SURVEILLANCE OF ANTI-BIOTIC RESISTANT *E. COLI* IN ISLAMABAD

By

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A thesis submitted to Bahria University, Islamabad in partial fulfillment of the requirement for the degree of MS in Environmental Sciences

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ABSTRACT

Escherichia coli (*E. coli*) is a Gram-negative, facultatively anaerobic, rod-shaped, coliform bacterium of the genus *E. coli*. It is commonly found in the lower intestine of warm-blooded animals and expelled into the environment within fecal matter and can cause serious food poisoning in their hosts. Extended spectrum beta lactamases (ESBL) producing *E. coli* confer resistance to most beta-lactam antibiotics, including third and fourth generation cephalosporins. It severely limits treatment possibilities for infections caused by these bacteria. The objective of this study was the Isolation and Identification of ESBL producing *E. coli* from the various water streams and to assess the prevalence of ESBL producing *E. coli* in the environment. For this purpose, samples were collected from various upstream and downstream locations of Islamabad. Spread plate and streak plate methods and different biochemical tests including; Indole test, Methyl Red test, Voges-Proskauer test, Simmon Citrate test and Triple Sugar Iron test were performed for the identification and confirmation of *E. coli*. Double disc synergy test and double disc diffusion test were performed for the confirmation of ESBL producing *E. coli*. The results show that ESBL *E. coli* was identified in Bharakahu downstream and its wet market, Korang stream G-6 and its wet market, I-9 waste water treatment plant and from Lai stream. No *E. coli* and ESBL *E. coli* were detected in the water samples of Sangrilla park upstream and Shahdara upstream. All of the samples were found positive for *E. coli* in Bharakahu downstream and 71% samples were found positive for *E. coli* in Bharakahu wet market and all of the samples were found positive for ESBL *E.coli* and 85 percent samples were found positive in Bharakahu wet market and all samples were found positive for *E. coli* in Korang G-6 aand 70 percent samples were positive for *E. coli* in Korang G-6 wet market. Similarly, in I-9 waste water treatment plant all of the samples were positive for *E. coli* and 100% for ESBL *E. coli*. All of the samples of Lai stream were positive for *E. coli* and ESBL *E. coli* in the collected samples.

Antibiotics FEP 30 (Cefepime) and CRO 30 (Ceftriaxone) were producing more synergistic effect towards AMC 30 (Amoxycillin + Clavulanic Acid) by giving zone of inhibition towards AMC 30 (Amoxycillin + Clavulanic Acid), while

CAZ 30 (Ceftazidime) has produced very little zone of inhibition. The remaining two CFM 5 (Cefixime) and ATM 30 (Aztreonam) are unable to give any zone of inhibition towards AMC 30 (Amoxycillin + Clavulanic Acid) which shows the synergistic effect of different antibiotics used in this study. The result show high prevalence of antibiotic resistant *E. coli* in environment.

ACKNOWLEDGEMENT

First and foremost, praises and thanks to Almighty Allah who is the supreme power, the most gracious and the most merciful, the Almighty, for His showers of blessings to complete this dissertation. My sincere gratitude to my supervisor Dr. Asma Jamil, Senior Assistant Professor, Earth & Environmental Sciences, Bahria University, Islamabad for her remarkable guidance throughout this study. I am thankful to Prof. Dr. Tahseen - Ullah Khan, Head of Department Earth and Environmental Science for providing all support. I also owe my gratitude to Dr. Farzana Altaf Shah; Director General Pak-EPA, Dr. Mohsina Tunio; Deputy Director lab Pak- EPA for providing all the support and facilities to carry out this study. I am thankful to Dr. Hamid Irshad; Senior Scientific officer NARC and Dr. Aitzaz Ahsan; Scientific officer NARC, for their assistance in collection of samples and for lab support. I am grateful to my parents who have provided moral support in my life. I am also grateful to all of my family members and friends who have supported me along the way. This accomplishment would not have been possible without them.

Thank you.

ABBREVIATIONS

E. coli	*Escherichia coli*
ESBL	Extended spectrum beta lactamases
ABR	Antibiotic resistance
WWTP	Waste water treatment plant
MR	Methyl red
VP	Voges Proskauer
TSI	Triple sugar iron
DDDT	Double disc diffusion test
DDST	Double disc synergy test
CAL 40	Ceftazidime + Clavulanic Acid
CAZ 30	Ceftazidime
CTL 40	Cefotaxime + Clavulanic Acid
CTX 30	Cefotaxime
AMC 30	Amoxycillin + Clavulanic Acid
ATM 30	Aztreonam
CRO 30	Ceftriaxone
CFM 5	Cefixime
FEP 30	Cefepime
CAZ 30	Ceftazidime
ABR	Antibiotic resistant bacteria

BLPB	Beta-Lactamase Producing bacteria
MIC	Minimum inhibitory concentrations
MPN	Most probable number
PCR	Polymerase chain reaction
MDR	Multiple drug resistance
MDDST	Modified Double Disc Synergy Test
CLSI	Clinical Laboratory Standard Institute

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CHAPTER 1

INTRODUCTION

Escherichia coli (*E. coli*) is a Gram-negative, facultative anaerobic, rod-shaped, coliform bacterium of the genus *Escherichia.* It is usually existing in the lower intestine of warm-blooded animals. *E. coli* is ejected into the environment within fecal matter and can be a reason of serious food poisoning in their hosts. A growing body of research, though, has inspected environmentally persistent *E. coli* which can animate for extended periods outside a host.

The rapid development of the bacteria which are resistant is happening globally, risking the use of antibiotics, that changes the medications and protected millions of lives. Several years when the antibiotic was used to treat the first person, the infection caused by the bacteria is again become the hazardous. The antibiotic resistance crisis has been credited to the overuse and misuse of these medications, in addition to a lack of new drug development by the pharmaceutical industry due to condensed economic incentives and interesting regulatory requirements. The Centers for Disease Control and Prevention (CDC) has classified a number of bacteria as presenting urgent, serious, and regarding threats, many of which are now responsible for placing a substantial clinical and financial burden on the health care system, patients, and their relatives (Sujatha et al., 2017).

1.1 History of Antibiotics

The current stage of antibiotics started with the detection of penicillin by Sir Alexander Fleming in 1928. Since then, advancement in medicines occurring and treat many people lives. Serious infections were treated by the antibiotics in the 1940's. bacteria treated by the antibiotics among World War II soldiers. But then penicillin was becoming a problem so in 1950's, a high number of advancements is done in the field of antibiotics. So, a new beta-lactam antibiotic was discovered.

However, methicillin resistant *Staphylococcus aureus* (MRSA) first case was examined during that same period, in the United Kingdom in 1962 and in the United States in 1968 (Kardos et al., 2011). Sir Alexander Fleming was very curious about the overuse of the antibiotics and he told that the demand of the drug will be increased and then it will be misused. The antibiotics overuse identifies the change of resistance (Venotla et al., 2015).

1.2 Benefits of antibiotics

Anti-microbials have not just protected patients' life but they have assumed a fundamental job in accomplishing real advances in medication and medical procedure. They have effectively anticipated or treated contaminations that can happen in patients who are getting chemotherapy medicines; who have endless ailments, for example, diabetes, renal illness, or rheumatoid joint inflammation. Likewise, in individuals who have had complex medical procedures, for example, organ transplants, joint substitutions, or cardiovascular medical procedure. Anti-microbials have additionally broadened expected life expectancies by changing the result of bacterial contaminations (Laxminarayan et al., 2011).

In both the industrialized and under developing countries, antibiotic agents are broadly utilized as development supplements in domesticated animals other than human use. An expected eighty percent of anti-toxins sold in the U.S. are utilized in animals and humans, essentially to elevate development and to forestall disease. Treating domesticated animals with antimicrobials is said to improve the general soundness of the creatures, delivering bigger yields and a higher-quality item. The anti-toxins utilized in domesticated animals are ingested by people when they devour sustenance. The exchange of resistant microorganisms to people by homestead creatures was first noted over thirty five years prior, when high rates of antibiotic obstruction were found in the intestinal greenery of both ranch creatures and ranchers. All the more as of late, atomic recognition techniques have exhibited that resistant microbes in homestead creatures achieve customers through meat items. This happens through a progression of antibiotics utilized in animals kills bacteria, permitting antibiotic resistant microorganisms to flourish, microscopic organisms are transmitted to people through the sustenance supply and these microorganisms can cause diseases in people that may prompt unfavorable wellbeing outcomes (Lee, 2015).

1.3 Agricultural use of Antibiotics

The rural utilization of antibiotic agents additionally influences the natural microbiome. Up to ninety percent of the antibiotic agents given to domesticated animals are discharged in urine and stool, at that point generally scattered through compost, groundwater, and surface overflow. Furthermore, antibiotic medications and streptomycin are splashed on natural product trees to go about as pesticides in the western and southern U.S. While this application represents a lot amount of in

general anti-microbial use, the resultant land spread can be significant. This training likewise adds to the introduction of microorganisms in nature to development hindering operators, adjusting the ecological environment by increasing the resistant amount against powerless microorganisms. The cleaning items also cause this issue, since they may confine the improvement of invulnerabilities to ecological antigens in the two youngsters and grown-ups. Subsequently, invulnerable outline litheness could be undermined, potentially increasing bleakness and death caused by diseases that wouldn't typically critical (Sarmah et al., 2006).

1.4 Use of Antibiotics and Antibiotic resistance

The utilization of antibiotic agents in human and animal has brought about occurrence of antibiotic resistant (ABR) microscopic organisms in people and faunas, yet in addition in the earth, for example in surface water and soil (Kummer et al., 2004). As an outcome, the likelihood of getting presented to ABR microscopic organisms outside a social insurance setting has expanded. A particular sort of anti-microbial obstruction that at present speaks to a noteworthy general wellbeing concern is the third era cephalosporin opposition initiated by ESBL *E. coli* are impervious to practically all beta-lactamase antibiotic agents, and frequently to different classes of anti-toxins too (Canton et al., 2008).

