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A Study of the Variation of *E. coli* Sequence Types Moderated by Bacteriophages in Environmental Sewage Samples from City of Islamabad

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Microbiology

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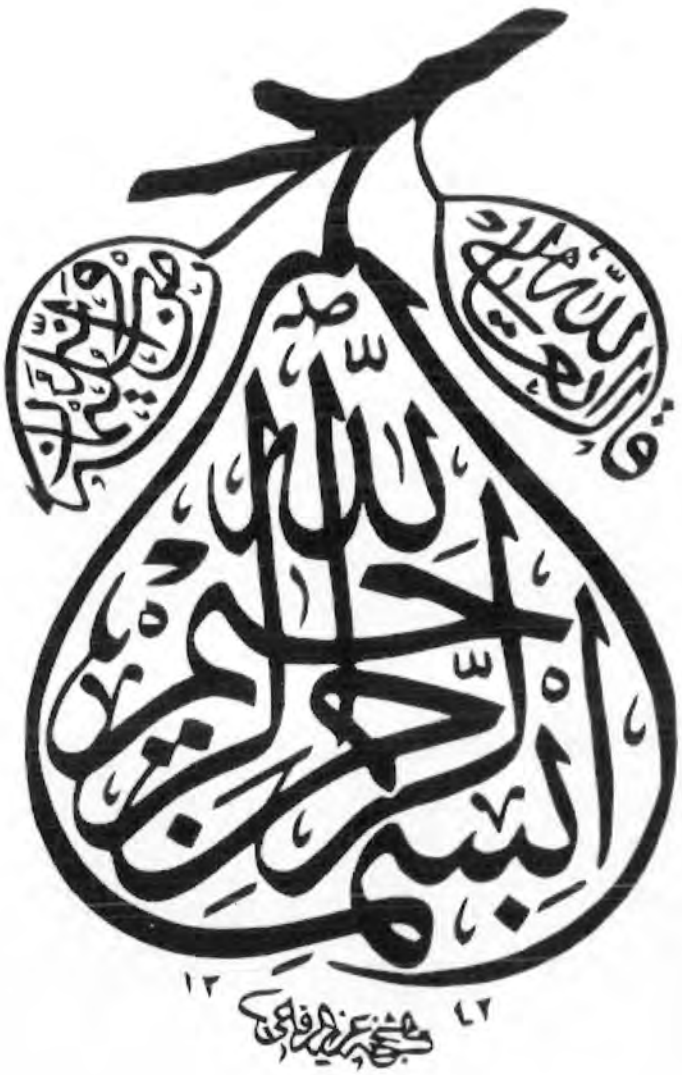

(We commence)
with the name of Allah
The most gracious (To begin with)*
The most Merciful (To the end)**

Dedicated

To

My beloved parents,

Without whom I am nothing

DECLARATION

I hereby declare that the work presented in the following thesis is my own efforts and that the thesis is my own composition. No part of the thesis has been previously presented for any other degree.

Saba Javeed

CERTIFICATE

This thesis by **Saba Javeed** is accepted in its present form by the department of Microbiology, Quaid-i-Azam University, Islamabad, for the requirement of the degree of Master of Philosophy in Microbiology.

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List of Abbreviation

%	Percentage
µl	Microlitre
E.coli	*Escherichia coli*
ESBL	Extended Spectrum Beta Lactamases
MBLs	Metallo-Beta Lactamases
MDR	Multi-Drug Resistance
NDM-1	New Delhi metallo-β-lactamase-1
PCR	Polymerase Chain Reaction
EHEC	enterohemorrhagic *E. coli*
EPEC	enteropathogenic *E. coli*
EIEC	enteroinvasive *E. coli*
EAEC	enteroaggregative *E. coli*
ETEC	enterotoxigenic *E. coli*
DAEC	diffusely adherent *E. coli*
ExPEC	Extraintestinal pathogenic *E. coli*
CPEc	Carbapenemase-producing *E. coli*
MLEE	Multilocus Enzyme Electrophoresis
MLST	Multi locus sequence typing
ST	Sequence typing
rRNA	Ribosomal RNA
AP-PCR	Arbitary primed PCR
*fim*H	minor component of type 1 fimbriae
*fum*C	fumarate hydratase (fumarase C)
UTIs	Urinary tract infections
HGT	Horizontal gene transfer

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Abstract

Abstract

E coli is one of the most variable organisms present in the environment as its genomic variations are numerous. Among many ways to study genomic variations, one is the method of sequence typing which can identify different types of an organism present in the environment. In this study, a total of 54 *E. coli* strains were isolated from sewage samples and identified and then sequenced by the two locus sequence typing method. Different frequencies of *fim*H and *fum*C gene alleles were also indicated. These sequence types were associated with the antibiotic resistance gene, $bla_{\text{CTX-M-15}}$ where 33.3% of the total population of the bacterial strains was positive for this antimicrobial resistant gene. Phage assay was done to check the effect of bacteriophages present in the sewage sample on the bacterial strains which showed the positive activity of phages from different sites against three bacterial strains. In summary, different sequence types of *E. coli* were found in the sewage samples and results suggest that the bacteriophages present in the environment may have a role to modulate the specific bacterial type in the environment.

Chapter 01

Introduction

Introduction:

The understanding of the microbial population biology is becoming very important to understand the mechanisms of antimicrobial resistance, causes of emerging infectious diseases and bioterrorism (1).

Escherichia coli have been evolved tremendously for previous many decades. Many innocuous microbial strains become pathogenic with the passage of time so it is very important to understand the diversity of the microbial community present in the environment. *E. coli* live in the vertebrate gut, which is considered its primary habitat. It's a facultative anaerobic organism and exists in symbiosis with the host. Although recombination events occur but clonal structure of whole population is maintained which allows the designation of the microbial population into phylogenetic groups. Commensal *E. coli* genetic structure has evolved due to the numerous environmental factors and multiple hosts and evolution of virulence determinants relate to the adaptation of the *E. coli* strain to the new environment. So characterization of microbial community is quite necessary to understand the process of becoming a commensal strain into pathogenic strain (2).

Pathogenic strains of *E. coli* are becoming serious threat due to their being causative agent of waterborne diseases. Water quality is monitored by these fecal indicator microbial species. *E. coli* is generally taken as harmless microorganism but some strains are virulent and are cause of intestinal and extraintestinal infections. Infection causing strains are : enterohemorrhagic *E. coli* (EHEC), enteropathogenic *E. coli* (EPEC), enteroinvasive *E. coli* (EIEC), enteroaggregative *E. coli* (EAEC), enterotoxigenic *E. coli* (ETEC), and diffusely adherent *E. coli* (DAEC). Strains producing Shiga toxin are called Stx strains. Different clinical symptoms are manifested by different strains as they have different pan-genome. Environmental water sample studies neglected ExPEC strains and focused only on InPEC-associated genes even though all strains in water samples were

ExPEC. ExPEC stains are cause of death and they are also an alarming public health hazard (3).

For identification of bacterial strains especially *E. coli* molecular and conventional approaches are being used which are important as they help in identification and diagnosis of infectious disease agents. But so far molecular approaches are more successful as they provide full picture of microbial community. So they are used for pathogenic *E. coli* strains identification as well as coli-form indicator recognition (4).

Numerous techniques are being used for the species identification and genome analysis. Among them four main techniques used especially for *E. coli* genetic entities study are: Serotyping which is based on combination of 173 O antigens, 80 K antigens and 56 H antigens, PCR which is used for molecular identification. After that in 1980s followed by the development of multilocus enzyme electrophoresis, bacterial strain typing was being revolutionized. In 1990s multilocus sequence typing became a powerful tool for studying microbial population genetics. For each isolate nucleotide sequence of 7 housekeeping genes were determined. Then this data could be analyzed in two ways. Either it is studied as MLEE (Multilocus Enzyme Electrophoresis) data such as on the basis of their sequences allele assigned at each locus or alleles present at different loci make the allelic profile which is also termed as sequence types (ST) as in MLST (Multilocus Sequence Typing).

Phylogenetic reconstruction could be done from nucleotides sequences with or without recombination events corrections. Currently three main MLST schemes are used. These schemes use different sets of housekeeping genes. Results from all these schemes are highly correlated and could be used to define and assign the clonal structure of a strain. *E. coli* could be placed in four phylogenetic groups. For this purpose Phylogrouping triplex PCR is used (2). Due to the high expense rate of MLST certain techniques have been evolved to replace the several loci to the few loci termed as two locus sequence typing (5).

These different sequence types of bacterial strains are considered as potential reservoir of the virulence and antibiotic resistant genes.

E. coli has adapted itself to different environmental niches so it has become necessary to unravel the genomic structure of species on global scale as the structure of bacterial population largely depend upon balance between recombination and mutation events which help in shifting of bacterial strain from clonal structure when recombination events frequency is low, to pathogenic structure when recombination rate is high (6). *E. coli* has been used as a model organism for decades, as it is easy to grow and isolate in the laboratory, so researchers have taken the advantage of latest technical advances to understand the pathogenic strains. In this respect genomic information played an important role to remove the ambiguities which could occur by phenotypical methods of identification (2).

E. coli is becoming antimicrobial resistant worldwide and this phenomenon is getting more frequent (7). For example only in an Irish hospitals about 75% of *E. coli* became antimicrobial resistant to eight or more of sixteen antimicrobials used from 1997 to 2007 (8).

Antibiotic resistance causes difficulties as it complicates the patient's treatment and these studies were carried out with respect to clinical setting. But it has now been established that source of antibiotic resistance in pathogenic bacterial strains not only comes from mutations but also conferred by antibiotic resistance determinants originated in non-clinical settings. So understanding of ecological and environmental processes required in acquisition of resistance gene has become necessary. Environmental bacteria possess a pool of unexplored genes some of which have the potential to be served as antimicrobial resistance gene and then to be passed on to the pathogenic bacterial strain.

Antibiotic resistance in environmental strain mainly comes from usage of antibiotics in human and veterinary medicine which is also responsible for its dissemination at large scale. Another reservoir for multiple resistance strains is the intestinal flora of healthy humans as resistant bacterial strains have also been isolated from feces of healthy

individuals. In a research study it was reported that 80.5% of healthy person's feces samples contained antibiotic resistant bacteria and 98% of these isolated microbes population were *E. coli* (9). After going through treatment from biological and treatment plants these bacteria entered into the environment through sewage. As it was found that effluent of treatment plants contain 10^3 CFU/ml of resistant coliform bacteria and 17% of them had shown resistance to antibiotics up to six- fold. Now it has become the growing public concern that antibiotic resistant bacteria have persisted and become prevalent in soil, surface water, municipal drinking water and sewage water (10).

Antibiotic resistance mechanism by which bacterial strain mostly become resistant to the beta lactam antibiotics, is the production of hydrolytic enzyme, other mechanisms could involve the mutations at the binding site of targets which has been found in quinolone resistant strains (11). But among all most frequently described mechanism of antibiotic resistance is due to beta lactamases against broadly used beta lactam antibiotics. This resistance can be disseminated by two general processes: through horizontal gene transfer and clonal expansion. Horizontal transfer occurs either by bacterial conjugation or through plasmid transfer which mobilized the mobile genetic elements like transposons and bacteriophages (12). One of the major class of beta lactamases are ESBLs and production of ESBLs is the most common mechanism of resistance against expanded-spectrum cephalosporins in *E. coli*. ESBLs has three key types which are SHV, CTX-M and TEM (13).

Among these three key types CTX-M enzymes are one of the quickly spreading clusters of enzymes dispersed worldwide. These enzymes are encoded by plasmids and are most predominant in *E. coli*. The major classes of CTX-M are CTX-M-25, CTX-M-9, CTX-M-8, CTX-M-2 and CTX-M-1. Reported CTX-M variants are about 165. Genetic environment of CTX-M genes reflect that naturally this gene has been transfer from

Kluyvera spp present in the environment. This gene has been transferred from chromosomes to the plasmids of *Enterobacteriaceae.* In the mid-2000s SHV-type and TEM-ESBLs were replaced by CTX-M–type ESBL as a most common type of ESBL

associated with *E. coli* (14). This CTX-M type is also considered common among isolated bacterial strains from European countries, which were responsible for community outbreak infections of multi drug resistant *E. coli* in UK (15).

