Gram negative rods	Gram negative rods	Gram negative rods	Gram negative rods	Gram negative rods	Gram negative rods	Gram negative rods	Gram negative rods	Gram negative rods
2	Motility	Positive	Positive	Positive	Positive	Positive	Positive	Positive	Positive	Positive	Positive	Positive
3	Indole	Positive	Positive	Positive	Positive	Positive	Positive	Positive	Positive	Positive	Positive	Positive
4	Citrate utilization	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative
5	Glucuronidase	Positive	Positive	Positive	Positive	Positive	Positive	Positive	Positive	Positive	Positive	Positive
6	Nitrate reduction	Positive	Positive	Positive	Positive	Positive	Positive	Positive	Positive	Positive	Positive	Positive
7	ONPG	Positive	Positive	Positive	Positive	Positive	Positive	Positive	Positive	Positive	Positive	Positive
8	Lysine utilization	Positive	Positive	Positive	Positive	Positive	Positive	Positive	Positive	Positive	Positive	Positive
9	Lactose	Positive	Positive	Positive	Positive	Positive	Positive	Positive	Positive	Positive	Positive	Positive
10	Glucose	Positive	Positive	Positive	Positive	Positive	Positive	Positive	Positive	Positive	Positive	Positive
11	Sucrose	Positive	Positive	Positive	Positive	Positive	Positive	Positive	Positive	Positive	Positive	Positive
12	Sorbitol	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative

Data revealed that all *E.coli* isolates showed the variable sensitivity against the different antibiotics used in the study. High rate of antibiotic resistance was found in Tazobactum against ten *E. coli* isolates i.e. 100% resistance indicating an alarming situation. Whereas the positive control of *E.coli*

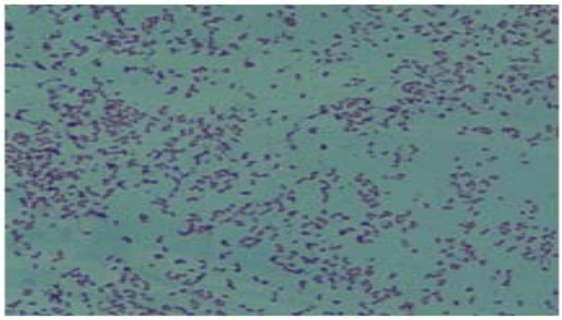

Figure 2: Gram negative rod shaped of *Escherichia coli* of Sample-2(S2)

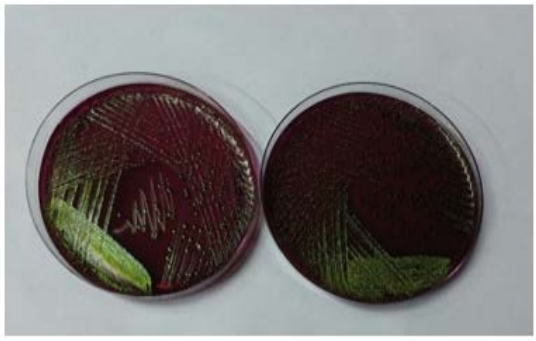

Figure 3: Green metallic sheen colonies of Positive control *Escherichia coli* and Sample-3(S3) on EMB Agar plate

Showed a zone of inhibition of 22.87mm. Sulbactum also found to be 70% resistant against isolated *E.coli*. Moreover A minimum of 20% resistance was also observed in Ampicillin and Streptomycin.
The study also revealed Susceptibility of Meropenem, Norfloxacin and Ciprofloxacin against all isolates of *E.coli*.