1.5 Introduction to Extended spectrum beta lactamases producing *E. coli*

ESBL *E. coli* creating *Enterobacteriaceae* are expanding in predominance around the world. ESBL *E. coli* gives protection from most beta-lactam anti-toxins, including third and fourth era cephalosporins, which seriously constrains treatment conceivable outcomes for contaminations brought about by these microorganisms. The improvement of antimicrobial opposition by *Enterobacteriaceae* is inescapable and is considered as a noteworthy issue in the treatment of bacterial diseases in the medical clinic and in the network. Beta-Lactamase-Producing microorganisms (BLPB) can assume a significant job in polymicrobial contaminations. They can have a direct pathogenic effect in causing the contamination just as a backhanded impact through their capacity to deliver the protein beta-lactamase (Reinthaler et al., 2010). These enzymes producing *Enterobacteriaceae* families are linked with a higher disease and mortality

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Treatment of *E. coli* creating strains of *Enterobacteriaceae* has risen as a noteworthy test in hospitalized patients. These chemicals speed up the break down of of β-lactam ring of antibiotic agents, in this manner obliterating their antimicrobial property. ESBL *E. coli* creating living beings are regularly impervious to a few different classes of antibiotic agents, as the plasmids with the quality encoding ESBL *E. coli* regularly convey other resistant determinants. The component of antibiotic opposition is delineated in figure 1.1

Source:https://www.reactgroup.org/toolbox/understand/antibiotic-resistance/resistance-mechanisms-in-bacteria/

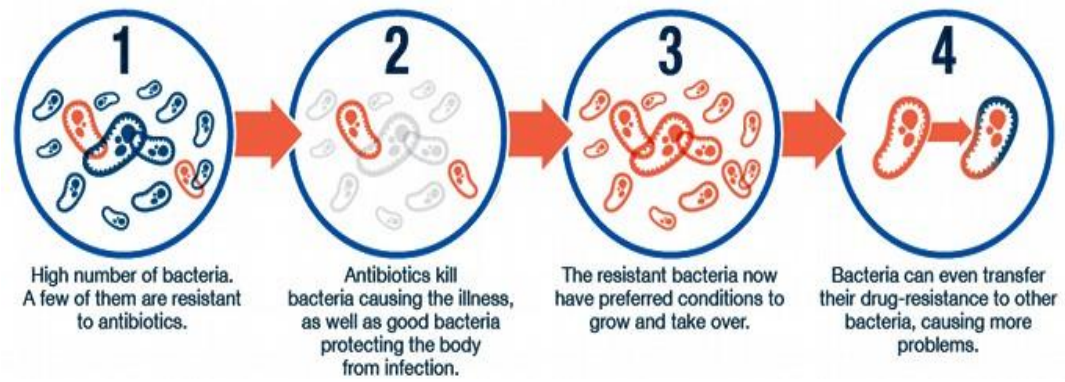

Figure 1.1. Antibiotic resistance mechanism.

Critically important antibiotics in humans list was made by WHO to confirm wise use of drug in both human and veterinary drug as shown in figure 1.2.

Source: http://proteininformatics.org/mkumar/lactamasedb/lactamase.html

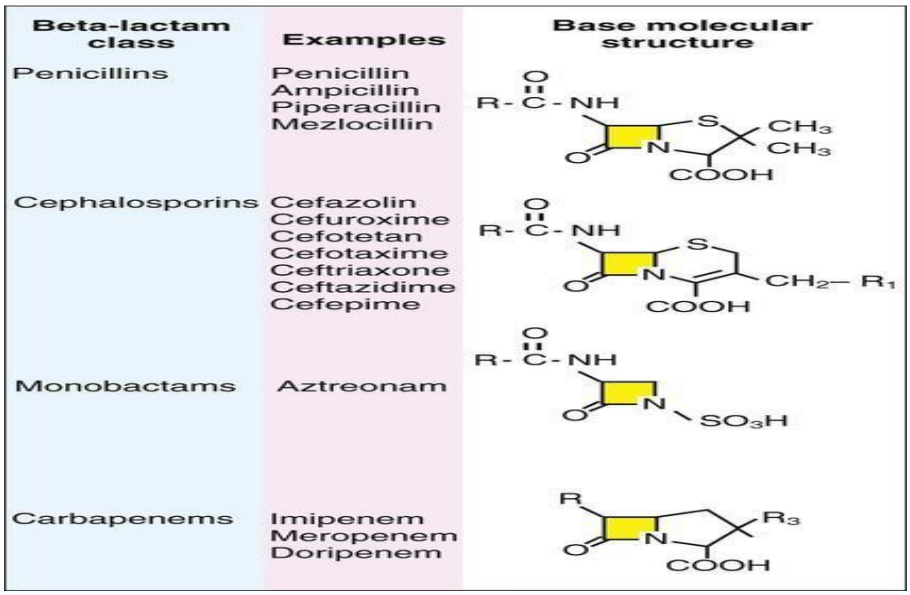

Figure 1.2. Different classes of antibiotics.

The third and fourth-age cephalosporins, fluoroquinolones, and macrolides are viewed as medications earnestly needing danger the executives of their utilization in nourishment creatures. The utilization of third generations cephalosporins represents a significant test. Numerous anti-toxins are discharged unaltered, are ecologically relentless, recognized downstream of wastewater treatment plants and nearby fields accepting creature composts. In treated effluents and sewage slime, anti-microbial deposits of a few classes extend in focuses from nanograms per liter up to low micrograms per liter. Except of the fact that these are minimum inhibitory concentrations (MICs), even low fixations give particular points of interest to certain resistant strains (Parish et al., 2001).

ESBL *E. coli* generation was chiefly seen in emergency clinic diseases brought about by Klebsiella pneumonia, yet today it is additionally regularly connected with network obtained contaminations, for the most part urinary tract diseases brought about by *E. coli*. Resistant diseases are winding up more difficult or even difficult to treat with flow anti-toxins, prompting contaminations causing higher grimness and mortality, forcing gigantic expenses on our general public. This expanding obstruction includes numerous basic human pathogens, including *Enterococcus faecium, Escherichia coli, Staphylococcus aureus, Klebsiella*

pneumoniae, Acinetobacter baumannii, Pseudomonas aeruginosa, and the *Enterobacter* species (Falagas et al., 2009).

Be that as it may, huge numbers of these microscopic organisms and their methods of obstruction originated from the regular habitat, including microorganisms inside soils and water. Anti-microbial obstruction improvement isn't only a neighborhood general medical problem however incorporates more extensive natural influences, which are amplified by universal travel and worldwide exchange staples. The (WHO, 2007) as of late reported a suite of strategies that, whenever actualized, ought to alleviate the development and further spread of antibiotic resistant life form. These activities have concentrated on antibiotics in the clinic and network settings, and diminishing antibiotic use in domesticated animals' generation. In any case, in the event that we are to all the more likely oversee anti-microbial obstruction, it is likewise essential that we think about the more extensive condition. Consequently, an enhanced comprehension of the effects of human exercises on antibiotic obstruction advancement is required, for example, non-human antibiotic use, medicinal assembling waste, household and farming waste discharges into the earth, and the influence of poor sanitation and perilous water supply (Finley et al., 2013).

Human action since the antibiotic advancements, creation after World War II has changed the dissemination and expanded the plenitude of opposition qualities. Qualities were two to multiple times progressively bounteous in 2008 contrasted with the 1970s in DNA separated from chronicled soil tests gathered somewhere in the range of 1940 and 2008 in the Netherlands. Specifically, qualities encoding protection from β-lactams and antibiotic medications were improved. Worrisomely, an expansion in ESBL *E. coli* of the CTX-M family was observed, which seems to originate before any clinical discovery of these catalysts (Pitout et al., 2010).

Besides, since industrialization, a large number of huge amounts of anti-toxins have been discharged into the earth, including by means of wastewater effluents, land utilization of creature squanders, action of yield sicknesses, aquaculture, and numerous different exercises. General wellbeing impacts from antimicrobial use in horticulture and aquaculture have officially attracted plentiful consideration the most recent period. Critically, antibiotic agents utilized in people and creatures frequently have a place with similar classes. The WHO has built up

a rundown of basically significant anti-toxins in people to guarantee reasonable medication use.

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Different studies have been carried out for the discovery and occurrence of antibiotic resistant *E. coli.* Hetty et al. (2008) described the isolation of ESBL *E. coli* making house flies and blow flies caught at two poultry farms, creating a different way of transfer of ESBL *E. coli* from poultry to humans.

Sandra et al. (2011). described the change of the most probable number (MPN) method for fast record of resistant *E. coli* bacteria in water body samples.

Anti-microbial resistant *E. coli* was not eliminated and *E. coli* resistant to cefotaxime including ESBL *E. coli* producers, ciprofloxacin, and cefotaxime was present in treated effluent samples.

Saba et al. (2011) examined the predominance of ESBL *E. coli* and Klebsiella disconnects from clinical and natural sources. They have discovered that *E. coli* is progressively predominant in both hospitalized and network obtained contaminations. Females were progressively inclined to diseases brought about by ESBL *E. coli* makers. Urinary tract contamination was increasingly normal in females tainted by ESBL *E. coli*.

Arne et al. (2017) Identified hazard factors that clarify systems and courses for spread of ESBL *E. coli* in a low predominance nation, which can be utilized to guide suitable treatment of CA-UTI and focused on contamination control measures. Another examination assessed the effect of initiated ooze and physicochemical waste water treatment forms on the commonness of conceivably destructive *E. coli*.