Because of the increasing resistance towards cephalosporins and other antibiotic classes, carbapenems have been started to use as a main treatment option for ESBL-producing *E. coli* infections. But due to the overuse of carbapenems there is an increasing number of Carbapenemase-producing *E. coli* (CPEc) reported. It has become extremely threatening because of the rapid increase in the prevalence of *E. coli* producing carbapenemases, particularly of New Delhi metallo-β-lactamase (NDM) (16) and *K. pneumoniae* carbapenemase (KPC). NDM was first isolated from Swedish patient in 2009 who has taken treatment from Indian hospital. And KPC was first reported in 2001 in USA (17). Because of the presence of the clonal expansion sequence type (ST) of KPC-producing *E. coli* 131, has become the most frequently reported, KPC-producing *E. coli* especially in China in East Asia (18).

The appearance of these MBLs in gram negative bacterial strains has become therapeutic trial, as these enzymes contain the higher capacity of hydrolytic activity which results in the failure of higher generation cephalosporin drugs. So treatment alternatives are becoming either unavailable or expensive with lower output results (19).

Among all the alternative approaches, phage therapy has the capacity to evade the resistance through antibiotics by attacking pathogenic bacterial strains. In Eastern Europe phage therapy has been successfully used for decades but it has been established quite recently in experimental models (20). Phages have the capacity to change the growth rate of antibiotic resistant and sensitive population simultaneously, As the lineages which grow more rapidly are affected usually more as compare to the rest of the population ('killing the winner'; (21);(22) Or this could also result from the direct interactions of phage replication and antibiotic resistance (23).

Because of these characteristics now trend has been shifted to the usage of bacteriophages against resistant microorganisms as an ideal treatment option. Bacteriophages, the natural enemies of bacteria are actually viruses that kill bacteria and are abundant in nature due to presence in different habitats like soil, sewage, human gut and sea water (24).

Large number of bacteriophages has been isolated from different environments and their number depends upon bacterial number and activity. High abundance of bacteriophages has been reported in soil fresh water and marine ecosystem (25). They are also associated with human and animal microbial communities (26), also in plant phylloshpere and rhizosphere (27), abundant in wastewater treatment plants (28) and also in extreme environmental conditions (29).

These are the most plentiful entities and are an important factor in controlling the populations of different hosts like algae fungi and bacterial communities. Therefore they could be used for removal of environmental microorganisms which are harmful for public health (30). Pathogens present in the sewage treatment plants could be removed by using bacteriophages for complete removal as reported in a research study conducted in India (31). Researchers have collected hospital wastewater from different location of Tamil Nadu and demonstrated that total coliform bacterial population ranged from 1.2×10^3 to 1.6×10^3/ 100 ml of sample.

Along with *Pseudomonas sp. Streptococcus sp* and *Bacillus spp* higher numbers of antibiotic resistant *E. coli* were isolated from all the places which indicated the amount of *E. coli* pollution in the area. *E. coli* specific phages were also isolated and their inoculation to the pathogenic strain resulted in the 100% removal of *E. coli* within 14 hours of application in the sewage.

Similarly a study had been done in which the researchers had reported eight bacteriophages. These phages were characterized and they were reported as lytic

bacteriophages as they were also showed similarity with T-even bacteriophages. But there was no lysogenic phage in the sewage (24).

These evidences further strengthen the notion of presence of lytic bacteriophages in the sewage samples isolated from the environment. Recently conducted research reported the power of bacteriophages to remove the pathogenic bacterial strains from the wastewater treatment (32). In this study the researchers had isolated two bacterial strains from the hospital waste drainage. Identification of bacterial strain was done at genus level and found to be *E. coli* and *Salmonella sp.* Titer of phages against isolated bacterial strains has shown a significant reduction in the total bacterial colonies so it could be deduced that bacteriophages could be utilized as bio control agent in wastewater treatment. The activity of the bacteriophages against bacterial isolates was resulted into 100% reduction for *E.coli* and *Salmonella sp.* This proves the presence of lytic phages along with the bacterial species in the same environment.

In Pakistan in a recently conducted study a lytic bacteriophage termed as P.E1 has been characterized to check its activity against multi drug resistant *E. coli* clinical isolates. This phage was isolated from sewage water supply which could be considered suitable for phage therapy as it has shown narrow host range for lytic activity. This phage reduced the host bacterial growth considerably. Its genome was double stranded and its size was larger than 12kb. Phages could be considered as unique option for the treatment against *E. coli* infections and could be used in phage therapy (33).

In Pakistan although there are research with respect to the lytic activity of phages isolated from the environment but there is a lack of information regarding the effect of bacteriophages on the bacterial communities and how these bacteriophages play their role in controlling the population of bacterial species present at the particular area. So these phages could be used in elimination of pathogenic strains and also could be practically employed to manipulate strains hence reducing antibiotic resistance. Therefore considering all these facts, present study has been design to investigate the killing effect

of bacteriophages on the bacterial community and controlling effect of these bacteriophages on different sequence types of *E. coli* strains present in the environment.

Aims and Objective:

The main aim of this study was to evaluate the major sequence types of *E. coli* present in the sewage samples of Islamabad and to analyze the effect of bacteriophages on these isolates. The objectives of the study were:

a. To isolate the environmental *E. coli* from sewage samples

b. Molecular identification of *fim*H, *fum*C and analyze the major sequence types by using two locus sequence typing

c. To study the antibiotic resistance genes $bla_{\text{NDM-1}}$ and $bla_{\text{CTX-M-15}}$ in isolated strains present in the environmental samples

d. To study lytic effect of bacteriophages on the isolated bacterial strains

Chapter 02

Literature Review

2. *Escherichia coli*:

Escherichia coli, a rod shaped bacterium is non-sporulating, facultative anaerobic gram negative microorganism. Its diameter is about 0.25-1.0 μm and length is about 2.0 micrometers (34). Strains having flagella are motile and flagella surround the bacterium in peritrichous arrangement (35). The optimum temperature for *E. coli* multiplication is 37°C (98.6°F) but few strain have shown growth at 49°C (120°F) (36).

Multiplication is carried out by reduction of certain substrates like fumarate, oxygen, dimethyl sulfoxide and trimethylamine N-oxide along with the oxidation of certain substances like pyruvic acid, amino acid, formic acid and hydrogen (37). *E. coli* can be divided into three major domains: commensal, pathogenic and extraintestinal strains. Commensal *E. coli* is commonly found in the intestine of the warm blooded organisms (38). About 0.1% of the gut microflora comprises of *E. coli* (39).

Bacterial pathogenic strains which cause disease have major route of transmission as fecal oral route. *E. coli* cells are used as an indicator of fecal contamination due to the fact that its cell can survive in the environment for limited amount of time (40, 41) and can tell the origin of contamination whether it's from human or birds (41). With respect to genetic and phenotypic diversity *E. coli* includes a vast population of diversified microorganisms. After looking at the genomic sequences of large number of isolates, taxonomic reclassification is essential (42).

E. coli is highly variable species as only 20% of the total genome is common in all the strains (43). *E. coli* have been classified on the basis of certain features into different groups. These groups are called as serotypes, pathotypes and phylotypes.

2.1 Serotypes:

In food, environment and clinical samples for the identification of a pathogenic *E. coli* strain, serotyping on the basis of antigen could be used as a standard to understand the epidemiology (43). For detection of pathogenic strains serotyping is considered as a well-known method (44). On the basis of its O (somatic), H (flagellar), and K (capsular) surface antigen profiles, *E.coli* is being serotyped (45). Many antigens of the O, H and K classes (56 H-antigens, 80 K-antigens, 173 O-antigens) have been stated, which can have any likely combination, making between 50,000- 100,000 serotypes (44).

2.2 Pathotypes:

E. coli normally live in the human host intestines as friendly bacterium causing no harm, but sometimes it causes very threatening diseases. Six possible pathotypes of the intestinal pathogenic *E. coli* are : Enteropathogenic *E. coli* (EPEC), Enterohaemorrhagic *E. coli* (EHEC), Enterotoxigenic *E. coli* (ETEC)(46), Enteroaggregative *E. coli* (EAEC), Enteroinvasive *E. coli* (EIEC), and Diffusely Adherent *E. coli* (DAEC).

I. Enteropathogenic E. coli (EPEC)

A common cause of infant diarrhea in developing countries is Enteropathogenic *E. coli*. In the manifestation of disease, loss of absorptive microvilli from the small intestine occur (47) which result from the colonization of EPEC in the epithelium of small intestine followed by lesion in the intestinal cell. This results in watery diarrhea (48).

II. Enterohaemorrhagic E. coli (EHEC)

Reported primary cause of haemorrhagic colitis is the Enterohaemorrhagic *E. coli* (49). The distinguishing feature of this group is, *E.coli* produces verocytotoxins, (50) which is also known as Shiga toxins (Stx). Bloody diarrhea result from the production of *stx* in the colon which damage its cell and results in bloody discharge (49). Renal inflammation also occurs as toxin travel to the kidney and damage renal endothelial cells (51).

III. Enterotoxigenic E. coli (ETEC)

A childhood diarrhea results in severe consequences on the children under five years old is caused by ETEC (52). This diarrhea is very common among travelers and in developing countries (49). Fatal effects of this disease result from the secretion of enterotoxins which are the heat-stable enterotoxins (53), the heat-labile enterotoxin (LT) or a combination of both (52).

IV. Enteroaggregative E. coli (EAEC)

As a causative agent of child and adult diarrhea EAEC (46), is recognized as a major source of epidemic and endemic worldwide (54). This pathogenic strain colonizes the colon first then damage the mucosal lining by secreting cytotoxins and enterotoxins (54).

V. Enteroinvasive E. coli (EIEC)

EIEC in genetic, biochemical and in pathogenic effect is similar to *Shigella spp.* (49). According to many studies these two microorganisms taxonomically shared the same origin and belong to same specie with respect to its many features, (55), as in having same mechanism of pathogenesis (52). Co-evolution of both microorganisms occurs mainly due to the presence and ability of invasive plasmid to invade the host tissue (56).

VI. Diffusely Adherent E. coli (DAEC)

DAEC name given to this strain due to its diffuse adherence pattern on cell line named as HeLa and HEp-2. In developing and developed countries this strain has been associated with watery diarrhea (57). Major infections caused by these strains are recurring urinary tract infection and diarrhea particularly in the children older than one year (54).

OXA-48 is commonly found in *K. pneumoniae* but absent in *E. coli* (94). KPCs are also disseminated among *K. pneumoniae*. In the countries like USA (95) and china (96) where KPC producing *K. pneumoniae* are highly prevalent, KPC producing *E. coli* has also been reported.

Dissemination rate of KPC is almost 10 years from the time it was first reported in 2001 and disseminated worldwide (97) while NDM has been disseminated in less than five years after its discovery in 2008 (98). NDM producers are resistant to all aminoglycoside antibiotics and it includes broad spectrum aminoglycoside like amikacin as well (99). This created a huge problem by limiting the treatment choice to only two drugs which colistin and tigecycline. Both of them are not considered as a best choice because of their potential toxicities and difficult pharmacodynamics (100). Twenty three variants of KPC are being reported mostly belonging to the *K. pneumoniae* (101).

Sixteen NDM variants are being reported. Most of the NDM variants are NDM-3, NDM-4, NDM-5, NDM-6, NDM-7, NDM-8 and NDM-12 which are mostly reported in *E. coli* instead of any other species of *Enterobacteriaceaen* (102). KPC-2 and KPC-3 variants of KPC are also reported in *E. coli* (103).

2.8 Epidemiology:

2.8.1 Overall scenario:

ESBLs have been increased in Europe as TEM-1, TEM-2 and SHV-1 were most prevalent ESBL in 1980's and 1990's (104). Among them most common was *K. pneumoniae* associated ESBL. In Turkey *E. coli* associated with UTIs was 21% which contained genes for ESBLs (105). In Spain ESBL-producing *E. coli* causing bacteremia, was 6.5 % (106).

