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BACTERIOPHAGE ISOLATED FROM THE ENVIRONMENT AND *E. COLI* OF MEAT ORIGIN AS A RESERVOIR OF ANTIBIOTIC RESISTANCE

by

MANISHA POLUR

THESIS

Submitted to the Graduate School

of Wayne State University,

Detroit, Michigan

in partial fulfillment of the requirements

for the degree of

MASTER OF SCIENCE

2014

MAJOR: NUTRITION AND FOOD SCIENCE

Approved by:

_____

Advisor Date

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DEDICATION

I would like to solely dedicate my Masters Thesis to my mother, Mrs. Polur Vijayakumari and my dear sister, Mrs. Honey Sompalli who have been the most wonderful guide in inspiring me to reach great heights. The moral support which my mom has given cannot be expressed through words. She has been there at my hard and happy times. I am very thankful to my sister for all the values that she has inculcated in me for the person I am today.

I would also like to dedicate this project to my guide Dr. Yifan Zhang, who has extended her support throughout my course of study. I am very grateful to her for letting me be a part of her lab and complete my research under her guidance.

ACKNOWLEDGEMENTS

"Teachers are those who use themselves as bridges over which they invite their students to cross, then having facilitated their crossing, joyfully encourage them to create bridges of their own." -- Nikos Kazantzakis

True to the above lines, my project mentor, Dr. Yifan Zhang, Assistant Professor, Department of Nutrition and Food Science, Wayne State University, has encouraged, guided and supported me from the initial to the final level, thus enabling me to develop an understanding of the subject. I am very grateful to have Dr. Kai-Lin-Catherine Jen, Professor and Dr. Kequan Zhou, Assistant Professor, Department of Nutrition and Food Science, Wayne State University for accepting to serve as the Committee members. I thank them for their valuable comments and feedbacks which has encouraged me to improve greatly.

I owe my deepest gratitude to Dr. Ahmed Heydari, Interim Chair, Department of Nutrition and Food Science and Dr. Pramod Khosla, Associate Professor, Department of Nutrition and Food Science for their enduring assistance and cooperation throughout my Masters.

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I would not have been able to finish my project successfully without the enduring support of my family , my brother Mr. Ashwin Kumar who has constantly encouraged me and appreciated me. This dissertation would not have been possible without my dear friends Ms. Sankara Gomathi, Ms. Sanjana Prabhakar , Ms. Poorva Divekar and Mr. Sumanth Dadam who had helped me immensely when I was in need and all my dear friends who had been there when I needed them.I cherish the help and support of my colleague, Mrs. Gayathri Gunathilaka, Ms. Mingyang Huang, Ms. Vasiana Tomco, Mr. Varun Tahlan and Mr. Ahmed.

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CHAPTER-1

INTRODUCTION

The continuous use of antimicrobial agents in the environment leads to the selection pressures which ultimately results in the survival of antibiotic-resistant bacteria. The routine practice of feeding the livestock with antimicrobial agents in order to treat infections and promote growth, results in the emergence of antibiotic-resistant bacteria. These resistant bacteria are then transferred from animals to humans through the food chain [1]. The wide usage of antibiotics in human and veterinary medicine has allowed the dissemination of antibiotic-resistant bacteria to the environment.

The severity of lateral gene transfer in bacterial evolution was not discovered until the 1950s when the multidrug resistance species emerged on a global scale. The multidrug resistance in *E. coli* has increased from 7.2% during the 1970's to 63% in 2000's. This broad increase in resistance is attributed to the introduction of new antimicrobial agents in the clinical medicine[2].

Bacteria and viruses play a significant role in causing diseases to humans. An important stage of bacteria evolution is the lateral gene transfer. Through the lateral gene transfer mechanism, the bacteria get adapted to the environment by acquiring genes from other organisms such as viruses. Horizontal gene transfer can take place through three ways: Transformation, Transduction and Conjugation.

Transformation:

Transformation in *E. coli* is one the ways through which extraneous genetic material is introduced into a bacterial cell resulting in the genetic alteration of the recipient cells. This

process takes place naturally when the bacterial cells undergo a stress due to starvation or high cell density [3]. This process of transformation may occur between bacterial cells or from a bacterial to a non-bacterial cell such as animal or plant cells. Transformation can also be artificially induced by the temperature shock treatment of the recipient cells by exposing them to cold conditions in the presence of Calcium or magnesium ions and subsequently to hot conditions [4].

Conjugation:

Conjugation in bacterial cells involves the process of transferring genetic material between two cells through a bridge like connection between two cells or when they come into contact with each other such as in the process of mating or sexual reproduction. The donor cells transfer their plasmid containing the genes into the recipient cells which usually lack the plasmid. These genes are then integrated into the recipient's chromosome and confer beneficial properties like the antibiotic resistance to the bacteria [5].

Transduction:

Transduction is another means of horizontal gene transfer where the genetic material is exchanged within bacterial cells through a virus as a vector. These viruses are called phages. This process does not need a cell to cell contact by the bacterial cells as in the process of conjugation. This process is one of the tools used for introducing a foreign genome into a host cell's genome [6]. The process of transduction may occur through either the Lytic cycle or the Lysogenic cycle.

Lysogenic Cycle:

In the lysogenic cycle the bacteriophages undergo a temperate viral replication by infecting the host cell and integrating into the host genome. The bacterium will continue to live without being destructed. The integrated phages, now called a prophage will be transferred to the daughter bacteria cells on each subsequent cell division. Later, due to environmental stress such as exposure to UV radiation or antibiotics, the phages replicate into many virions and cause proliferation of the new phages through the lytic cycle [7]. There is no production of phage progenies in the lysogenic cycle. A lysogenic cycle is converted to lytic cycle by some physical or chemical agents. A collection of the phage progenies is referred to as the lysate. A lysogenic bacteria becomes immune to subsequent infection as a result of the prophages.