An aggregate of 719 *E. coli* isolates gathered from four metropolitan plants

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The prevalence of ESBL *E. coli* and Klebsiella isolates from clinical and natural sources. Result introduced that *E. coli* is most basic in ESBL *E. coli* maker with Klebsiella, Enterobacter and Citrobacter. *E. coli* is progressively common in both hospitalized and network gained contaminations. Females were increasingly disposed to contaminations brought about by ESBL *E. coli* makers. Guys of senior age and females of conceptive age were increasingly disposed to of contaminations brought about by ESBL *E. coli* makers. In natural source, sewage was most extreme pervasive wellspring of ESBL *E. coli* makers following soil and standing water. This gives the commonness of this superbug in this piece of the world is high which needs unmistakable consideration of the general public (Riaz et al., 2011).

Gao et al. (2014) stated that the spread of medication resistant microbes from creature homesteads to oceanic conditions can represent a potential danger to general wellbeing. In this examination, antimicrobial obstruction, opposition qualities, and hereditary closeness of ESBL *E. coli* of various causes like chicken defecation and area 5and downstream waterway waters were broke down to follow the spread of medication resistant microorganisms of creatures. The outcomes demonstrated that a sum of 29 ESBL *E. coli* was gotten from 258 examples.

Korzeniewska et al. (2013) identified nearness of ESBL *E. coli*-positive *Enterobacteriaceae* in city sewage and their emanation to the encompassing air and the stream getting effluent from WWTP was researched. In the gathering of 455 segregated strains, up to 19.8% were phenotypic ESBL *E. coli*-makers. They were identified in the 100% of sewage tests investigated.

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Ahmed et al. (2016) assessed the predominance of ESBL *E. coli* and anti-microbial example of *Pseudomonas* gathered during 2010 to 2014 from tertiary consideration emergency clinics of Peshawar, Pakistan. Out of 3450 examples, 334 Pseudomonas spp. disengages contained 232 indoor and 102 open air patients were acquired from various examples and their powerlessness example was resolved against 20 anti-toxins. Antimicrobial helplessness testing was done utilizing the

Kirby-Bauer agar dispersion technique and ESBL *E. coli* generation was recognized by Synergy Disk Diffusion strategy in this investigation.

Microbial pathogens are one of the significant wellbeing dangers related with water and wastewaters. Current techniques for the identification of pathogenic infections, microbes, protozoa and helminths will in general be mistaken, tedious and costly. Thus, pointer microbes are ordinarily used to decide the general danger of fecal sullying and the conceivable nearness of pathogens in water and wastewaters. Subsequently, techniques to legitimately identify microbial pathogens in water and wastewaters were explored (Simmon, 2014).

Elsayed et al. (2017) described the multidrug resistant strains among UTI brought about by *E. coli* disengages against ordinarily utilized antimicrobial operators and the conceivable job of ESBL *E. coli* in *E. coli* production from anti-microbials Hundred *E. coli* isolates from 260 in patients remained recognized. Powerlessness to different antibiotic agents was checked utilizing standard techniques. All Isolates were analyzed for the nearness of ESBL *E. coli* utilizing mix plate technique, showing ESBL *E. coli* generation. Waste runoffs cause microbial pollution of surface water as well as result in pollution of groundwater, especially in zones where the water table is shallow. For instance, in Lahore city of Punjab, Wah and other near zones, the shallow groundwater was observed to be exceedingly polluted with coliforms and fecal coliforms because of the addition of sewage water from lacking sewage transfer procedures (Ahmad et al., 2012).

Seedy et al. (2017) detected the genes responsible for the multiple antibiotic resistance *S. aureus* isolates from food of animal origin in Egypt by polymerase chain reaction (PCR). An aggregate of 125 examples were haphazardly gathered from milk, meat, and their items from Giza and Beni-Suef Governorates markets. The *S. aureus* separates were exposed to antimicrobial affectability tests utilizing four antibacterial plates (Oxoid), and after that the PCR was performed for location of anti-microbial opposition qualities. Out of 125 examples, 19 *S. aureus* segregates were distinguished. All distinguished segregates were various medication opposition (MDR).

Walther et al. (2018) reported Pathogen normally related with multi drug resistant (MDR) phenotypes, including ESBL and *Acinetobacter baumannii* taken from horses and given to horse clinics and were found positive for ESBL producing

E. coli.

Franz et al. (2015) found pathogenic *E. coli* producing ESBL isolated from surface water and wastewater. 17.1% of all ESBL creating *E. coli* were suspected pathogenic variations. Their investigation exhibited that the sea-going condition is a potential supply of *E. coli* variations that join ESBL qualities, an abnormal state of multi-sedate resistance and destructiveness elements, and represent a danger to people.

Haberecht et al. (2019) quantified and described ESBL and KPC producing *E. coli* in Northern Colorado from sewerage, surface water, and influent and effluent wastewater treatment sources. Total detected bacteria and *E. coli* abundances, and the percentages that contain ESBL. Their results show that total *E. coli* abundance decreased through the water treatment process.

1.1 Objectives of the study

Keeping in view the use of antibiotics and prevalence of antibiotic resistant bacteria in environment, this study was designed with the following objectives:

- Isolation and Identification of ESBL *E. coli* from the water samples of various origins.
- To assess the prevalence of ESBL *E. coli* in selected regions of Islamabad.

CHAPTER 2

MATERIALS AND METHODS

The present study was conducted to evaluate the prevalence of ESBL *E. coli* in the water streams and from the wet markets. Microbiological analysis and biochemical tests were conducted for the identification of *E. coli* and ESBL *E. coli*.

2.1 Sample collection

The water samples were collected from various locations of Islamabad during the month of July to September 2018 for microbiological study. Sampling bottles were washed, sterilized in an autoclave from 15 to 20 min at 121 ˚C at 1500 psi, heat dried in hot air oven and properly labeled. A total of 56 samples were taken from all the locations. These samples were taken in 1000 ml sterilized glass bottles according to standard method (APHA, 2005) and were brought to the laboratory in ice coolers. These sample bottles were accurately labeled. Microbiological examination of the water samples was conducted within 24 hours of collection of the sample. Location of these sampling points and their coordinates are shown in table 2.1.

Table 2.1. Sample location.

Sample ID	Sample location	Coordinates
1	Upstream Sangrilla park	33°53'38.7"N 73°22'15.8"E
2	Downstream Bahrakahu	33.7068° N, 73.0870° E
3	Wet market Bahrakahu	33.7068° N, 73.0870° E
4	Upstream Shahdara	33.7068° N, 73.0870° E
5	Downstream G-6	33°38'32.6"N 73°07'46.7"E
6	Wet market G-6	33°38'32.6"N 73°07'46.7"E
7	I-9 Treatment plant	33°39'09.7"N 73°03'20.6"E.
8	Lai stream	33°36'10.4"N 73°04'25.1"E

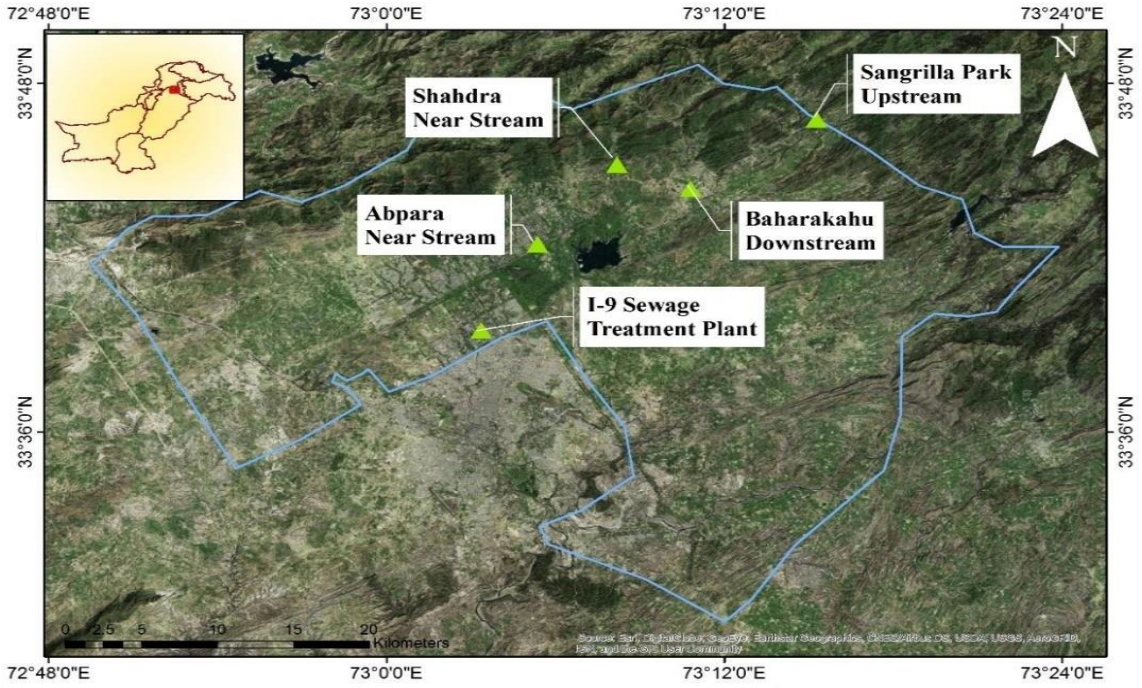

Figure 2.1. Map showing sampling locations.

Figure 2.2. Sampling from upstream Sangrilla park.

Figure 2.3. Sampling from Bharakahu downstream and its wet market.

Figure 2.4. Sampling from Shahdara upstream.