In Latin America, ESBL producer *E. coli* ranged from 8.5% to 18% (107). It has reported that SHV-7 and SHV-12 as the most prominent ESBLs in US hospitals (108). Data from the MYSTIC program conducted during 1999–2004, showed a low level of ESBL

prevalence among *E. coli* and *K. pneumoniae*, with *E. coli* strains producing less than 1.5% of ESBLS from 2001–2004. In overall Asian countries, ESBL-producing *E. coli* especially CTX-M–producing are the most important multiple drug resistant *E. coli* reported. CTX-M producing *E. coli* produce major problem in populated cities (109). CTX-M– producing *E. coli* is also reported from rural areas of china showed that this problem is not just limited to urban areas (110). In the central parts of Asia KPC- and NDM-producing *E. coli* has also been reported (111). While in Indian subcontinent NDM producing *E. coli* are most prevalent (112).

2.8.2 Epidemiology in South Asia:

In India ESBLs in *E. coli* prevalence could be ranged from >45 and 79% (113). For the monitoring and keeping check and balance in the spread of antimicrobial resistance, certain surveillance studies were conducted like Study for Monitoring Antimicrobial Resistance Trends (SMART) for the Asia-Pacific region which provided data regarding ESBL- producing *E.coli* and carbapenemase producing *E. coli* from Indian region. Among them CTX-M was about 95%, most abundant among all (114). In India a study involving *E. coli* samples taken from pediatric patients, ESBL-producing *E. coli* among infants comprised of 47% of all urinary tract infections (UTIs) (113).

In neonate patients colonization of CTX-M-15 producing *E. coli* was reported as 55 % (115). In the south India among 104 strains of *E. coli* the prevalence of CTX-M gene was 63.7%. Among the entire CTX-M strains CTX-M-1 group was reported as 100% prevalent (116).

Bangladesh has very high CTX-M among ESBL *E. coli* prevalence rate. It has prevalence rate up to the 76% (117). Molecular characterization study in Bangladesh showed that 11% of 339 strains of *E. coli* were ESBL producers and 100% of them were of CTXM- 1 group category (118). Among clinical isolates NDM-producing *E. coli* were associated with the diarrhea patients in Bangladesh (119).

In Nepal multidrug resistant *E. coli* are the reported cause of UTI in adults (120). While infections due to *E. coli* in children were about 67% (121). In Nepal *E. coli* prevalence was 13.5 % (120). But NDM-producing *E. coli* strains are the only carbapenems isolated from this country. From this region also NDM-8 and NDM-12, two variants of NDM gene has also been reported (122, 123).

2.8.3 Epidemiology in Pakistan:

As a common pathogen CTX-M producing *E. coli* have been described since 2000s in Pakistan and usually these isolates are uropathogenic (124). In Pakistan CTXM- 1 group has been reported as the most common strain (125). In Islamabad the rate of Amp C isolates were 35% while the ESBLs were 64% (126). ST 131 has also been reported from Pakistan. In the hospitalized patients in Pakistan NDM-producing *E. coli* were more frequent as compared to the rest of the strains (125). This result was collected by screening of carbapenemase-producing *Enterobacteriaceae* by taking the rectal swabs of the patients. This data also showed that from Pakistan, among *Enterobacteriaceae* family, the most common among fecal carriage were NDM-producing *E. coli* strains as compare to the rest of species (127).

A strain is specie subgroup that has specific characteristics which make it distinguished from other strains. These variations could only be found at genetic level as its genomic environment which gives the ability to the strain to utilize specific source for energy utilization, demonstrate virulence and show resistance towards particular class of antibiotics. Due to its host specificity *E. coli* strain could be used as an indicator species for detection of fecal contamination. To understand the process of conversion of an innocuous bacterial strain into pathogenic strain population genetic study is very important (2).

2.9 *E. coli* sequence types:

Globally *E. coli* has been considered as most frequently producing (ESBL) species which could be estimated by the fact that its prevalence is upto 91% in certain countries like Ireland (128). In other countries its prevalence is 61% as in Spain (129), in Japan 41% (130), in USA from 47% to 56% (131), in Canada 78% (85) and in Israel 41% (132) respectively.

E. coli sequence type O25b:H4-B2-ST131 which is a set of pandemic clone belongs to group B2, has globally caused a major threat due to its prevailing feature. Phylogenetic group B2 present in human asymptomatically but it has association with extraintestinal infections (133). It has been reported that among all the groups B2 has diverged earlier in *E. coli* and is comprised of nine subclasses and in that the basal group is ST131 (72). O25b ST131 has been reported in many countries like in Spain, Australia, China, UK and USA (129) (134).

CTX-M-15 belong to the group CTX-M-1 group, is considered as most prevalent ESBL around the world. It was discovered in India in 1999 for the very first time (135). Then in mid-2000s its prevalence enhanced worldwide (92). The most successful strain of *E. coli* ST131 is responsible for the spread of CTX-M genes through acquisition and horizontal transfer of plasmids encoding this gene (136).

ST131 is actually *E. coli* bacterial strain O25b: H4 ST131. It has emerged in North America, Asia and Europe in 2008. In the same years there were reports of this strain in Kuwait, India, Canada and several European countries. This strain belonged to the virulent phylogenetic lineage B2 (137). Later it was assigned as most successful clone of *E. coli*. The success lies in its ability to produce the ESBLs (136).

2.9.1 Antimicrobial resistance genetically defines successful ST131:

Deeper studies are required to understand the evolutionary epidemiology of ST131 which has become universal problem now. With regard to its spread and evolution at the local scale, information is very scarce (138). It is causing high rate of infection in neonates (139) and also at childcare facilities (140). The rate of ST131 infection in the long-term healthcare facilities is 76% which is quite high as compare to the public (15%) and hospitals (49%) (141). This is because of CTX-M elements carried by the gene. These elements are beta-lactamases (*bla)* which has the ability to hydrolyze the rings of beta lactam antibiotics (142). In long term care facilities, hospitals and community $bla_{\text{CTX-M}}$-positive ST131 causing UTIs are becoming common (143-145).

ST131 has been reported in healthy people as well but it was not of ESBL producer that belong to multidrug resistant lineage. It has now been established that capability to be antimicrobial resistant, virulence characteristics and phylogenetic features has attributed to the success of this bacterial clone throughout the world (146).

To understand the factors which contribute to the success of a particular strain it is important to look into the genetic environment of the strain. As ST131 is a clonal lineage so to understand its genetic definition two closely related clonal complexes (ST1876 and ST95) has been studied which helped in comprehending the genes associated with this lineage. A great number of pathogenic and virulence genes are associated with the ST131 clonal type (129). Most important among them is *fim*H gene associated with type 1 fimbrial adhesion protein. On the basis of serotypes this lineage is divided into three different Clades. Serotype O16:H5 having *fim*H*41* allele is placed in Clade A. Serotypes O25b:H4, encoding *fim*H*22* allele id placed in Clade B. *fim*H*22* containing strain were fluoroquinolone-sensitive as genomic analysis of different strains collected in 1967, 1990, 1992 and 1997 has revealed (85).

Clade C is named as group H30 as it contained *fim*H*30* allele and all remaining ST131 belonged to this group. H30 appeared in around 1997 (147). A very small number of ST131 belong to H30 has lost *fim*H*30* for *fim*H*22/35*. Before 2000, Fluoroquinolone-sensitive ST131 was very common but now it is quite scarce. Reason for its rarity lies in the mutational changes occurring in two genes: *gyrA* gene *which* encode for DNA gyrase subunit A and *par*C encoding DNA topoisomerase 4 subunit A (148). H30-R was reported previously as always fluoroquinolone-resistant but this quality could be lost (149).

In H30-R a subgroup named as H30-Rx which has been emerged in around 2002 and has always been observed as *bla*CTX-M-positive. Three single nucleotide polymorphisms are responsible for attributing fluoroquinolone-resistance and they also differentiate the H30-R from H30-Rx. *Bla*CTX-M-15 is the CTX-M element type considered as most frequently isolated type (150), as about 49% of *E. coli* were *bla*CTX-M-15-positive ST131 from 2004-2006 in long term care facilities (151). This gene was not found in ST38, ST405 and ST 648 also problematic strain (152).

ST131 has been categorized on the basis of its virulence genes, which place them into different virotypes (153). Its virulence genes are mostly located on Mobile genetic elements and pathogenic islands which contain gene related to invasion, adhesion, proteases, lipopolysaccharides and polysaccharide capsules (154). According to recent surveillance data overall prevalence of *E. coli* clinical isolates are 12.5% to 30%, fluoroquinolone-resistant isolates is 70% to 80% and extended spectrum beta lactamase producing isolates was from 50% to 60% (155).

2.9.2 *E. coli* major sequence types and their significance:

Importance of specific sequence types of *E. coli* lies in the fact that some of these sequence types are associated with the particular extraintestinal syndromes like ST73 and ST127 are associated with pyelonephritis (156). Some of the sequence types are reported to be involved in emergence of antibiotic properties of the bacterial strains for example ST69 is associated with trimethoprim sulfamethoxazole resistance (157) and ST131 with the production of beta lactamase and also with resistance to fluoroquinolone (158).

But with respect to genetic properties and pathological behavior sequence types are not always uniform. In ST95, for instance North American Clade OMP6 with serotype O18: K1:H7 was reported to be associated with UTI and newborn meningitis because of encoding genes of P fimbriae and hemolysin (159), whereas European Clade OMP9 with serotypes O18:K1:H7 lack these genes and only associated with newborn meningitis. Furthermore serotypes O1:K1 and O2:K1 occur in ST95 are only reported in UTI cases instead of neonatal meningitis (160).

Similarly ST73 comprises of two kinds of strains like virulent strains CFT073, which has been reported to cause extraintestinal infections in various animal models and was also reported as an archetypal human pyelonephritis strain and a nonvirulent type Nissle 1917 and ABU83972 which are commensal strains that not only nonpathogenic in nature but also protect the host against symptomatic infection (161). Different typing schemes are required as they could provide differentiation between closely lineated strains and thus increase the understanding regarding clinical strains behavior and evolutionary changes which give rise to different sub lineages between them.

2.9.3 *E. coli* sequence types from environment:

For the first time the presence of $bla_{CTX\text{-}M\text{-}15}$ was reported in 2014 in the river sediments of UK which has the ability to readily transmit to other gram negative bacterial species (162). After sequencing it was realized by the researchers that most of the bacterial strains isolated were pathogenic as in like *E. coli* ST131, ST167 and ST38.

In a research regarding the molecular homology analysis of different bacterial strains from humans and environmental waters by pulse field gel electrophoresis and MLST techniques it was confirmed that different isolated *E. coli* sequence types belong to B2-ST131, D-ST648, D-ST38, or A-CC10. Presence of these strains demonstrated that there is a transmission of resistant genes from human to environment. In this study several novel strains has also been reported. It was the first study to report CTX-M producing *E. coli* from healthy and hospitalized human , water and swine which further confirmed the notion that pathogenic strains in the environment originally comes from the animals and humans (163).

In a study conducted in Bangladesh to check the trainability of New Delhi metallo-β-lactamase variant 1 (NDM-1) drug resistance between hospital and communities, it was reported that most prevalent strain was ST101 and also three more sequence types ST648, ST101, and ST405 were observed and these three sequence types are responsible for NDM-1 encoding *E. coli* in the UK upto 80% (164). And also it was reported that ST101 is most common type of strain present in Bangladesh environment up to 10.3% and in United Kingdom up to 50% in clinical isolates. (165).

studied through DNA sequencing based method. (3) With the help of nucleotide probe the third methods are categorized as DNA hybridization based method (169).

Methods that come under the category of DNA banding pattern are: (i) Pulsed-field gel electrophoresis (PFGE) in which larger molecules of DNA are separated on agarose gel. (ii) Restriction fragment length polymorphism (RFLP) which could separate the smaller fragments as well. RFLP analysis could be made simpler by subjecting its partial restriction fragments to Southern blotting along with labeled probes.

(iii) Ribotyping, which involves using rRNA probes (170). In this typing method ribotypes are gained which are the fragments of DNA banding pattern related to relevant rRNA. (iv) Arbitrarily primed PCR (AP-PCR), which involved the use of arbitrary primers to amplify the unknown genomic sequence of DNA. It is also called random amplification of polymorphic DNA (RAPD), (v) REP-PCR which is Repetitive sequencing-based PCR in which interspersed repetitive consensus sequences are amplified which are reported to be dispersed throughout the genome of bacterial specie (171) and DNA fragment is collected. (vi) Multiple-locus variable number tandem repeat analysis (MLVA) which involves the polymorphic analysis of Variable number tandem repeat loci located on chromosome (172). (vii) Denaturing gel electrophoresis, which involves the separation of same length PCR amplicons on the basis of their sequences on the polyacrylamide gels is another method.