Table 5: Zone of inhibition (in mm)* of antibiotics against *E.coli* isolated from water samples

Sample Code	Streptomycin	Kanamycin	Meropenem	Norfloxacin	Ciprofloxacin	Ampicillin	Sulbactum	Tazobactum
S1	18.47	17.50	28.37	27.51	32.38	17.80	0	0
S2	17.96	16.57	29.81	27.73	29.25	15.24	14.33	0
S3	20.64	17.12	31.42	29.52	32.47	15.68	15.50	0
S4	16.00	15.49	31.88	23.73	24.35	22.78	0	0
S5	0	0	41.72	35.04	40.52	0	0	0
S6	17.13	17.22	25.85	27.52	28.62	14.98	0	0
S7	19.01	16.66	26.78	28.33	28.04	15.46	0	0
S8	16.89	16.74	30.64	26.98	28.45	20.43	15.06	0
S9	18.08	16.64	28.34	21.12	34.44	0	0	0
S10	0	14.72	31.78	18.51	22.00	14.15	0	0
Positive Control	22.10	14.05	24.65	20.25	27.34	25.14	17.81	22.87

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

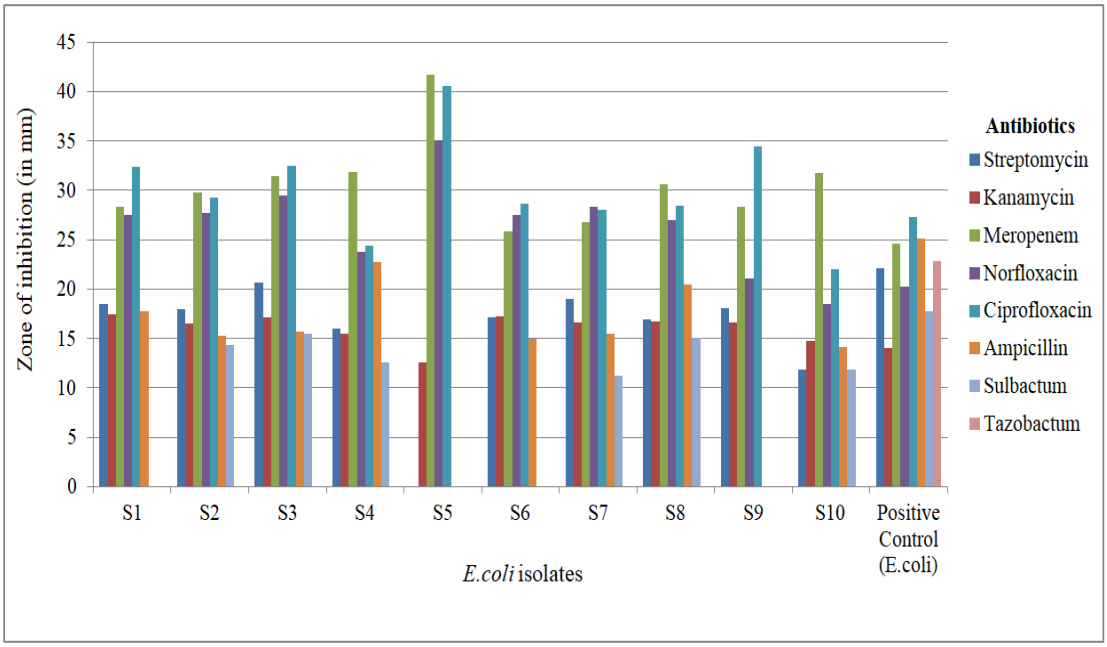

Figure 4: Graphical representation of Antibiotic sensitivity against *Escherichia coli*

Conclusion

The results show high rates of antimicrobial resistance to Tazobactum and Sulbactum. Meropenem, Norfloxacin and Ciprofloxacin is considered appropriate for empirical treatment of *E. coli* in the study area. Periodic monitoring of antimicrobial susceptibility in the community is recommended.it can pose major health threat to human community. The resistance to certain antibiotics may be due to the frequent discharge of the chemicals into the river which make the antibiotics ineffective against the microorganisms present. In the present study we determined the antibiotic resistant and susceptibility pattern of 10 *E.coli* isolates from river Ganga water flowing in different cities of India.

The cumulative Resistance of the antibiotic as obtained in the study is Tzobactum> Sulbactum>Ampicillin>Streptomycin>Kanamycin>Meropenem, Norfloxacin and Ciprofloxacin; so that our study can beneficially assist in the identification of alternate drug to kill these multi drug resistant *E.coli* and treat the disease caused by these drug resistant *E.coli.*

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