Figure 2.5. Sampling from Korang river at G6 (abpara) and its wet market.

Figure 2.6. Sampling from I-9 wastewater treatment plant.

Figure 2.7. Sampling from Lai stream.

2.2 Experimental Design

Experimental design of this study has been described in the figure 2.8.

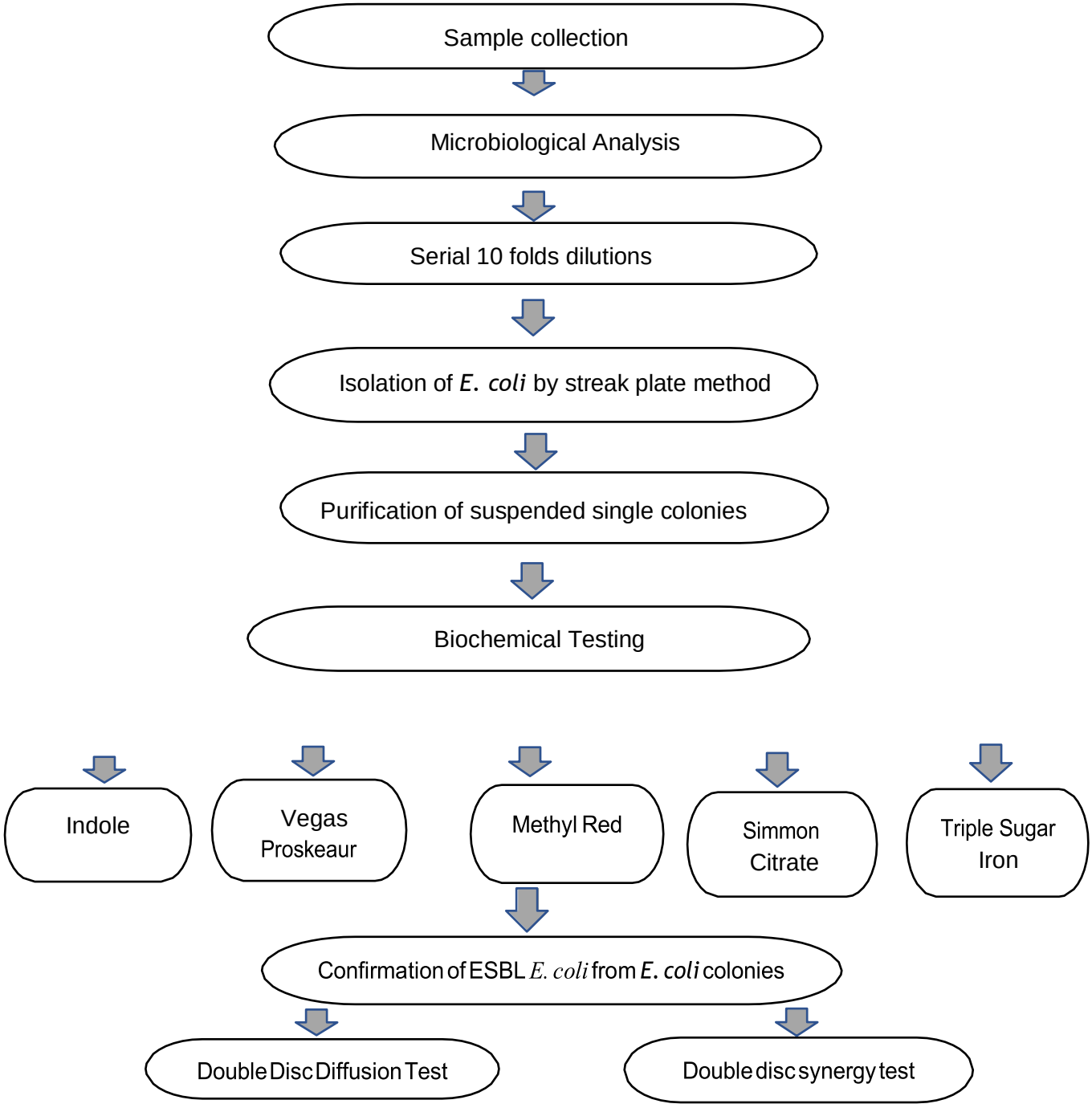

Figure 2.8. Experimental design of the study.

2.3 Microbiological analysis

Microbiological analysis of water sample was done by spread plate count technique and streak plate method. Two different media including MacConkey agar and Muller Hinton agar were prepared according to standard protocols. The petri dishes and these media were sterilized in an autoclave at 121°C for almost 15 minutes so that any contamination even at the microbial level can be eliminated. Media was poured in disinfected dry plates in a sterile laminar flow. The agar was permitted to cool and solidify. Then these plates were tightly sealed and were put in an incubator for the period of 24 hours at 37°C so that the chance of error or any unwanted bacterial growth should be identified. After 24 hours, the water sample was spread over media to determine the *E. coli*. for this reason,100 µl of water samples from different location points were spread over the MacConkey agar plates (APHA, 2014). These samples were then incubated at 37℃ for 24 hours as shown in figure 2.9.

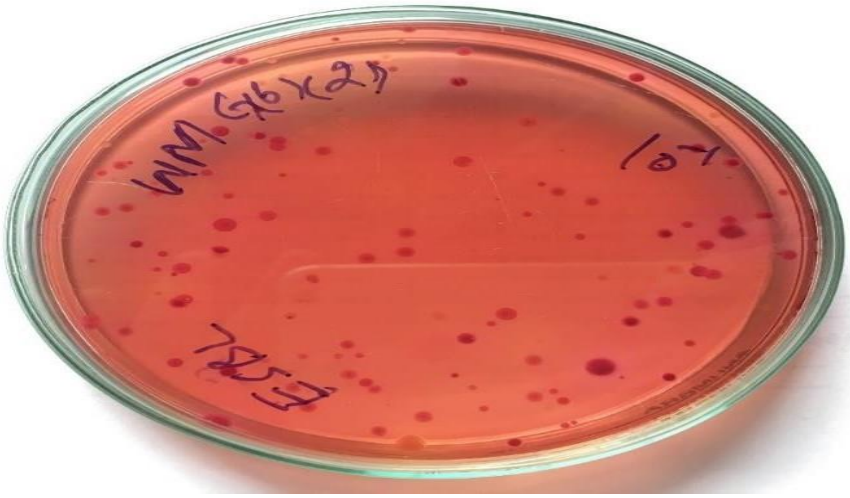

Figure 2.9. Bacterial colonies on MacConkey agar.

2.4 Streak Plate Method

Streaking is a method used to separate, pure strain from mixed cultures, different type of microorganisms was streaked individually. Samples were then taken from the subsequent colonies and a microbial culture were grown on another plate which indicated the presence of an individual microbe. New plates of MacConkey agar were set up for

streaking of the subsequent colonies and microbiological culture for individual microorganisms as per standard procedures (Bergey's Manual of Determinative Bacteriology, 1994).

The inoculating loop was disinfected on the Bunsen burner by putting the circle wire loop into the fire until it was cooled. A secluded colony was picked from the agar plate culture and spread over the main quadrant. Instantly marked the loop delicately finished a fourth of the plate. The loop was blazed again and was permitted to cool. From edge of the zone that was streaked, expanded the streaks into the second quarter of the plate. The loop was blazed again and was permitted to cool. From the edge of the zone that was streaked, expanded the streaks into the second quarter of the plate. The loop was blazed again and was enabled it to cool. Back to the region that was simply streaked zone, expanded the streaks into the second from the last quarter of the plate, blazed the loop again and cooled it before streaking again, broadened the streak into the inside fourth of the plate. Agar plates that were streaked were marked properly with date and time and then incubated at 37°C for 24 hours. The colonies developed in the plates were carefully analyzed following 24 hours. This method was repeated if there is in excess of one sort of colony; each composes ought to be streaked again on a different plate to acquire a totally pure culture (Bergey's Manual of Determinative Bacteriology, 1994) as shown in figure 2.10.

Figure 2.10. Bacterial colonies on MacConkey agar.

2.5 Colony Morphology

Basically, colony morphology is a technique that is used to perceive shape, shading, and outward presentation of individual colonies of microorganisms on a plate, these attributes can often be utilized to identify or possibly limit the species shown on the plate. The simple physical examination was completed by naked eyes or sometimes by using the microscope for appropriate identification of colony morphology (Fatima et al., 2011). Colony morphology on MacConkey agar is shown in figure 2.11.

Figure 2.11. Colony morphology on MacConkey agar.

2.6 *E. coli* confirmatory test

Different biochemical tests were performed in the laboratory for the identification of *E. coli.*

i. Indole test.

ii. Methyl Red test.

iii. Voges-Proskauer test.

iv. Simmon Citrate test.

v. Triple Sugar Iron test.

2.6.1 Indole test

This test is used for the bacterial species and to control the ability of the organism to alter tryptophan into indole. This division is done by a number of different intracellular enzymes, a classification usually mentioned to as “tryptophanase”.

Four micro liters tube of tryptone water was Inoculated with 3 to 4 colonies from purified culture growth. A negative control was also run. The tubes were incubated at 37°C for 24 hours. Added 0.5ml of indole reagent directly to the tube and agitated and allowed tube to stand for 5-10 minutes. Formation of red colored ring at the top showed a positive indole reaction and absence of red color ring indicated negative reaction (Fatima et al., 2011) as shown in figure 2.12.

Figure 2.12. Indole test.

2.6.2 Methyl Red Test

In this test, a broth medium containing glucose is used to grow the test bacteria. If glucose is used by the bacteria with production of a stable acid, by adding in broth culture its color changes from yellow to red.