Methods based on DNA sequencing are of two main types one of them is Sanger method, being used traditionally and second recently used method is pyrosequencing technique. Sanger Method involves the chain termination step so it is termed as chain termination sequencing of DNA. In this method template DNA sequence is determined by the use of dideoxynucleotides which during amplification disrupt the elongation of DNA. A nonelectrophoretic sequencing technique which is based on detection of pyrophosphate by real time quantitative method, released during DNA synthesis by incorporation into DNA chain, is known as pyrosequensing.

Gene sequencing methods are 16S rRNA gene sequencing and multilocus sequence typing, in which allelic variants of several housekeeping genes are uncovered and it is currently most widespread method used for bacterial strain characterization (173).

For detection of DNA mutations DNA hybridization is most widely used. The reason for its intensive used lies in the fact that it needs complementary sequence to proceed in the reaction. In DNA hybridization two main elements are: targets and probes. Targets are actually free DNA whose identity is to be determined with the help of labeled probes. This is carried out with the help of DNA arrays. Recently two types of DNA arrays are used named as macroarrays and microarrays. Main difference between them is the number and size of spots used on the supports (169).

2.10.2 Multi locus sequence typing:

Among all the techniques MLST is considered as the best because of detailed analytic picture of the genomic sequences of the bacterial strains provided by this technique. In MLST conserved sequence holding housekeeping genes, usually seven genes are examined to find out the exact genomic picture of the strain. These genes encode for essential functioning protein so there is very small chance of variation in the sequence of these genes. Usually 450-500 bp segment of seven genes is sequenced and a number is assigned to each different sequence if it occurs in the gene. So each strain is sequence type when it has been assigned an allelic profile comprises of seven numbers.

These number are assigned in order of their unique sequences discovery so sequence type is allelic profile of the strain, basically a combine effect of the numbers given on the basis of alleles present on each loci designated by MLST for a bacterial strain (173) these numbers are same as used in 'electrophoretic type' (ET) assigned in MLEE (174).

Relationship among the strains could be found out by comparing the allelic profile of the strains. Strains which are closely related are given same STs. MLST is widely used

because of number of advantages this technique provide, like material (DNA preparation) which is used to assign the sequence type is easily portable and transported through mail without any difficulty. MLST is readily automated because of primer sequences and protocol availability through electronic source (175).

This method is fully scalable even for thousands of samples. As for designing of new MLST system three basic elements are required which are: isolates choice for evaluation, genetic loci selection for characterization and primer design for amplification and determination of nucleotide sequence. For comparison of international isolates this approach has become rapidly a routinely used tool (176). This technique has been applied for number of diverse purposes like resistant variants emergence, disease caused by variants associated with the particular virulence genes and also global dispersal of the particular virulent strain (16). This data can also be used to assess the mutational rate and recombination which are occurring in the population and bringing the evolutionary changes in the bacterial communities (177). This information has also been used to check the evolutionary relationships among those bacteria which belong to same genus (178).

Three MLST schemes are used with three different data bases depending upon different set of gene. *Ec*MLST database which was concerned originally only with enteropathogenic *E. coli* (179). This data base was created by Thomas Whittam whereas the second data base created by Mark Acthman is placed at Warwick medical school (180), and third data base founded by Sylvain Brisse and Erick Denamur and placed at Pasteur Institute (Paris, France) (181), this database was not restricted to particular strain sequencing of the *E. coli* .

There is not evident reason behind the choice of genes for each scheme except for the fact that they were conserved genes and encode for primary function of the *E. coli* strain. Lowest level of horizontal gene transfer has been observed in genes of Pasteur Institute

scheme which were selected, out of eleven of housekeeping genes (182). Lowest diversity of the genes has been observed in Warwick scheme then Michigan scheme followed by Pasteur Institute scheme genes having the highest nucleotide diversity among all schemes (183).

2.10.3 CH Typing:

Despite of many advantages of MLST some of the researchers have developed sequencing scheme utilizing fever genes than the seven housekeeping genes used in MLST. CH typing utilizing two loci (*fum*C/*fim*H) for typing, which has provided same results as MLST based profile results (184). CH typing scheme for *E. coli* has been established by utilizing internal fragment of *fim*H gene comprising of 489-nucleotide and 469-nt internal fragment of *fum*C gene as used in the standard multilocus sequence typing technique. CH typing have an edge over the MLST scheme in this regard that it is economical but has excellent clonal discriminatory power of separating the strains and also closely resemble to MLST based grouping of clonal strains.

*fim*H gene was selected because large majority of *E. coli* strains have gene for type 1 fimbriae. The *fim*H cluster is located at the region where usually pathogenic islands are located and it is highly recombinogenic region, situated downstream of leuX, tRNA locus. For the functional mutations *fim*H gene is under positive selection (185). and bacterial adhesion properties regarding the pathogenesis could be changed intensely as single nucleotide polymorphism produce amino acids substitutions which govern these changes (186).

In a research conducted previously it has been reported that clinically important ST95 strain has high level of allelic diversity in *fim*H gene region. Twelve unique alleles were reported in 44 isolates due to amino acid substitution and *fim*H cluster recombination due to strong positive selection pressure (185).

This genetic diversity in the *fim*H gene gives an advantage so it could be used in number of typing methods. But typing method should have the power to give reproducibility, efficiency with respect to time and cost and also discriminatory power (82). In clinical microbiology 7-locus MLST usage is limited because of its high cost and labour, comparable results with lesser number of loci are very attractive until the discriminatory power can be kept constant or increased. *fim*H internal typing region of about 489-bp has been identified which could distinguished 58 alleles as compare to 67 alleles distinguished by full *fim*H gene.

Among all the housekeeping genes the best suited candidate chosen for pairing against *fim*H was *fum*C as it has demonstrated the greatest discriminatory power in numbers. In practical *fim*H with *fum*C pairing produces the highest value among all the pairings of housekeeping genes which make it best suited for typing. Another characteristic which make is better suited for pairing was its highest congruence with major phylogenetic groups and ST profiles. Between CH typing and MLST scheme the overall match rate was 95.8%.

High discriminatory power of *fim*H locus in CH typing is due to two main mechanisms. Firstly because of homologous recombination there is replacement in *fim* cluster. And secondly point mutations which lead to the amino acid substitution. These single nucleotide polymorphisms occur with rapid speed and gather in *fim*H allelic backgrounds. This phenomenon occurs during successful clonal strains diversification at the population level and even in a single host infection period (5).

To devise a typing scheme having pairing of *fim*H and *fum*C loci , Researchers in 2012, had selected *fum*C over the seven housekeeping loci due to the reason, as it has provided greatest discriminatory power, highest level of nucleotide polymorphism and also phylogenetic grouping prediction can also be done by it. This combination has provided such a greater discriminatory power than MLST that it has taken the place of multilocus

enzyme electrophoresis (187) as a standard method for predicting *E. coli* population structure.

MLST profile can be predicted by CH typing method. CH typing has correlated with STs and ST complexes by MLST and other techniques for more than 90%. This fact has increased its utility as molecular tool for studying population structure of *E. coli* strains and also its application in epidemiological investigations (5).

2.10.4 Relevance to clinical samples:

Genome sequencing of environmental *E. coli* strains provides insight into the relationship based on evolution, virulence, pathogenic lifestyles which in turn favor adaptations and process of antimicrobial resistance. These sequenced strains are associated with antimicrobial resistance (188).

Antibiotics after administration in environment are discharged unchanged without metabolizing in the external surroundings (189). Therefore wastewater plants act as a reservoir for mixture of pharmaceuticals as these entities enter into them (190) and each of this could reach the environment. Antibiotic contamination has been known as a major environmental pollution of aquatic ecology as they have potential harmful effects on human health and ecosystem (191).

The resistome which are known as environmental reservoir of antibiotic resistance genes, along with its precursors are being changing due to the human activities like sewage discharge, development of drug and agricultural livestock (192). Thus the possibility of transfer of antibiotic resistant genes into pathogenic microorganisms has been increasing; (192). As when encountered with the clinically important isolates these environmental bacteria can act like a source of resistance genes (193). For the purpose of managing antibiotic resistance environment has got prime importance as it happens to be the source of spread of resistance and has become danger to the public health. To elucidate the movement of resistant genes between clinically pathogenic microorganism and

environmental bacterial strains, a thorough understanding of prevalence of antibiotic resistance and diversity should be developed (194).

Antibiotic resistance genes in the environment are the source of transfer of resistance genes in clinical bacterial strains, as the source of antimicrobial resistance lies in environmental resistome (195). By two ways the movements of microbial genome is possible: vertically by the microbial population cell division and horizontally across genera and species (196). This transfer is possible by the mobilome which are the genetic elements that contribute to horizontal gene transfer (197).

For HGT the three major principle mechanism involves are: conjugation (direct transfer through cell), transduction (phage mediated transfer), and naturally occurring transformation (transfer of DNA to cell). These mechanism help in mobilization of the genetic elements like pathogenicity islands,, plasmids and prophages containing resistance gene in their genome (197).

Moreover resistance gene cassettes can be analyzed by integrons that make possible the movement of tandem genes from the single promotor element thus offer splendid phenotypes carrying multi drug resistance (172). Furthermore transposable elements also mobilized the resistance genes within the chromosomes and to the plasmids (197).

Genes encoding resistance to antibiotic drug, heavy metals and virulence factor for clinical isolates are spread by mobilome which is assisted through the movement in the environment (198). Therefore no region is considered safe or can avoid the introduction and mobilization of antimicrobial drug resistant genes and organisms (197). Furthermore this movement is augmented by the selection pressure imparted by the anthropogenic activities. This provides the chance to the naturally occurring bacterial species and bacteria introduced by human activities into the environment to form multiple resistant phenotypes (197).

One greatest example of transfer of antimicrobial resistance from environmental resistomes to the clinical microbes is in the class-A extended-spectrum –lactamase CTX-M, which is present on plasmids contained by the globally acknowledged pathogenic strains and it could be traced back to environmental strain *Kluyvera spp.* (199), and *qnr* gene which encode resistance to quinolone, present on the conjugative plasmid in the ciprofloxacin resistant strain of *K. pneumoniae* could be traced back to the water-born species like *Vibrio*, *Shewanella*, and *Aeromonas* present in the environment (193).

Resistance exchange between the two backgrounds has been reported by some researchers (200). They isolated the multi drug resistance *Proteobacteria* from the soil which possess resistance against five major classes of antibiotics like (beta-lactams, aminoglycosides, amphenicols, sulfonamides, and tetracyclines), having perfect match of nucleotide sequence of a gene present in the pathogenic disease causing bacteria. Therefore environmental resistome is the reservoir of those antimicrobial drug resistance elements. That can be readily transmits into the human disease causing strains which have been proving enormously difficult to treat (198).

2.11 *E. coli* in sewage water:

In the environment there is an increase in the emergence of antibiotic resistant bacteria which are disseminating rapidly and this is because of use of human and veterinary medicine, growing trend of animal farming and industrial effluent which released into the wastewater and wastewater treatment plants which are contributing in the spread of resistance in the environment (201).

In treated wastewater runoff various microorganisms survive and multiply even after treatment because of inadequate disinfection processes (202). Presence of pathogenic bacterial strains in the environmental water has become the major concern for the public health official around the world. Effluent discharge from sewage treatment plant is the source of number of enteric pathogenic bacterial strain emergence in environmental

surface water. Therefore agricultural runoff and surface water can play a significant role in spread of resistant and pathogenic strain in the environment (203).

Microbial population quantification is currently done by measuring the level of fecal indicator bacterial strain like enterococci and thermotolerant *E. coli* which gives the information about the presence of pathogens and quality of water (204). Nevertheless some protozoa, viruses and bacterial strain have higher survival rate in the aquatic water than these microorganisms (205). These surviving strains actually harbor virulence genes which could not be investigated in sewage treatment plant and thus are releasing into the outside environment. *E. coli* quantification is very important as it serve as a pathogenic strain by causing number of infections either internal or extraintestinal (206).

It has been demonstrated previously that certain clonal strains of *E. coli* persist in the sewage treatment plants which harbor the virulence genes, characteristic of uropathogenic strains (207) which suggest that clones carrying the pathogenic and virulence gene persist in the environment especially in the sewage water and could find their way in the other sources of water (208).

2.11.1 Antimicrobial resistance in water as a threat:

Dissemination and emergence of antibiotic resistance has become a global phenomenon. These resistant organism are released into the aquatic environment from different sources like manure, man and animal through the means like feces, manure and sewage water. Along with these, waste water from the hospitals could also be the source of antibiotic resistant microorganism in the environment. The resistant genes acquired in the ecosystem can be easily transferred to the other organisms in different ecosystem. There is also mobility of living microbes as in travelling which also enable the spread of resistant genes throughout the world (209).

Now this is contributing to the endemic and epidemic spread of multi- drug resistance among microorganisms. Due to the misuse of antibiotics and mismanaged infrastructure

of health-care resistant microbes are found in all the compartment of environment (210). In treated and untreated waste water contamination is added through antibiotic compounds used in the households (211). Antibiotic resistant bacteria has been isolated and reported in different places like livestock production farms, sewage, hospital drains and wastewater of cities (212, 213).

One of the major class of antibiotics resistant enzymes which is CTX-M ESBL types has been reported in poultry, wild bird and in sewage water as well (214-216).

2.12 Role of bacteriophages:

According to the "killing the winner "hypothesis (21), cross infection by different types of phages naturally limits the abundance of successful strains and thereby increases bacterial diversity (217).

Bacteria infecting, viruses are known as phages or Bacteriophages. Due to their large abundant in nature, Bacteriophages are considered the most profound entity on earth. They have very important role in maintaining microbial ecology as they can control the number of bacteria in an environment either by lysing and infecting certain bacteria like up to 15% in bacterioplanton case (218) or by selecting certain bacteria which are resistant to them. So far cell lysis has contributed to significant extent in the context of recycling of organic compounds released by bacteria in the biosphere (219). But selection of resistant bacteria by bacteriophages has changed the number of bacterial strains in a certain environment which lead to the evolution of bacterial genome by transduction through horizontal gene transfer. Bacteriophages are specific to cell they attack and it is done due to the presence of certain receptors on the bacterial cell which are identified by the bacteriophages.

Bacteriophages can attack a bacterial cell in two different mode of action, known as lytic and lysogenic life cycles. On the basis of these two modes bacteriophages can be categorized as virulent and temperate phages. Virulent bacteriophages follow lytic cycle

in which the cell machinery is regulated by the bacteriophages in such a way that through cell lysis new phages are released in the environment. While in lysogenic cycle the genome of phage integrates in the bacterial host cell either in the cell chromosome or as an independent replicon. The bacteriophage at this stage is known as prophage which has the ability to induce the lysis of cell. Those bacteriophages which follow lytic cycle are called temperate bacteriophages. Induction of the lytic cycle could be spontaneous or by certain inducers. Lysogenic inducers could be anthopogenically introduced or naturally present in the environment. Among natural inducers, are UV light and host starvation and due to human activities like excessive use of antibiotic, the best known among them is quinolone by which the lytic cycle could be induced in the host bacterial specie.

Transduction is the phenomenon through which some of the phages can mobilize genetic material among different bacterial strains. Although virulent bacteriophages can also transduce but this process is usually associated with temperate phages probably because of their replication process it is easy to detect transductants (220).

Through transduction genetic material from the phage can be incorporated into the bacterial strain in which either it will packaged as random DNA fragment or at specialized sites for prophage attachment. Size range for DNA fragment is limited but it could reach upto 100 kb. Transduction of genetic material through bacteriophages could be in any form like it could be a linear fragment of chromosome or in the form of mobile genetic element like transposons, plasmids and insertion sequences (221).

2.12.1 Bacteriophages-Bacterial interaction in the environment:

Large number of bacteriophages has been isolated from different environments and their number depends upon bacterial number and activity. High abundance of bacteriophages has been reported in soil, fresh water and marine ecosystem (25). They are also associated with human and animal microbial communities (26), also in plant phylloshpere

and rhizosphere (27), abundant in wastewater treatment plants (28) and also in extreme environmental conditions (29).

In a given setting relationship between bacteriophages and bacteria can be numerically calculated as virus to bacteria ratio. Minimal number of bacteria at any place indicated the strong bacteriophage-bacteria interaction at that place.

This ratio mainly depends upon the sample source and it is variable. Reported values are from 0.01 to 100 while in a dominant state this ratio could range between 1 and 10 (25). In some areas phages could outnumber the bacterial strains then this ratio lies from 1 to 10. But it is not necessary that all phages could find their host bacterial specie but due to large number of phages present in the environment and persist in number of biomes it is assumed that phage could affect bacteria in different biomes (222).

2.12.3 Phages can alter bacterial population:

Phages have the capacity to change the growth rate of antibiotic resistant and sensitive population simultaneously, As the lineages which grow more rapidly are affected usually more as compare to the rest of the population ('killing the winner'; (21). Or this could also result from the direct interactions of phage replication and antibiotic resistance (23, 223).

Frequencies of presence of different lineages of bacterial strains over a long period of time depend on rates at which phage resistance is acquired by the bacterial strains. For stronger strain selection phage resistance mutations are becoming common. This phenomenon depends upon relative mutation provision rate that would be governed by the antibiotic resistance pressure. Thus phages have the potential to modify the selection of antimicrobial resistance elements (224).

According to recently conducted study a lytic bacteriophage termed as P.E1 has been characterized to check its activity against multi drug resistant *E. coli* clinical isolates. This phage has been isolated from sewage water supply. This phage could be considered

suitable for phage therapy as it has shown narrow host range for lytic activity. This phage reduced the host bacterial growth considerably. Its genome was double stranded and it has shown activity against the host bacterium at 70^0C. Genome size was larger than 12kb. This phage could be considered as unique option for the treatment against *E. coli* infections and could be used in phage therapy (34).

In a study the power of bacteriophages was implied to remove the pathogenic bacterial strains from the wastewater treatment. In this study researchers had isolated two bacterial strains from the hospital waste drainage. Identification of bacterial strains was done at genus level and found to be *E. coli* and *Salmonella sp.* Titer of phages against isolated bacterial strains has shown a significant reduction in the total bacterial colonies so it could be deduced that bacteriophages could be utilized as bio control agent in wastewater treatment. In this study, activity of the bacteriophages against bacterial isolates was resulted into 100% reduction for *E.coli* and *Salmonella sp.* This proved the presence of lytic phages along with the bacterial species in the same environment (33).

Chapter 03

Materials and Methods

3. Materials and Methods:

The study was carried out to check the variation of *E. coli* sequence types moderated by bacteriophages isolated from environmental sewage sample collected from city of Islamabad.

3.1 Study sites and sample collection:

Islamabad is the capital city of Pakistan. It is divided into five zones out of which Zones I and II comprise of developed and under developed residential sectors which include commercial and residential areas. Sectors are named with letters from A-I. Most developed sectors are G and F. G sector is divided from G-5 to G-17. For this study, sewage points were selected from sector G to check the variation in the sequence types of *E. coli* and effect of bacteriophages on these sequence types.

A total of eight sites were selected. For isolation of coliform population from these sewage points, samples were taken approximately 30 cm below the water surface from each site. Samples were taken in a sterile falcon tube (approximately 30 ml) and transported to the laboratory for further analysis.

3.2 Isolation and identification of *E. coli*:

For isolation of *E. coli*, the sewage samples were spun in a swing out centrifuge large enough to accommodate 15ml tubes at 4500 rpm. Supernatant was separated and saved for phage assay work. Bacterial pallets were suspended in 1ml of sample. It was a concentrated sample suspension so before further processing of sample on selective media, it was diluted to measure a single colony. For that purpose three dilutions of that suspension were made as 10^{-1}, 10^{-2}, and 10^{-3} respectively. Fifty µl of each dilution were spread out on MacConkey Agar media plates.

Media was prepared according to manufacturer direction in 500 ml flask. Media was autoclaved then cooled to bring its temperature to normal. Once normal, antibiotic vancomycin was added at final concentration of 25µg/ml of media, it was poured and plates were made. Sample dilutions each of quantity 50µl were spread on each of the plates and incubated at 37°C for 24 hours. Bacterial colonies were counted for each of the media plates by colony counter. Total bacterial counts and lactose fermenters were counted. From each site 10 individual colonies of *E. coli* were randomly picked from each plate and streaked on a fresh media plate for further purification. These colonies were purified by single colony streaking method. These 10 isolates from each site comprises of environmental bacterial sample collection.

3.3 Strain morphological identification and confirmation:

Isolates were identified as *E. coli* on the basis of colony morphology. As *E. coli* colony appeared on MacConkey agar as circular, umbonate and entire (form, elevation and margins) in pink color which helped in identification of theses microorganisms so 10 isolates were selected from each sample for further confirmation. Phenotypic and genotypic confirmation was carried out for these isolates.

First of all gram staining was done.

3.3.1 Gram Staining:

A smear was made on glass slide by adding a single bacterial colony and a drop of autoclaved distilled water. This smear was heat fixed on flame by passing the slide to and fro over the surface of flame. Then this smear was first treated with primary stain solution which is crystal violet staining reagent for one minute then washed the slide with indirect flow of water for at least two second then mordant was added which is iodine solution for one minute after this again slide was washed with indirect flow of water for two seconds. Then slide was flooded with decolorizing solution (95% alcohol) for 15 seconds or until the slide appear clear. Then counter stain safranin was added for 30 seconds to one

minute. Then slide was washed in the end with water. Then blot dry and observed under the microscope. All isolates appeared pink rods which indicated that they were gram negative isolates.

Further biochemical test were performed for recognition of bacterial species

3.4 Biochemical analysis:

3.4.1 Citrate utilization test:

Citrate utilization test could be used to distinguish fecal coliforms such as *E. coli* whose presence would be suggestive of fecal contamination. The test is based upon the ability of microorganisms to convert citrate into oxaloacetate, which is indicated in the form of color change in the media. The test was carried out with Simon citrate media slants which were made by pouring autoclaved media in the test tube tilting the tubes and allowing it to settle. Then these slants were inoculated by fresh culture of microorganisms. Single isolated colony was picked and lightly streaked on the surface of slant. Then these test tubes were incubated at 35°C (+/- 2°C) for 18 to 48 hours in aerobic conditions. Tubes were examined for color change which indicates the utilization of citrate as only carbon source provided for microorganisms. Negative results were indicated by no color change in the media while the positive result showed in the form of growth along with change of media color of the slant. If bacteria utilize the citrate then color of media will change from forest green to blue which indicate positive results.

3.4.2 Triple Sugar Iron (TSI) test:

Triple sugar iron agar is a tubed differential medium used in determining carbohydrate fermentation and H_2S production. Gas from carbohydrate metabolism can also be detected. Bacteria can metabolize carbohydrates aerobically (with oxygen) or without oxygen. TSI differentiates bacteria based on their fermentation of lactose, glucose and sucrose and also on the production of hydrogen sulfide. TSI is frequently used in the

identification of the *Enterobacteriaceae*. TSI contains three carbohydrates: glucose (0.1%), sucrose (1%), and lactose (1%).When any of the carbohydrates are fermented, the drop in pH will cause the medium to change its color from reddish-orange (the original color) to yellow.

A straight needle was used to pick the single isolated colony of the microorganism and streaked the TSI slants. Butt was stabbed by the needle while the slant was slightly streaked. Then the tubes were incubated with caps loosened at 35±2°C in aerobic conditions. The tubes were examined after 18-24hrs for carbohydrate fermentation, gas production and hydrogen sulfide production. Results could be interpreted in the light of these facts that Acid butt, alkaline slant (yellow butt, red slant) indicated that glucose has been fermented and sucrose and lactose are not. Acid butt, acid slant (yellow butt, yellow slant) showed that lactose and/or sucrose has been fermented. Alkaline butt, alkaline slant (red butt, red slant), neither glucose, lactose, nor sucrose has been fermented. Gas production is indicated by bubbles in the butt. With large amounts of gas, the agar may be broken or pushed upward. Both slant and butt are acidic for *E. coli*. Gas production has also been seen in *E. coli* case.