Inoculated each 5 ml tube of MR-VP broth with 2-3 colonies from purified growth culture. A negative control was also run. The tubes were incubated at 37°C for 24 hours. Added 2-3 drops of Methyl Red indicator, as a result, red coloration indicated positive reaction (Mcdevitt et al., 2009) as shown in figure 2.13.

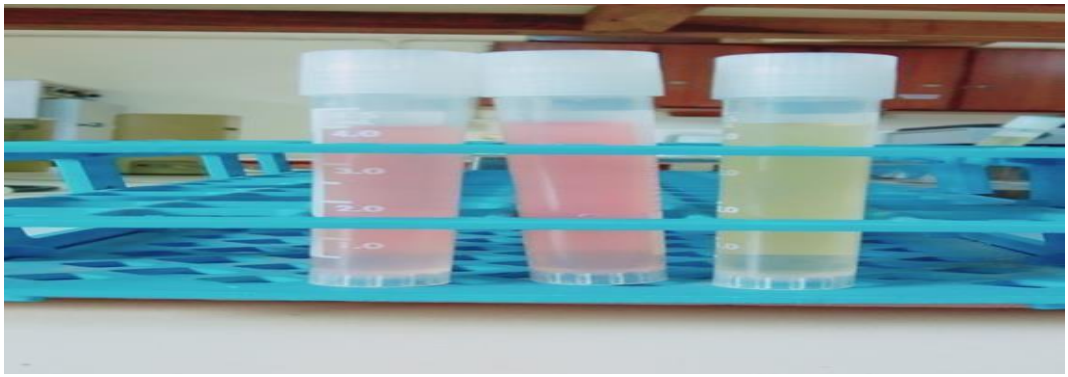

Figure 2.13. Methyl red test.

2.6.3 Voges-Proskauer Test

Inoculated each 3 ml tube of MR-VP broth with 2-3 colonies from purified growth culture. A negative control was also run. The tubes were incubated at 37°C for 24 hours. Added 15 drops of Reagent A and 5 drops of Reagent B. Development of distinct red/pink color within 5 minutes indicated a positive reaction (Mcdevitt et al., 2009) as shown in figure 2.14.

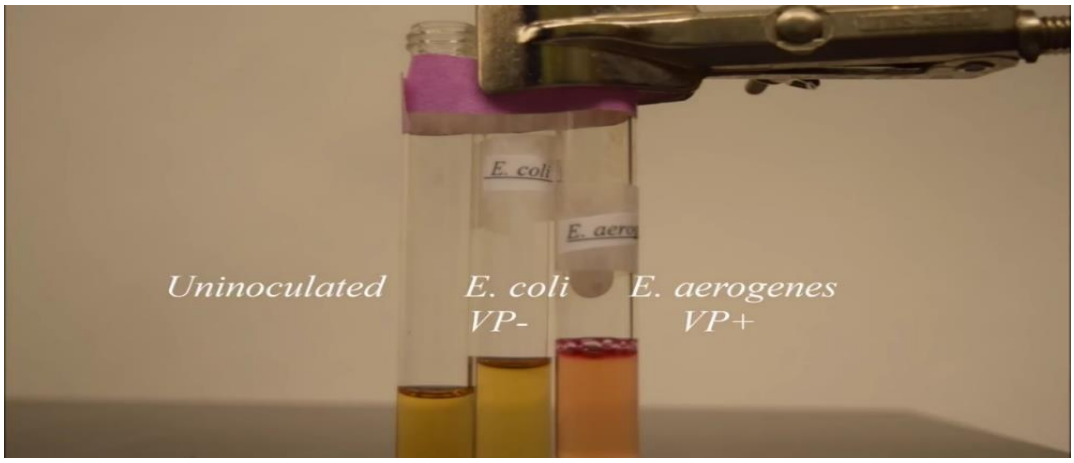

Figure 2.14. Voges Proskauer test.

2.6.4 Citrate test

Inoculum was taken from purified culture growth inoculate Citrate tubes by stabbing the butt and streaking the slant. A negative control was also run. The tubes were incubated at 37°C for 24 hours. Blue color of slant indicates a positive reaction (Malik et al., 2015) as shown in figure 2.15.

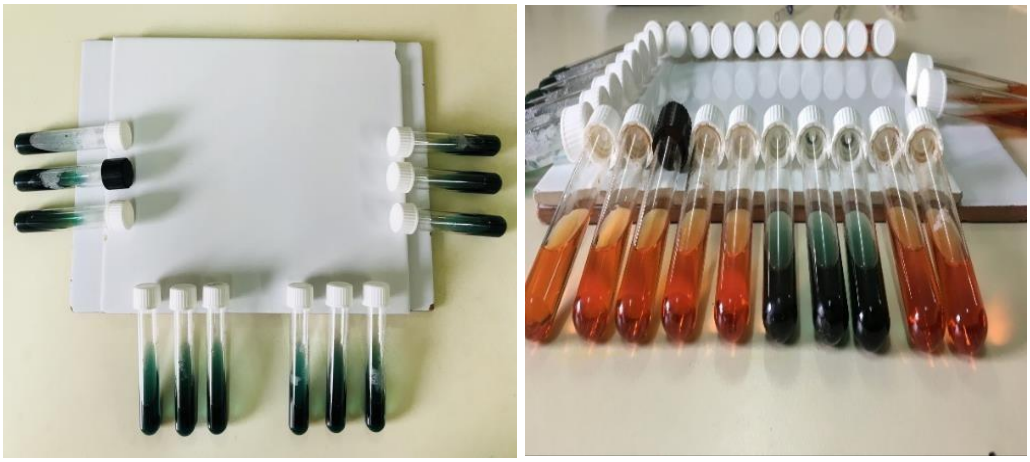

Figure 2.15. Citrate test.

2.6.5 Triple Sugar Iron Test

By taking inoculum from purified culture growth inoculated citrate tubes by stabbing the butt and streaking the slant. A negative control was also run. The tubes were incubated at 37ºC for 24 hours. Yellow color of slant, Gas production, Cracks and Motility indicated a positive reaction for *E. coli* (Arshad et al., 2011) as shown in figure 2.16.

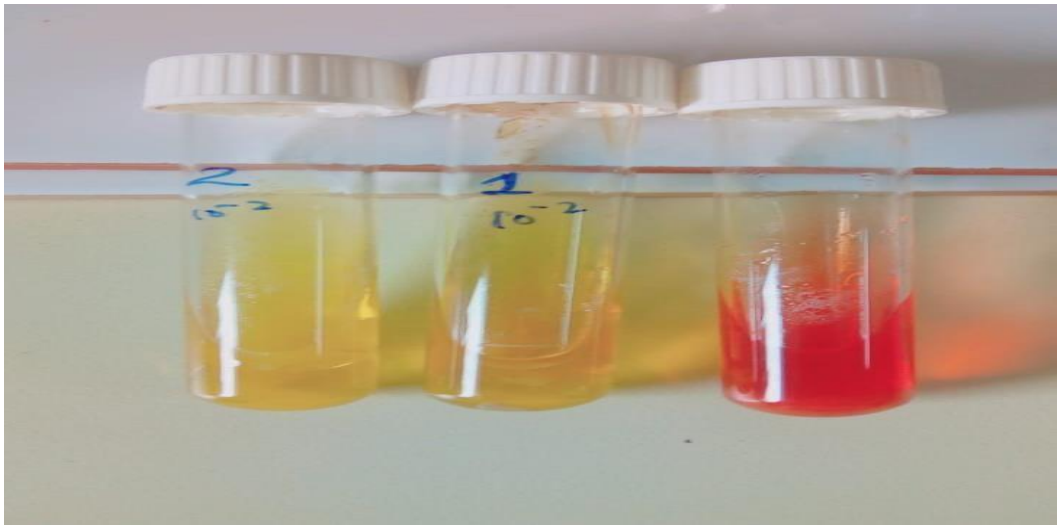

Figure 2.16. Triple sugar iron test.

2.7 Antibiotic Resistance Assay

Antimicrobial susceptibility was determined by Kirby-Bauer disk diffusion technique, recommended by clinical and Laboratory Standards Institute CLSI, guidelines. ESBL *E. coli* colonies can be confirmed from the following two confirmatory test.

1. Double disc diffusion test.
2. Double disc synergy test.

2.7.1 Double Disk Diffusion Test

Materials used in this test are:

- Muller Hinton Agar Plates
- Purified Culture Plates
- Screw Capped Test Tubes
- Normal Saline
- McFarland standard (0.5)
- Wire loop
- Swabs (Cotton Bud)
- Test Tube Stand

- Vortex
- Burner
- 5ml Pipette
- Ceftazidime (antibiotic disk) (CAZ 30)
- Ceftazidime with Clavulanic acid (antibiotic disk) (CAL 40)
- Cefotaxime (antibiotic disk) (CTX 30)
- Cefotaxime with Clavulanic Acid (antibiotic disk) (CTL 40)

Method

38g Muller Hinton Agar Media powder was suspended in 1L of distilled water and boiled to dissolve the powder completely. Then autoclaved at 121^{o}C for 15 minutes. After that let it cool to 55^{o}C in water-bath set at 55^{o}C. Then media was poured in the plates at 25-30ml per plate. Incubated the media plates overnight at 37^{o} C to check for any contamination. Poured 5ml of Normal Saline (4.5) in test tube as per sample and dispense 2-3 colonies and compared the turbidity to the 0.5 McFarland standard. This preparation is used to swab the Muller Hinton Agar plate. After swabbing was applied, all four-antibiotic disk at the distance of 25-30mm apart. Incubated the plates for 24 hours at 37^{o} C. The sample was considered positive if the diameter of inhibition zone of the disk with clavulanic acid is 5mm larger than the diameter of inhibition zone of the disk without clavulanic acid (Sujhata et al., 217) as shown in figure 2.17.