3.4.3 Indole test:

Indole test is carried out to check the ability of microorganisms to degrade tryptophan and produce indole. With the help of tryptophanase enzyme bacteria hydrolyze the tryptophan. Indole detection depends upon chemical reaction between indole and p-dimethylaminobenzaldehyde (DMAB). Reaction will be carried out under acidic conditions. Red dye will be produced at the end of reaction. To perform indole test tube containing trypton broth was inoculated with small amount of pure bacterial culture and incubated at 35°C (+/- 2°C) for 24 to 48 hours. After that to check the indole production Kovacs reagent about 5 drops was added to the tube. Appearance of red color in the form of ring indicated positive test while negative test result indicate cloudy sample appearance.

3.5 Molecular analysis:

For genotypic identification PCR was done for that DNA extraction was required.

3.5.1 DNA extraction:

DNA extraction was carried out through Phenol chloroform method by following procedure

- Bacterial cultures were refreshed by culturing on nutrient agar plate and incubated overnight at 37°C to get isolated colonies. Then heavy suspension of isolated colonies were made in 2ml eppendorf tube containing sterile normal saline and centrifuged for three minutes at 13,000 rpm. Supernatant was removed and bacterial pellet was obtained for DNA extraction.

Further extraction comprises of following steps:

- Pellet was resuspended in 450µl TE Buffer, then 45µl SDS (10%) and 5µl of proteinase K (20mg/ml) was added and this mixture was incubated for one hour at 37°C.
- After incubation Phenol-Chloroform (1:1) 500µl was added and mixed properly
- Now this tube was centrifuge for 20 minutes and upper aqueous phase of this mixture was transferred to new tube and again phenol-chloroform 500µl was added to this tube and centrifuged at 10,000 rpm for 5 minutes.
- Upper aqueous phase was separated and 50µl sodium acetate along with 300µl isopropanol was added and mixed well until DNA precipitated out for that purpose this mix was centrifuged at 10,000 rpm for 5 minutes, liquid phase was removed and this tube contents were washed with 1ml of 70% ethanol by centrifuging at 10000 rpm for 1 minute.
- Ethanol was removed and 200µl TE buffer was added and this DNA was stored at -20°C.

3.6 Polymerase Chain Reaction:

Polymerase chain reaction for individual gene was carried out with the following conditions.

3.6.1 PCR for *fim*H gene:

For molecular identification PCR was carried out for *fim*H gene. The reaction mixture for PCR comprises of total 50 µl volume having 2 µl of template DNA, 5 µl of 10X reaction buffer, 2 µl of dNTPs, 2 µl of $MgCl_2$, 2 µl of each primer and 1U of pfu DNA polymerase. Primers for this gene were 5′-CACTCAGGGAACCATTCAGGCA-3′(F) and for reverse primer the sequence was 5′-CTTATTGATAAACAAAAGTCAC-3′(R). For amplification the conditions were initial denaturation was carried out for 5 minutes at 94°C, then for 10 cycles, each cycle consisting of 60 seconds at 94°C, 60 s at 45°C, and 60 s at 72°C and this was followed by 20 cycles each one consisted of 60 s at 94°C, 60 s at 55°C, and 60 s at 72°C with final extension at 72°C for 5 min (225).

3.6.2 PCR for *fum*C gene:

PCR reaction mixture for this gene contained total 50 µl of reaction volume having 2 µl of template DNA, 4 µl of each Primers, 5 µl of 10X buffer, 15 mM MgCl2, 5 µl of dNTPs and 0.5 U Ampli*Taq* Gold. Primers sequence for this gene were: *fum*C (fumarate hydratase) TCACAGGTCGCCAGCGCTTC (F) and GTACGCAGCGAAAAAGATTC (R). Thermocycler conditions were 2 min of initial denaturation at 95°C; then 30 cycles of 1 min of denaturation at 95°C, followed by 1 min of annealing at 52 °C, then 2 min of extension at 72°C; and a final additional 5 min at 72°C (226).

3.6.3 PCR for CTX-M-15 gene:

PCR amplification of $bla_{CTX\text{-}M}$ alleles was carried out in the reaction mixture containing 50 µl total volume of reaction mixture contacting 1 µl of DNA extract, 30 pmol of each primer, 100 µM each dNTPs, 1.25 U Taq polymerase and buffer containing 1.5 mM

$MgCl_2$. Primers for this gene were 5′-ATGTGCAGYACCAGT AARGT -3′ (F) and for reverse primer the sequence was 5′-TGGGTRAARTARGTSACCAGA -3′(R). Thermocycler reaction parameters were initial denaturation at 94°C for 7 min then denaturation at 94°C for 50 sec followed by annealing at 50°C for 40 seconds. Elongation had taken place at 72°C for 60 sec; this was repeated for 35 cycles; and final extension was at 72°C for 5 min (227).

3.6.4 PCR for NDM-1 gene:

PCR amplification of *bla* $_{NDM-1}$ gene was carried out in the reaction mixture containing 20 µl total volume of reaction mixture contacting 2 µl of DNA extract, 3 µl of each primer, 10 µl master mix and rest was PCR water. Primers for this gene were 5′-ATTAGCCGCTGCATTGAT-3′ (F) and for reverse primer the sequence was 5′-CATGTCGAGATAGGAAGTG-3′(R). Thermocycler reaction parameters were initial denaturation at 94°C for 5 min then denaturation at 95°C for 30 sec followed by annealing at 58°C for 30 seconds. Elongation has taken place at 72°C for 30 sec; this was repeated for 35 cycles; and final extension was at 72°C for 10 min (228).

3.6.5 Gel electrophoresis for Identification of PCR product:

After PCR product were analyzed by electrophoresis using 1 % (w/v) agarose gel in TBE buffer. For that 2% Agarose Gel (Sigma) was prepared by melting 2g agarose in 100mL of 0.5X TBE buffer. 10µL of PCR products were resolved in 2% gel along with 1kb DNA marker (Fermentas) for 45min at 110V in a horizontal gel electrophoresis apparatus.

As these DNA will be sent for sequencing so DNA was extracted from the gel by Qiagen gel extraction kit.

3.7 Sequencing:

3.7.1 DNA extraction from gel:

Qiagen Gel Extraction kit was used for the DNA extraction from the gel. This protocol was used for DNA purification and extraction up to the size of 70 bp to 10 kb from standard agarose gels in TBE or TAE buffer. Per spin column 400mg agarose can be processed. Adsorption of DNA occurs at pH ≤7.5 and yellow color of Buffer QG was an indicator of that pH, and all steps were carried out at 13,000 rpm at room temperature, these points were considered to carry out the procedure.

3.7.1.1 QIAquick Gel Extraction Kit Protocol:

i. Processing of DNA gel

- DNA fragment was excised from the agarose gel with the help of scalpel.
- Gel was weighed in a clear, colorless tube then QG buffer was added to the 3 volume to the 1 volume of the gel. If gel was 100mg then 300µl of QG Buffer was added.
- This mixture was incubated for 10 minutes at 50°C so that the gel slice would be melted. During incubation this mixture was vortexed for 2-3 minutes to completely dissolve the gel.

ii. Binding of DNA

- Then 1 gel volume of isopropanol was added to this mixture and mixed it without centrifugation.
- QIAquick spin column was placed in 2ml collection tube.
- Then sample was applied to QIAquick column and centrifuged for 1 minute. This step is necessary for the binding of DNA to the column membrane. The maximum volume of the column was 800µl if more than this limit sample was used than it was loaded again and then was spin.

iii. Washing

- Flow-through was discarded and QIAquick column was placed back in the same collection tube.
- Then 0.5ml of QG Buffer was added to QIAquick column and centrifuged for 1 minute. This step removed all the traces of agarose attached to the sample.
- For washing 0.75ml of PE Buffer was added to the QIAquick column and centrifuged for 1 minute.
- Flow-through was discarded and QIAquick column was centrifuged for additional 1 minute to remove all the residual ethanol from PE Buffer. This additional centrifugation was also carried out at 13,000 rpm.

iv. Elution

- QIAquick column was placed in 1.5 ml tube.
- 50µl of EB Buffer was added to the center of QIAquick membrane and centrifuged the column for 1 minute. Water at pH (7.0–8.5) could also be used for this step. As Elution efficiency depends upon pH, maximum elution efficiency could be achieved between pH 7.0 and 8.5. When using water, it was made sure that the pH value was within this range.
- DNA was stored at –20°C as DNA could be degraded in the absence of a buffering agent.

After all this process these DNAs were sent for sequencing.

3.7.2 Compilation of sequencing data:

Sequencing was done at Cardriff University, UK. Then strain sequences were analyzed and particular sequence type was assigned to them.

3.8 Phage assay:

To check the effect of bacteriophage on the strains, phage assay was done in which virulent phages were identified. Agar overlay method was used. The purpose of the phage assay was to grow isolated plaques of particular phage particles on a bacterial lawn.

Phage assay consist of two steps: phage enrichment and double layer agar overlay plaque assay

First enrichment experiment was done which include addition of 10ml of supernatant from processed samples and 10ml of LB broth containing exponential phase *E. coli* strain (OD, 600mm). These test tubes were incubated at 37°C for 24 hours to allow phage enrichment. After incubation bacteria were centrifuged at 11,000 rpm for 20 minutes at 4°C. The supernatant obtained was filtered through 0.22µm cellulose acetate syringe filter and stored at 4°C.

To check the lytic activity of the phages, double agar overlay assay was carried out. Soft agar was prepared by making the amount of agar half in the media. Bacteriophage dilutions were made in LB broth. Then 100µl of dilution of filtrate was added to the 50µl of *E. coli* culture grown overnight. Then this mixture was incubated for 20 minutes to allow phage adsorption. Then 3 ml of the soft LB agar was added to it. This test tube was rolled in the hand then this mixture was added on the hard agar plates prepared before. Soft agar was allowed to settle for 2-3 minutes then this plate was

inverted and incubated at 37°C for 24 hours. Numbers of plaques were counted afterwards.

Chapter 04

Results

Results:

Present study was conducted to assess the variation in sequence types of *E. coli* moderated by bacteriophages in environmental sewage samples from city of Islamabad.

4.1 Study Sampling sites:

A total of 8 sewage sample were taken from the sector G area of Islamabad during April to June, 2015. Approximately 30 ml of total sample was taken from these areas which also contained some amount of sediments in it. Sampling sites comprised of residential as well as commercial areas. There was a difference in the sewage samples taken from only residential areas as water sample was clear as compared to the sample collected from areas having commercialized places like hospitals, shops etc. One sample was taken right from the main hole which was the most turbid of all samples. Locations of all the sampling sites are mentioned in Table 2 and Figures 1 and 2 represent some of the actual sites while Figure 3 shows all sites highlighted on an image of google map.

Table 2: Study sampling sites location

Serial Number	Sample No.	Location
1	Sample 5	G-8 Markaz
2	Sample 6	G-8 Residential Area
3	Sample 8	G-9/4 Man hole
4	Sample 9	G-8/2 Residential Area
5	Sample 10	G-7/3 Residential Area
6	Sample 16	G-6 Markkas Residential and Commercial Area
7	Sample 17	G-6/1 Residential and Commercial Area
8	Sample 18	G-7/1 Kachi Abadi

Figure 1: G-6/1

Figure 2: G-7/3 near Residential Area

Figure 3: Sampling sites on the map

4.2 Isolation and Identification of *E. coli* strains:

The *E. coli* isolates were identified by colony morphology and their ability to ferment lactose on MacConkey agar, Gram staining and biochemical tests. Colonies on MacConkey showed pink color and were circular elevated and slightly raised as shown in the Figure 4.

Figure 4: Colony Morphology of *E coli* colonies on the MacConkey Agar plate

4.3 Colony forming units counts (CFU/ml):

After culturing of sewage samples on selective media plates, CFUs were counted which showed the number of bacterial cell grown in the form of colony on the media plate. Plates containing colonies about 30-300 were selected for the CFU count. Culture plates exceeding the counting limit were labeled as too numerous to be counted (TNTC). Three dilutions of samples were made before and on these three dilutions different CFU counts were observed. Highest numbers of CFU were observed as 2.0×10^{4} CFU/ml was observed in sample number 10 at dilution 10^{-2} (Table 3). Samples taken from different location (residential/commercial) areas had shown different number of colonies on the plate as sample 5 taken from residential area (Table 2) had shown large colonies forming units which presented higher number of viable cells present at the site while sample 1 (Table 2) taken from Markaz had shown lowest number of colonies on the media plate (Table 3). These both CFU/ml, were at dilution 10^{-1}. Lactose fermenters were also counted along with the total number of colonies on the plate. Lactose fermenters on 10^{-1} dilution for sample 5 and sample 1 were 5.1×10^{5} and 2.3×10^{3} respectively. These results are presented in detailed form in Table 3.