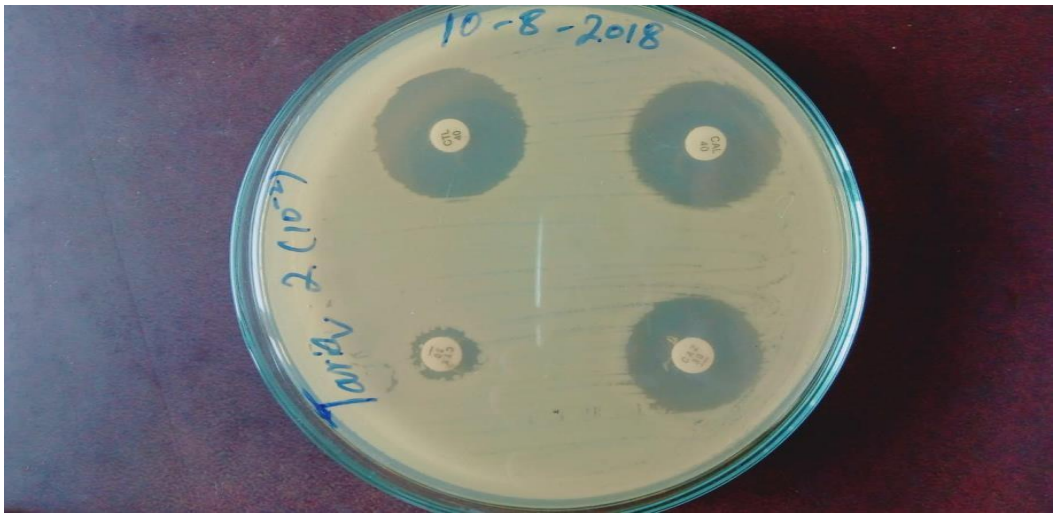

Figure 2.17. Double disc diffusion test.

2.7.2 Double Disk Synergy Test

This test is performed for the confirmation of ESBL *E. coli*

- Muller Hinton Agar Plates
- Purified Culture Plates
- Screw Capped Test Tubes
- Normal Saline
- McFarland standard (0.5)
- Wire loop
- Test Tube Stand
- Vortex
- Burner
- Swabs (Cotton Bud)
- 5ml Pipette
- Amoxycillin + Clavulanic Acid (antibiotic disk)
- Ceftazidime (antibiotic disk)
- Aztreonam (antibiotic disk)
- Ceftriaxone (antibiotic disk)
- Cefixime (antibiotic disk)
- Cefepime (antibiotic disk)

Method

38g Muller Hinton Agar Media powder was poured in 1L of distilled water and boiled to dissolve the powder completely. Then autoclaved at 121° C for 15 minutes. After that let it cool to 55° C in water-bath set at 55° C. Then media was poured in the plates at 25-30ml per plate. Incubated the media plates overnight at 37° C to check for any contamination. 5ml of Normal Saline was poured in test tube as per sample and dispensed 2-3 colonies and equated the turbidity to the 0.5 McFarland standard. This preparation was used to swab the Muller Hinton Agar plate. After swabbing applied Amoxycillin + Clavulanic Acid (antibiotic disk) in the center of petri plate. Then apply all five- antibiotic disks mentioned above at the distance of 20mm apart each other and from central disk. Incubated the plates for 24 hours at 37°C. After incubation synergism was checked between the Amoxycillin with

Clavulanic Acid (antibiotic disk) and other antibiotic disks. The synergistic effect was considered when any antibiotic disk out of five antibiotic disks mentioned above gives zone of inhibition towards Amoxycillin with Clavulanic Acid (antibiotic disk) in the center (Kaur et al., 2013) as shown in figure 2.18.

Figure 2.18. Double Disk Synergy Test.

After swabbing applied Amoxycillin + Clavulanic Acid (antibiotic disk) in the center of petri plate. Then apply all five-antibiotic disks mentioned above at the distance of 20mm apart each other and from central disk. Incubated the plates for 24 hours at 37^{O} C. After incubation synergism was checked between the Amoxycillin + Clavulanic Acid (antibiotic disk) and other antibiotic disks. The synergistic effect was considered when any antibiotic disk out of five antibiotic disks mentioned above gives zone of inhibition towards Amoxycillin with Clavulanic Acid (Yousef et al., 2016).

CHAPTER 3
RESULT AND DISCUSSION

This study was conducted for detection and surveillance of specific indicator microorganism *E. coli,* producing a specific resistance mechanism, ESBL *E. coli* of water streams in Islamabad.

3.1 Detection of *E. coli* in and ESBL *E. coli* in water samples

Five biochemical tests were performed for the confirmation of *E. coli* in the water samples. The results of biochemical tests are given in table 3.1.

Table 3.1. Biochemical test performed for identification of *E. coli* test.

Sample ID	Indole	MR	VP	Citrate	TSI
Upstream Sangrilla park	Not detected	Not detected	Not Detected	Not detected	Not Detected
Downstream Bahrakahu	Positive	Positive	Negative	Negative	Yellow, gas, Cracks
Wet market bahrakahu	Positive	Positive	Negative	Negative	Yellow, Gas, cracks,
Upstream Shahdara	Not detected	Not detected	Not Detected	Not detected	Not Detected
Downstream G-6	Positive	Positive	Negative	Negative	Yellow Gas and cracks
Wet market G-6	Positive	Negative	Negative	Negative	Yellow, cracks, Motility
I-9 treatment plant	Positive	Positive	Negative	Negative	Yellow, Gas, cracks
Lai stream	Positive	Positive	Negative	Negative	Yellow, Gas, cracks

This study aimed to investigate the surveillance of anti-biotic resistant *E. coli* in Islamabad from a variety of sources including water streams, wet markets and waste water treatment plant. Samples were taken from location 1 *i.e.* upstream Sangrilla park for the detection of *E. coli* and ESBL *E. coli* in it. The surrounding environment of Location 1 was very pleasant and clean and it is on the way of Murree and mountainous area. Intervention of people is minimum therefore water stream is very pure and free from the contamination. Samples were taken from Location 1 to check the presence of *E. coli* in that water. But, as the water was very pure and free from any contamination, no *E. coli* was detected in water samples. Samples taken from the location 1 were free from total *E. coli* and were also free from ESBL *E. coli.*

Samples were taken from the location 2 *i.e.* downstream Bharakahu to check *E. coli* and ESBL *E. coli* in it. Water stream passing from Location 1 crosses from Bharakahu. The surrounding environment of Bharakahu was very unclean. Most of infectious diseases are caused because of unhygienic environment and a large number of viral fever cases in the capital are reported from rural areas. Indication of insignificant anthropogenic consequence is also found in groundwater. Samples taken from Bharakahu was analyzed in the laboratory and found *E. coli* in it due to prevalence of bacteria in water. In Pakistan, 20-40% of the hospitalized patients are suffering from ailments resulting from pollution of drinking water e.g. dysentery, diarrhea, cholera and typhoid due to prevalence of bacteria in water (Qadir et al., 2017; Anwar et al., 2017). Samples also taken from wet market for detection of *E. coli.*

Samples taken from Location 2 were analyzed for ESBL *E. coli* after the confirmation of *E. coli* in it. Double disc synergy test and double disc diffusion test were performed to check the presence of ESBL *E. coli* in the *E. coli* positive samples. 5/7 samples were found positive for ESBL *E. coli.*

Gao et al. (2014) checked ESBL *E. coli* in the chicken feces, location 5and downstream river and were found 16/150, 1/27 and 12/81 samples positive for ESBL *E. coli.* Figure 3.1 shows percent prevalence of Total *E. coli* and ESBL *E. coli* in Bharakahu samples.

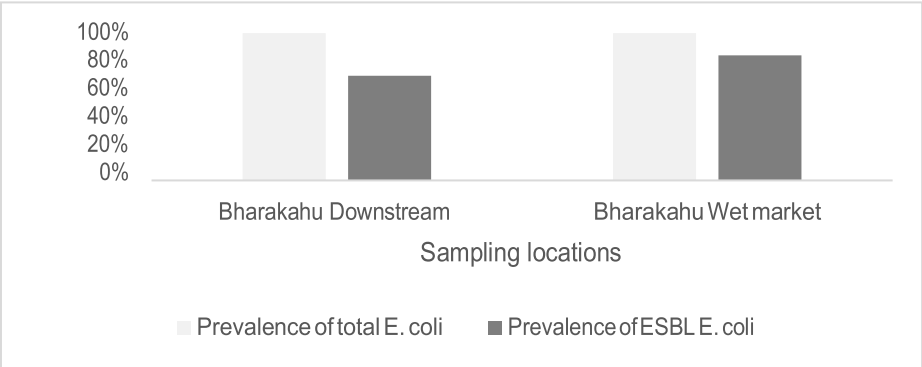

Figure 3.1: Percent prevalence of *E. coli* and ESBL *E. coli* in Bharakahu

Shahdara upstream for detection of *E. coli.* It's a valley which is surrounded by mountains and a water Canal come from Hills and pass from the middle of the valley. This view of lush green mountains and water make it very beautiful valley from other locations of Islamabad. As the water of Location 4 is very clear and human intervention from nearby areas were minimum, no *E. coli* was detected in its water samples.

Water stream coming from Location 4 *i.e* Korang river downstream is passing from the G-6 area of Islamabad. Samples were taken from G-6 area to analyze the water stream and its wet market; containing meat shops and poultry slaughter shops. Surrounding environment of Korang stream was very polluted. People threw their waste near the stream and this waste ultimately contaminate the water stream. All samples were found contaminated with *E. coli.* Figure 3.1 shows percent prevalence of *E. coli* and ESBL *E. coli* in Korang stream G-6 and its wet market samples as shown in figure 3.2.

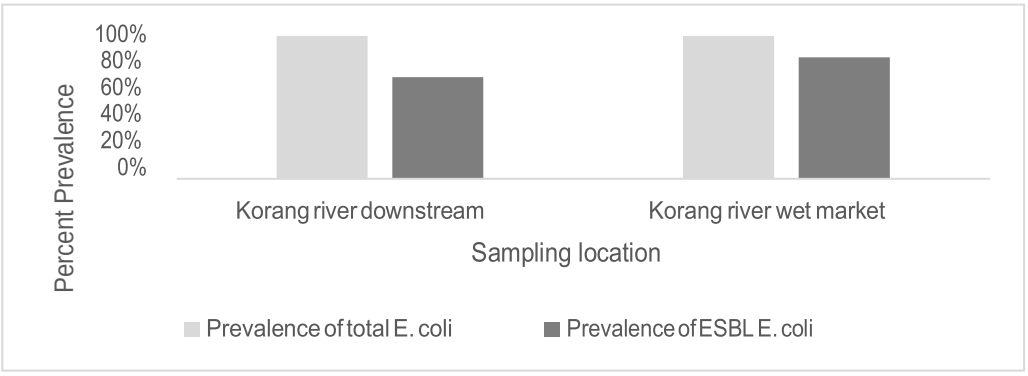

Figure 3.2: Percent prevalence of *E. coli* and ESBL *E. coli* in korang river G-6 and its wet market.

Frigon et al. (2012) stated that pathogenic bacteria were released from the effluents of treatment plants, including *E. coli*, in the freshwater environment, and defining the possible selection of pathogens. Samples collected from the waste water treatment plant to determine the prevalence of *E. coli* showed their presence in all of the samples because all the samples were heavily contaminated. (Ewa et al., 2013) also determined *E. coli* and ESBL *E. coli* in the waste water samples and found both of them were found in the effluent of waste water treatment plant. (Toze, 2008) stated that microbial pathogens are one of the major health risks associated with water and wastewaters. Pathogenic microorganisms are the basis of fecal pollution, with *E. coli*, in wastewater. Wastewater treatment procedures are planned to reduce the contaminants concentration which includes pathogens, in the treated water before release to receiving water bodies, but, numerous WWTPs discharge such effluents without decontamination (Frigon et al., 2012). Samples taken from I-9 waste water treatment plant were all found positive for ESBL
E. coli as shown in figure 3.3.

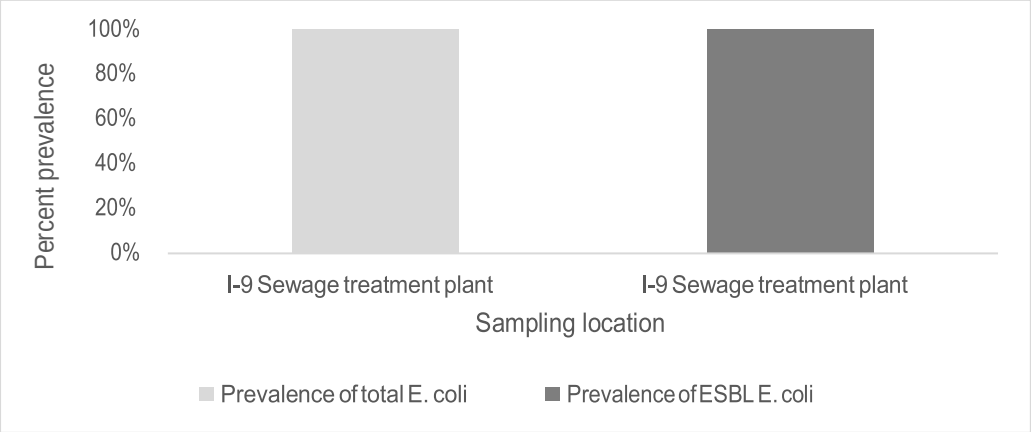

Figure 3.3: Percent prevalence of *E. coli* and ESBL *E. coli* in I-9 sewage treatment plant.

Samples collected from Location 8 *i.e.* Lai stream were found positive for *E. coli*. The surrounding environment of Location 8 was very polluted as the people living in nearby areas through their garbage near the streams and the municipal authority is not doing their proper job in picking up the garbage. Gao et al. (2014) also found *E. coli* in the nearby river water contaminated with garbage. ESBL producing *E. coli*

identified from 150 manure samples and 108 water samples, including 16 strains from manure and 13 isolates from water. Samples taken from Location 8 were positive for *E. coli* and further these samples were found to confirm ESBL *E. coli* in it. All of the samples were found positive for ESBL *E. coli* as shown in figure 3.4.

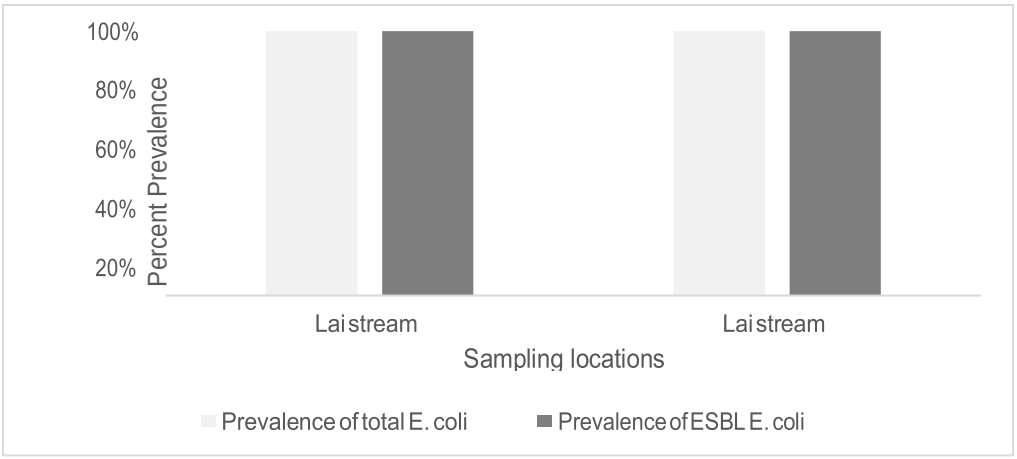

Figure 3.4: Percent prevalence of *E. coli* and ESBL *E. coli* result at Lai stream.

3.2 Result of individual Antibiotics

The isolates were tested for their antimicrobial susceptibilities by the disc diffusion method according to the Clinical Laboratory Standard Institute (CLSI) guidelines (Wayne, 2010). 27mm for cefotaxime and less than 25mm for ceftriaxone are the diameters required for determining the ESBL *E. coli* production. The ESBL *E. coli* production was tested by the Modified Double Disc Synergy Test (MDDST) by using a disc of amoxicillin-clavulanate (20/10 µg) along with four cephalosporins; 3GC-cefotaxime, ceftriaxone, aztreonam cefixime, 4GC-cefepime and ceftazidime. Mueller- Hinton agar plate was used from the bacterial culture growth, as was recommended by Clinical & Laboratory Standards Institute (CLSI, 2010). Amoxicillin- clavulanate (20/10 µg) disc was placed in the center of the plate. The discs of 3GC and 4GC were placed 20mm apart respectively, center to that of the amoxicillin-clavulanate disc. Increase of zone towards amoxicillin-clavulanate disc was considered as positive for the ESBL *E. coli* production (Kaur et al., 2013).

They used Double disc synergy method for the detection of ESBL *E. coli* in their urinary isolates. ESBL *E. coli* production was seen 63.4% *E. coli* and in 67.3% *Klebsiella pneumoniae* isolates. In twelve *E. coli* and five *K. pneumoniae* strains, \ synergism was shown only by cefepime with amoxicillin-clavulanate. In the following figure FEP 30 (Cefepime) and CRO 30 (Ceftriaxone) are producing more synergistic effect towards AMC 30 (Amoxycillin + Clavulanic Acid) by giving zone of inhibition towards AMC 30 (Amoxycillin + Clavulanic Acid), while CAZ 30 (Ceftazidime) was producing very little zone of inhibition. The remaining two CFM 5 (Cefixime) and ATM 30 (Aztreonam) are unable to give any zone of inhibition towards AMC 30 (Amoxycillin + Clavulanic Acid). Figure 3.5 is showing synergism between different antibiotics.

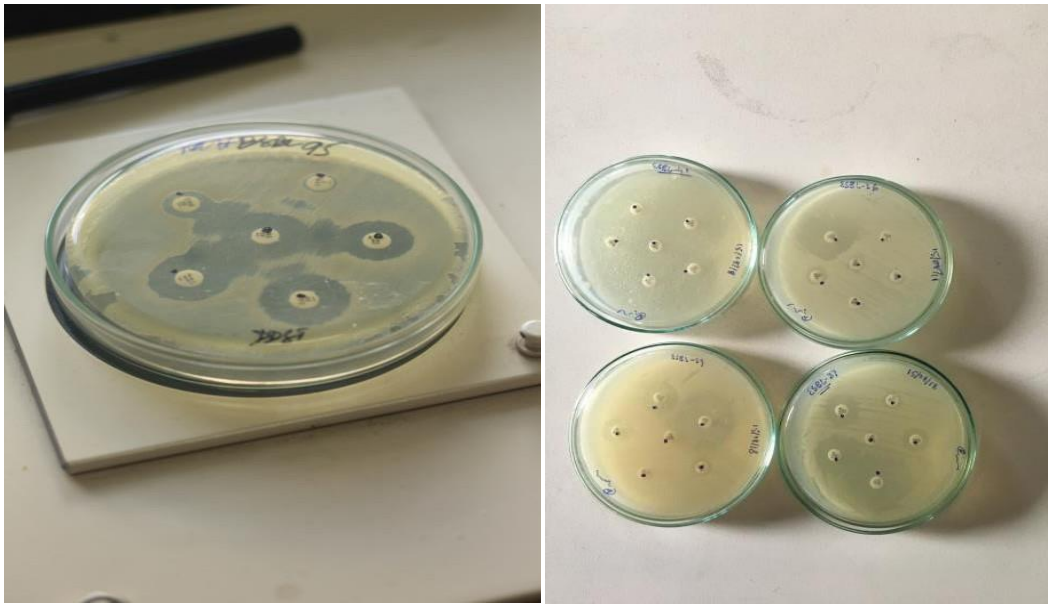

Figure 3.5: Synergism between different antibiotics in double disc synergy test.