Table 3: Colony Forming Units of the samples

		Colony forming units counts of sampless											
		Number of colonies at 10-1 dilution plate		Number of colonies at 10-2 dilution plate		Number of colonies at 10-3 dilution plate		Number of colony forming units at 10-1 dilution plate		Number of colony forming units at 10-2 dilution plate		Number of colony forming units at 10-2 dilution plate	
S.No	Sample No.	Total colonies	Red Colonies	Total colonies	Red Colonies	Total colonies	Red Colonies	Total colonies	Red Colonies	Total colonies	Red Colonies	Total colonies	Red Colonies
1	5	80	13	12	2	3	3	1.6×10^{4}	2.6×10^{3}	2.4×10^{4}	4.0×10^{3}	6.0×10^{4}	6.0×10^{4}
2	6	TNTC	TNTC	132	90	45	30			2.6×10^{5}	1.8×10^{5}	9.0×10^{5}	6.0×10^{5}
3	8	TNTC	TNTC	261	251	239	150			5.2×10^{5}	5.0×10^{5}	4.8×10^{6}	3.0×10^{6}
4	9	TNTC	TNTC	80	74	150	38			1.6×10^{5}	1.5×10^{5}	3.0×10^{6}	7.6×10^{5}
5	10	275	254	100	93	26	25	5.1×10^{4}	5.1×10^{4}	2.0×10^{5}	1.9×10^{5}	5.2×10^{5}	5.0×10^{5}
6	16	135	57	29	22	16	9	2.7×10^{4}	1.1×10^{4}	5.8×10^{4}	4.4×10^{4}	3.2×10^{5}	1.8×10^{5}
7	17	TNTC	TNTC	65	54	91	52			1.3×10^{5}	1.1×10^{5}	1.8×10^{6}	1.8×10^{6}
8	18	105	64	11	6	3	3	2.1×10^{4}	1.3×10^{4}	2.2×10^{4}	1.2×10^{4}	6.0×10^{4}	1.8×10^{7}
9	19	91	54	11	5	5	4	1.8×10^{4}	1.1×10^{4}	2.2×10^{4}	1.0×10^{4}	1.0×10^{5}	1.8×10^{8}

4.4 Biochemical identification:

Biochemical tests were carried out to confirm the isolates as *E. coli* and results are as following:

4.4.1 Citrate utilization test:

Citrate utilization test results were negative for all the isolates as *E. coli* do not utilize citrate so color of Simon citrate media did not change from forest green to blue.

4.4.2 Triple Sugar Iron (TSI) test:

Both slant and butt were acidic for *E. coli*. Gas production was also been seen as breakage of agar in the slant. Acidic butt and slant (yellow butt, yellow slant) showed that lactose and/or sucrose had been fermented.

4.4.3 Indole test:

Reddish brown ring was formed by all the isolates which confirmed the Gram negative *E. coli* presence (Figure 5).

Figure 5: Positive Indole Test with ring

4.5 Molecular analysis:

4.5.1 Molecular identification of *fum*C gene:

PCR was performed for detection of *fum*C gene in each DNA sample. All 54 strains were found positive for *fum*C gene as it was detected in all isolated DNAs (Figure 6).

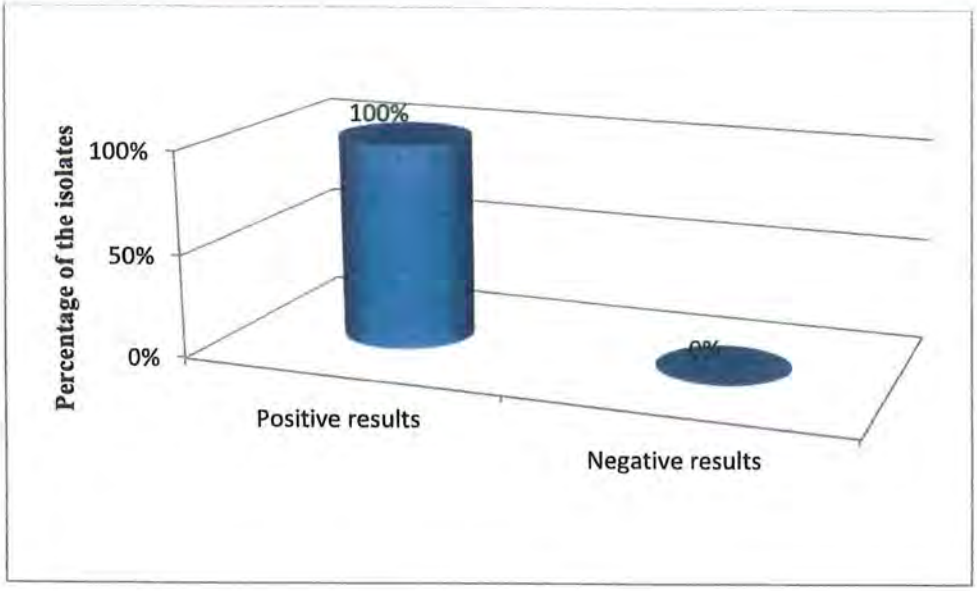

Figure 6: Percentage of *fum*C gene in the all isolates. Entire isolates showed 100% positive results for *fum*C gene.

4.5.2 Molecular identification of *fim*H gene:

PCR was performed for detection of *fim*H gene in each DNA sample. *fim*H gene was detected in 49 out of 54 isolates, which showed its prevalence among the isolates to be 90.7 % (Figure 7).

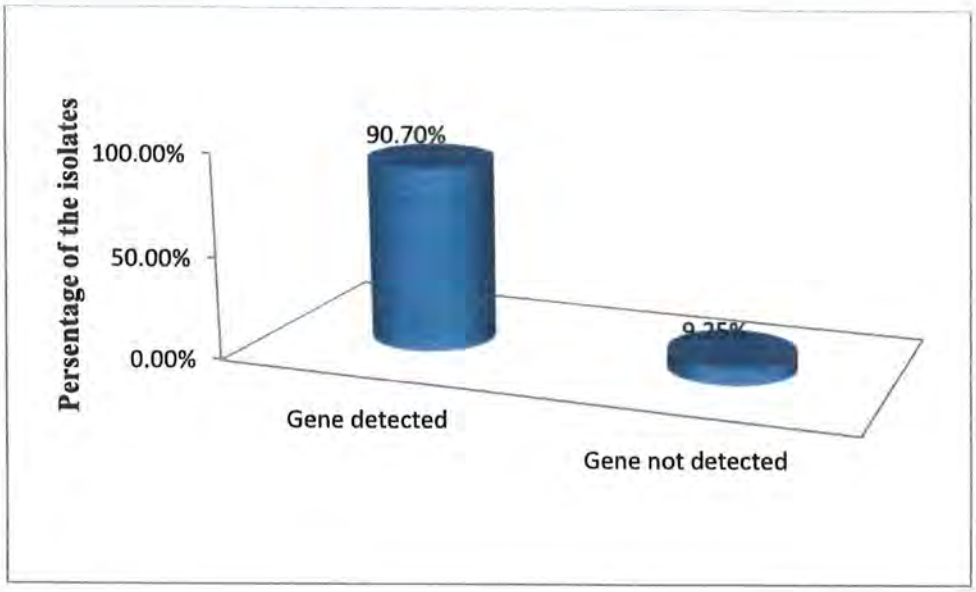

Figure 7: Presence of *fim*H gene in isolates of *E. coli*. Out of 54 isolates 49 were being detected to have *fim*H gene while 5 strains were negative for this gene. 90.7% were positive while rest of the isolates had shown negative result

4.6 Detection of antibiotic resistance genes:

4.6.1 Molecular detection of bla_{NDM-1} gene:

All the isolates were tested for the presence of bla_{NDM-1} gene by polymerase chain reaction using specific primers (Table 1). The amplified products were run on the 2% agarose gel. All the isolates were negative for bla_{NDM-1} gene, which could be depicted in Figure 8.

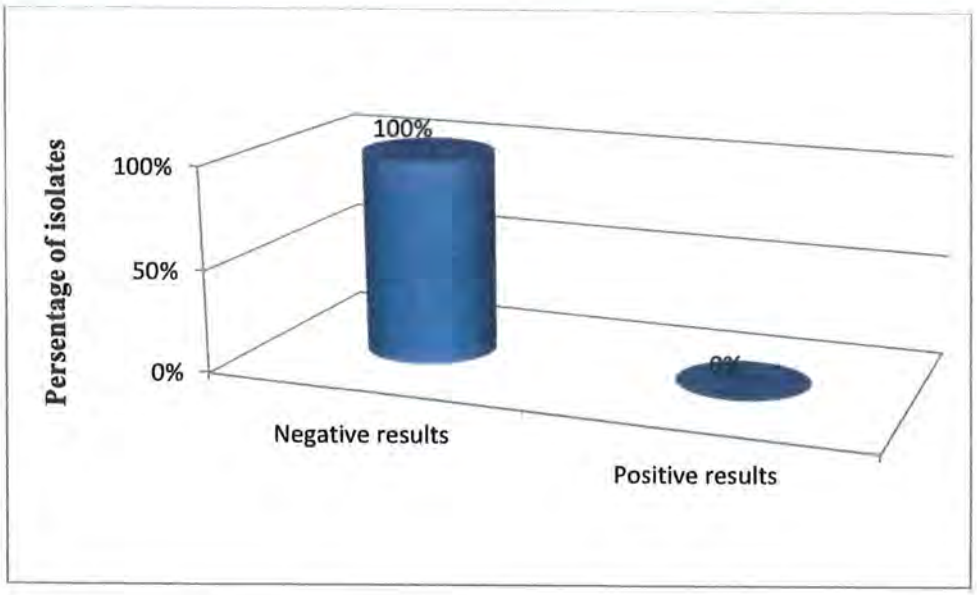

Figure 8: The presence of NDM-1 gene in isolates of *E. coli*. It shows 100% negative results for $bla_{\text{NDM-1}}$ gene.

4.6.2 Molecular characterization of $bla_{\text{CTX-M-15}}$ gene:

For the detection of CTX-M-15 gene, PCR was done and results were out of 54 isolates, 18 were positive for the CTX-M-15 gene while 36 were negative (Table 4). In terms of percentages these results could be stated as 33.3% were positive while 66.6% were the negative (Figure 9).

Table 4: Presence of CTX-M-15 gene in the *E. coli*

Serial No.	Presence of CTX- M-15 gene	Results	Percentage of CTX-M-15 gene isolates
1	18	Positive	33.3%
2	36	Negative	66.6%

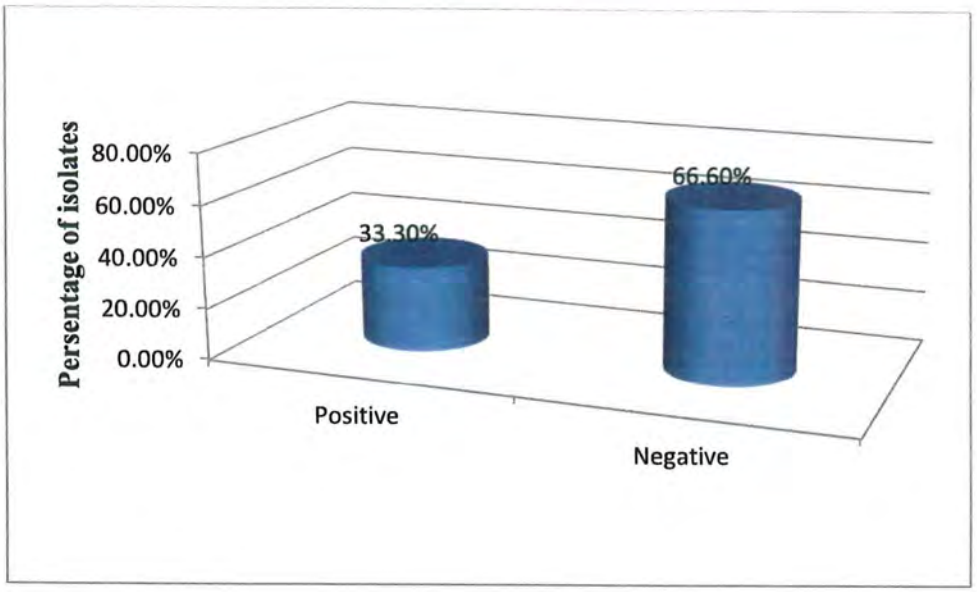

Figure 9: Percentage results for the presence of the antibiotic resistant gene CTX-M-15 in all isolates.