3.3 Inhibition zone in double disc diffusion test

This test was done using two combination of antibiotics along with clavulanic acid i.e. Ceftazidime (30µg)/ ceftazidime with clavulanic acid (30/10µg) (CAZ/CAZC) and Cefotaxime (30µg)/cefotaxime with clavulanic (30/10µg) (CTX/CTXC) acid. Both the discs have the distance of 25mm on Muller Hinton agar plate. A more than 5 mm increase

in zone diameter for either antimicrobial agent tested in combination with clavulanic acid versus its zone when tested alone was designated as ESBL *E. coli* positive (Chandra and Goswami, 2014). In this study, figure 3.6 shows Ceftazidime (30µg)/ ceftazidime with clavulanic acid (30/10µg) (CAZ/CAZC) and Cefotaxime (30µg)/cefotaxime with clavulanic (30/10µg) (CTX/CTXC) acid, both the discs were giving a minimum of ≥5 mm increase in zone diameter for either antimicrobial agent tested in combination with clavulanic acid versus its zone of inhibition. Sujatha et al. (2016) also used double disc diffusion test for their urinary isolates. The use of ceftazidime (30 µg) and ceftazidime with clavulanic acid (30µg/ 10µg) detected 46 isolates as ESBL *E. coli* positive as compared to cefotaxime (30 µg) and cefotaxime with clavulanic acid (30µg/ 10µg) which detected 33 isolates as ESBL *E. coli* positive as shown in figure 3.6.

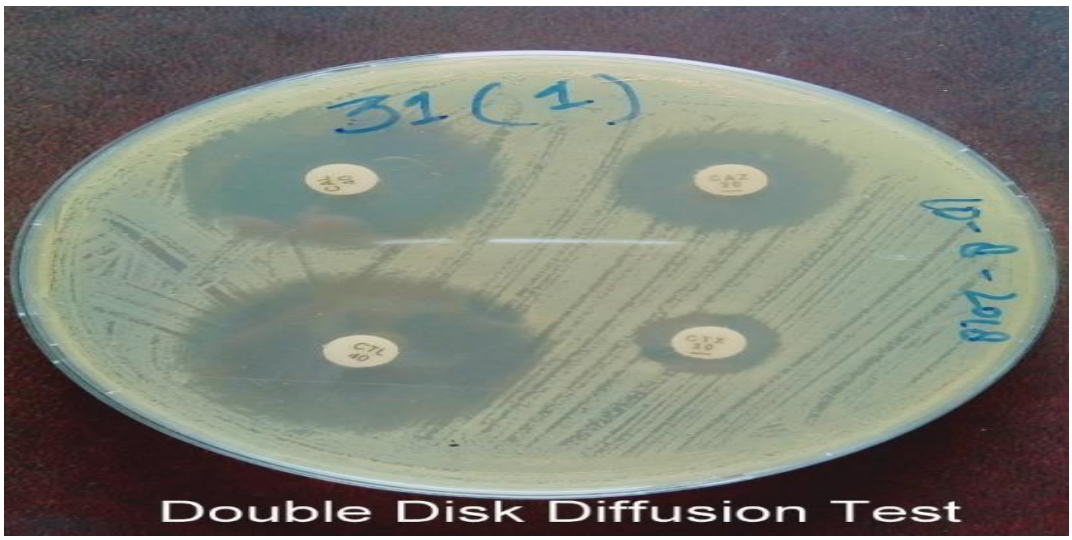

Figure 3.6.: Formation of inhibition zone in double disc diffusion test.

Table 3.2 demonstrates the sizes of inhibition zones formed in the experiments of double disc diffusion test.

Table 3.2. Zone of inhibition of different antibiotic discs in double disc diffusion test

Sample ID	AL 40 diameter	AZ 30 diameter	TL 40 diameter	TX 30 diameter
ESBL/B//1	28 mm	20mm	30mm	3mm
ESBL/B//2	25mm	18mm	8mm	12mm
ESBL/B//3	25mm	16mm	26mm	8mm
ESBL/B//4	24.5mm	18.5mm	27mm	8mm
ESBL/B//5	25mm	19mm	26mm	12mm
ESBL/B/WM/1	27mm	24mm	30mm	15mm
ESBL/B/WM/2	25mm	14mm	28mm	4mm
ESBL/B/WM/3	26mm	18mm	29mm	10mm
ESBL/B/WM/4	23mm	15mm	25mm	13mm
ESBL/B/WM/5	25mm	16mm	28mm	30mm
ESBL/B/WM/6	27mm	19mm	40mm	28mm
ESBL/G6/1	30mm	25mm	34mm	17mm
ESBL/G6/2	25mm	17mm	28mm	3mm
ESBL/G6/3	25mm	16mm	26mm	8mm
ESBL/G6/4	24mm	18.5mm	26mm	10mm
ESBL/G6/5	25mm	19mm	26mm	12mm
ESBL/G6/WM1	27mm	24mm	30mm	15mm

ESBL/G6/WM/2	25mm	14mm	28mm	4mm
ESBL/G6/WM/3	26mm	18mm	28mm	10mm
ESBL/G6/WM/4	23mm	15mm	25mm	13mm
ESBL/G6/WM/5	25mm	16mm	28mm	30mm
ESBL/G6/WM/6	26mm	19mm	37mm	25mm
ESBL/I9/WWTP /1	31mm	25mm	32mm	17mm
ESBL/I9/WWTP /2	25mm	17mm	21mm	3mm
ESBL/I9/WWTP /3	28mm	16mm	28mm	8mm
ESBL/I9/WWTP/4	27mm	15mm	26mm	8mm
ESL/I9/WWTP/5	25mm	19mm	6mm	12mm
ESBL/I9/WWTP/6	25.5mm	24mm	30mm	15mm
ESBL/I9/WWTP/7	25mm	16mm	28mm	30mm
ESBL/Lai/1	29mm	19mm	39mm	30mm
ESBL/Lai/2	32mm	25mm	32mm	17mm
ESBL/Lai/3	26mm	18mm	28mm	10mm
ESBL/Lai/4	23mm	15mm	25mm	13mm
ESBL/Lai/5	25mm	16mm	28mm	30mm
ESBL/Lai/6	26mm	19mm	37mm	25mm
ESBL/Lai/7	31mm	25mm	2mm	7mm

This table shows the results of double disc diffusion test off all positive *E. coli* samples. Zone of inhibition in each of the sample result is more than 5mm which according to CLSI guidelines confirms that it is ESBL *E. coli.* The synergistic effect of positive pairs of antibiotics might be owing to action on persisters *i.e.* bacterial population member which resist with the contact with single drug disc, perhaps while metabolic activity was reduced, and their offspring do not show enhanced resistance (King et al., 2012). ESBL detection methods are the general principle that the activity of extended spectrum antibiotic against ESBL *E. coli* was enhanced. Carbapenems are used to treat serious infections caused by ESBL-producing organisms (Rawat et al., 2010). ESBL producing bacteria therefore present at all of the locations and the synergistic effect has been identified.

Conclusions

Following are the conclusion drawn from the study:

i. *E. coli* and ESBL *E. coli* was identified at Bharakahu downstream and its wet market, Korang stream G-6 and its wet market, I-9 waste water treatment plant and from Lai stream in the collected samples.

ii. No *E. coli* and ESBL *E. coli* was detected in the water samples of upstream Sangrilla park and Location 4 upstream Shahdara.

iii. Percent prevalence of *E. coli* and ESBL *E. coli* in Location 2 *i.e.* Barakahu was 100% and 71% and its wet market was 100% and 85%, Korang G-6 was 100% and 71% and its wet market was 100% and 85% in the collected samples.

iv. Prevalence of I-9 waste water treatment plant was 100% for *E. coli* and 100% for ESBL*E. coli* and Location 8 Lai stream were 100% for *E. coli* and 100% for ESBL *E. coli* in the collected samples.

v. FEP 30 (Cefepime) and CRO 30 (Ceftriaxone) have produced more synergistic effect towards AMC 30 (Amoxycillin with Clavulanic Acid) by limiting the growth of microbes, while CAZ 30 (Ceftazidime) has produced very little zone of inhibition depicting less synergism between the two antibiotics and less limiting the growth of microbes.

vi. The two antibiotics CFM 5 (Cefixime) and ATM 30 (Aztreonam) were unable to give any zone of inhibition towards AMC 30 (Amoxycillin with Clavulanic Acid) showing no synergism between the tested antibiotics in our experiments and were not able to limit the growth of microbes.

Recommendations

Following are the recommendations of this study:

i. Detection and surveillance of antibiotic resistant bacteria should be done in other areas and water reservoirs also.

ii. Other antibiotics e.g. ampicillin, erythromycin, ceftizoxime can be used for the detection of ESBL *E. coli* and the synergism between various other antibiotics should be checked in future studies.

iii. Detection of antibiotic resistant genes in the isolated bacteria can be done for surveillance in the environmental samples.

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