Figure 10: A representative gel of *bla*$_{\text{CTX-M-15}}$ gene PCR. Lane 1-7 and 9-15 shows samples results while Lane 8 shows 100bp DNA Ladder (M) (vivantis). Lanes 1, 5,7,10 and 15 show the positive results for the presence of gene.

4.7 Two locus tying

For determining the sequence types of *E. coli* isolates, the *fum*C and *fim*H genes were sequenced and their different alleles were assigned to the isolates.

4.7.1 *fim*H Allelic variability:

Out of 54 strains, 21 were assigned different allele types following the *fim*H sequence database. The prominent allele type was 54 found in eight isolates thus having highest percentage. Some of the allele types were in only one strain. These allelic types were 65, 61, 41, and 30. Their percentages were 4.76% individually. Allele types 27, 32 and 34 appeared twice and their percentages were 9.52% individually. Appearance of single allelic number implied the abundance of that allele in the isolates sample (Table 5).

Table 5: Variability in *fim*H allele type in isolates

Serial Number	*fim*H allele Type	Number of isolates carrying the particular allele	Percentage of particular allele in total isolates
1	27	2	9.52%
2	30	1	4.76%
3	32	2	9.52%
4	34	2	9.52%
5	41	1	4.76%
6	54	8	38%
7	61	1	4.76%
8	65	1	4.76%

4.7.2 *fum*C Allele variability:

All strains were positive for *fum*C allele and different allele types were found after sequencing. Allele type 11 appeared more frequently and it was found in 15 strains and its percentage among all 54 strains was 27.77%. Allele type 4 was detected in 11 strains and its percentage was 20.37%. Twenty-nine allele type appeared in three strains and its percentage among all was 5.55%. *fum*C allele 35 was assigned to 10 strain and its percentage was 18.51% among all isolates. In 5 isolates *fum*C allele 7 was found and its percentage was 9.25%. Some allele types were present in one isolate only and their percentages were 1.85% respectively. These were allele types 23, 36, 88 and 636. Allele types 6, 26 and 65 appeared in two strains each and its percentages were 3.70% among all 54 strains (Table 6).

Table 6: Variability in *fum*C allele type in isolates

Serial Number	*fum*C allele Type	Number of isolates carrying the particular allele	Percentage of particular allele in total isolates
1	4	11	20.37%
2	6	2	3.70%
3	7	5	9.25%
4	11	15	27.77%
5	23	1	1.85%
6	26	2	3.70%
7	29	3	5.55%
8	35	10	18.51%
9	36	1	1.85%
10	65	2	3.70%
11	88	1	1.85%
12	636	1	1.85%

4.8 Phage assay result:

Phage assay was done for three *E. coli* strains which had shown maximum activity against pooled phage supernatant.

Different numbers of plaques were observed in three tested strains (Table 7). These selected strains had shown maximum number of plaques against multiple sites like strain 17 showed 8 plaques against site 9, 2 plaques against site 10 and 35 plaques against site 17. Similarly strain No. 22 showed 56 plaques against site 17. Strain 62 showed 1 plaque against site number 9 and it was hazy as well but it showed 64 plaques against site 17 which was maximum number of plaques in all the strains. Clear, round and transparent plaques were observed against strain 17 while other plaques were hazy in appearance (Table 7).

Table 7: Phage assay results for different sites

Serial No.	Strain Number	Sites	Number of plagues
1	17	9	8
		10	2
		17	35
2	22	17	56(hazy)
3	62	9	1(hazy)
		17	64(hazy)

4.9 Sequence type determination:

On the basis of molecular identification and sequencing of *fum*C and *fim*H genes different sequence types of *E. coli* were determined. The predominant type was sequence type 88. The types of many of the isolates could not be finalized due to higher variability in the allele types of both genes studied. It requires the sequencing of more candidate genes in order to give a clear result.

Chapter 05

Discussion

5. Discussion

To study the variation in the sequence types of *E. coli* in the environmental samples and how bacteriophages moderate their different sequence types, *E. coli* from the sewage samples were taken and study was carried out which involved the growth of isolates on differential media containing antibiotic.

MacConkey agar has been used for differentiation and selection in a number of studies. Adams and Moss in 2000, isolated *E. coli* from human and animal feces reported smooth pink colonies on the solid MacConkey agar indicating lactose fermentation. It is important to understand the changes in the genomic structure of *E. coli* strains present in the environment as significant changes keep occurring from time to time. A study showed a significant increase in the antibiotic resistance against 11 to 15 antimicrobial agents in *E. coli* strains isolated from animals (229). In order to understand the diversity of *E. coli* strains in this study, two locus sequence typing was done which has been done by number of researchers (230, 231), since its introduction in 2012.

The strategy based on the two locus sequence typing involved sequencing of *fum*C and *fim*H genes. In the present study *fum*C gene was present in all strains as *fum*C is the housekeeping loci commonly used for MLST (173) and was expected to be present in all *E. coli* isolates. Variation in this gene is considered to be the neutral nature because of conservation of encoded protein zone (232). Allelic variations exist in *fum*C gene and different alleles were found in the study isolates where most common was *fum*C allele 4 present in the sequence type 88.

The second gene tested in two locus typing was *fim*H which encodes the type 1 fimbrial adhesion, and is under positive selection for functional mutations so useful for the successful survival of the bacterial strain (185). In the same study it was reported that presence of *fim*H gene was only associated with the pathogenic stains rather than commensal strains so in the present study *fim*H gene was absent in 5 isolates but it was present in 49 out of 54 total strains which showed that its prevalence was very high

(95%) (Figure7), which could imply that these strains could be potential pathogenic strains.

In the present study a great allelic diversity in the *fim*H gene was observed (Table 5). Similar kind of diversity in the strains has also been reported by Steen, Stahlhut et al. in 2009, but it was reported with *K. pneumoniae* isolates obtained from different sources (241). Another study by Weissman ,et al. in 2006, has also reported similar results in the clinical isolates where they observed a notable level of allelic diversity in *fim*H gene (188). According to different studies (233, 234), the genetic diversity of *fim*H could be used in typing applications. It has been reported that this variability of *fim*H provides virulence to extraintestinal *E. coli* strains (235). In the present study a lot of genetic variability among the *fim*H alleles has been observed.

High diversity among the isolates demonstrates the genetic variability in the bacterial strains as Table 5 shows the percentages of allelic variation among the strains in the current study where *fim*H allele 30 has also been seen in only one strain having its prevalence of about 4.26% (Table 5) but Johnson, Tchesnokova et al. in 2013, has observed allelic diversity in *E. coli* ST131 with *fim*H allele 30 in 67% of isolates among all the other *fim*H variants (89). The difference in both results could be because of different sequence types reported in both studies.

The global increase of *E. coli* producing CTX-M β-lactamases, mainly CTX-M-15 has chief public health implications (15). So detection of this gene was carried out in the studied isolates where 18 (33.3%) were CTX-M positive (Table 4). Similar study has been conducted by Sowmiya, Malathi et al. in 2012, in which they reported the presence of CTX-M-15 in 14%, along with other resistant genes but $bla_{\text{NDM-1}}$ was absent among all their isolates (245). While in present study all isolates were negative for the NDM-1 gene which shows that most prevalent resistant gene was CTX-M-15 in the strains in present study. The possible reason for the higher percentage of CTX-M-15 in the present

study could be that the samples were collected from the environment and this gene is highly prevalent in the environment.

In the present study detection of CTX-M-15 gene in the strains collected from the environment corresponds with the results of Yan-Yan Hu, Jia-Chang Cai et al. in 2013, who reported for the first time the presence of CTX-M-producing *E. coli* isolates collected from water, swine, and healthy and hospitalized humans, suggesting that pathogens in the environment might originate both from human and animal sources (166). According to them $bla_{\text{CTX-M-15}}$ and $bla_{\text{CTX-M-55}}$ were the dominant CTX-M-1 group genes present in the sample just like the predominance of CTX-M-15 gene in the current research (Table 4). Their reported sequence types were ST131, ST648, and ST38 while the reported sequence type in the present study was ST88. Difference in the reported sequence types could be attributed to the fact that study areas for both the researches were different so environment was different.

As there were many variations in the allele types of two loci, the sequence type of only few isolates could be deciphered. The one sequence type was sequence type 88 which has been reported in literature. In one study in 2008, 11 hospital-acquired or health care-associated infections were of this type, with in most cases prior antibiotic administration (236). Ortega et al. in 2012, has also reported ST88 previously in association with c-AmpC production in a French hospital (247). On the University College of Cork (Ireland) database, 23 ST88 *E. coli* isolates are registered that were recovered from infections of humans and domestic animals (http://mlst.ucc.ie/mlst/dbs/*Ecoli*; data last accessed on 20 July 2011). Previously these strains were recovered from the urine but in the present study this sequence type has been detected in the sewage samples collected from the environment.

In all the sequence type 88 strains, *fumC* allele is constant which also complies with the literature of the fact that housekeeping gene has less variability and low number of the mutant as compare to the other alleles (173).

Along with the sequence types, effect of phage population was also tested to study the notion that phages could moderate the bacterial population of a specific site. Phages were isolated as indicated in Table 7. Similar isolation was done by Ackermann and Nguyen, 1983, in which *E. coli* phages were isolated from the sewage samples containing fecal content of human and animal source (248). Likewise number of studies have reported the isolation of phages from fecal and environmental samples (237, 238).

Phages can kill the population of bacterial strains as it is indicated in the form of appearance of plaques on the plate treated with the phage suspension. There is the difference in the number of plaques in different samples (Table 7). The difference in the number of plaques obtained from the sewage water of different sources may be due to the fact that the area yielding more PFU contents might be having bacterial host in greater number as compared to the others. The presence of host cells in greater number may be attributed to the presence of more organic matter in the sewage. These results are comparable with those observed by Dhillon et al. in 1970, who investigated sewage from urban and rural areas (251).

These phages have shown the killing ability against the bacterial strains present in the samples. These results could be compared to all the experiment which showed the isolation and presence of lytic phages from the sample. Bibi, Abbas et al. in 2016, recently conducted a study in which lytic bacteriophage termed as P.E1 has been characterized by them to check its activity against multi drug resistant *E. coli* clinical isolates (230). This phage has been isolated from sewage water supply which further strengthens the findings of the present study about the controlling nature of the phages present at the particular site.

There is a need to do more research in this area so the lytic capacity of these phages could be utilized in phage therapy and also these phages could be used to check the diversity of bacterial population at the particular site.

Conclusion

6. Conclusion

Few strains are involved in the spread of antibiotic resistance but reason for this phenomenon is poorly understood. In the present research it has been shown that bacteriophages could control the sequence types of *E. coli* which is associated with antibiotic resistance. It will be helpful to understand the linkage between the individual sequence types with antibiotic resistance and also it has been proposed that bacteriophages could be applied to reduce these particular sequence types in the environment. So the following conclusions can be drawn:

- There is number of different sequence types of *E. coli* present in the environment.
- These sequence types have different allelic profiles and they also carry the antibiotic resistant gene like CTX-M-15.
- These bacterial strains could be considered as the potential reservoir of spread of antibiotic resistance gene in the environment.

Future Aspects

Future Aspects

The present project can be carried on in different directions such as:

- Determination of sequence types of *E. coli* at different locations of Islamabad city
- Determination of other families of antibiotic resistance genes in all the samples
- Sequencing of these gene to find out the differences at the allelic level

Chapter 07

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7. References

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