Lytic Cycle:

In this stage of viral production, the infection with the phage will destroy the bacteria genome and will result in the lysis of the bacteria cells. In the lytic cycle, the phages exist as separate molecule in the host. They replicate using the host DNA machinery separately from the bacterial DNA. These phages are called virulent viruses which can multiply only on bacterial cells, use the bacterial cell mechanisms to assemble components and at the end of the cycle, lyse the bacterial cells and kills the host bacteria [8]. These phages might then move on to infect the other bacterial cells and indulge in the lysogenic cycle. Hence, this process continues and thus the spread of antibiotic resistance among the organisms occurs in the environment. Studies indicate that lytic cycle is mostly prevalent in an environment with high host abundance and activity [9].

The process of transduction was primarily studied by Zinder and Lederburg [10] in Salmonella, to show that the phage particles act as vectors for the transfer of genetic material in bacteria. Transduction could be seen when the phage sensitive bacterial indicator strains are infected with phages grown on one or more of the bacterial donor strains which differ from the indicator by a few genetic characters. Some of the bacteria which survive, transfer the genetic traits to their progeny from the donor strains. This transducibility is limited to only certain strains of phages [11].

Foreign genetic material is introduced into the bacterium via viruses that have replicated in the bacteria and have packaged DNA fragments into the bacterial chromosome [12]. Phage particles are the most abundant biological entities in the world [13]. They outnumber the bacterial cells in the open ocean by a factor of ten [14]. It is previously said that considering the phage transduction frequency to be 10^{-8} plaque forming units, then each phage multiplies at the rate of 20 million billion times per second in the oceans [15]. Having such a high multiplication frequency, phages could be considered as the very potent mediators of Horizontal gene transfer occurring in the environment.

E. coli as the host bacterium:

Escherichia coli is a commensal bacterium of humans and animals. Most types of *E. coli* are harmless commensals present in the intestinal region of humans and animals whereas some intestinal and extra intestinal genetic variants cause infections such as gastroenteritis, peritonia, meningitis and septicemia [16]. Some infections might be treated by using antimicrobial drugs, whereas some cannot be treated by the drugs. *E. coli* is one of the organisms that show a high

resistance to a wide range of antimicrobial agents that have been used since a long time in human and veterinary medicine [17].

Retail poultry products are a major source of antibiotic resistant *E. coli*. During slaughter, the resistant strains from the gut are readily passed on to the carcass and thereby the resultant meats are often contaminated with the resistant strains. Other meat samples such pork chops and ground beef also carry resistant strains of bacteria [18]. The various *E. coli* outbreaks are associated with the meat products particularly of bovine origin [19].

Bacteriophages:

Bacteriophages (or phages) are bacterial viruses that invade the bacteria cells. The history of the discovery of bacteriophages is dated back to 1896 when a British bacteriologist observed an unknown source in the waters of the rivers Ganges and Jamuna in India which led to limiting the spread of cholera epidemics [20]. Phages are obligate intracellular parasites that are host-specific and lack their own metabolism. They have a protective protein shell (capsid) which encases the densely compacted genetic material (DNA). The double-stranded DNA might contain few genes to several thousand genes. These remarkable organisms have an additional feature called the tail and its associated fibers which aid as DNA transfer devices. They replicate in the bacteria, either by disrupting the bacterial metabolism and lysing the cells or integrating in their host genome to multiply and form various progeny [21].

A latent form of bacteriophage called a prophage, can be present in the bacterial cell either in the integrated form or as an extra chromosomal plasmid. These phages exist as separate viral genomes without causing the disruption of the bacterial cell. These bacterial cells under environmental stress such as exposure to antibiotics and ultra-violet radiations, release the

phages in a process called prophage induction. These prophages then undergo the lytic cycle, where they harness the bacterial genomic machinery and replicate to produce many phage progeny by disrupting the bacterial cells resulting in the appearance of clear regions or plaques. Hence in this process of transduction, they transfer the genetic material acquired from the donor strains to the indicator strains [22]. Thereby, these phages play an important role in the process of Horizontal gene transfer (HGT), by transferring antibiotic resistance genes to the bacterial strains and resulting in the emergence of new resistant strains. A bacterium with the prophage may possess antibiotic resistance genes, virulence factors and the pathogenecity to cause the disease [23].

Antibiotic Resistance:

Antibiotic-resistant bacteria are an increasing threat to humans and animals. The accumulation of antibiotic resistance genes on the mobile elements such as plasmids, integrons has led to the point where multidrug resistance phenotypes can be transferred to the other bacterial recipients in a single genetic event. The bacteria exhibit many common resistance mechanisms such as mutation in the antimicrobial target site, change in cell wall permeability to antimicrobials, alteration of the antimicrobial [24]. The antibiotic resistance is acquired by the bacteria either through the process of conjugation where the genes are transferred from bacterium to another by a simple mating process or through virus particles (phages). The resistance genes from one bacterium are packaged into the heads of the phages. On infection, these viral particles inject the resistance traits into the new bacterium through their transfer devices. Thereby the bacterium acquires the resistance traits. These bacteria then pass on these traits to their successive generations either through horizontal or vertical gene transfers. Vertical gene transfers occur when bacterium pass the resistance genes to their successive population

while horizontal gene transfer occurs when the bacteria exchange genetic material with other bacteria.

The resistant strains of bacteria can be passed on even to people through several environmental media such as solid and liquid manure of animals as well as through human excretions. These resistant bacteria may be present even in the feces and intestinal flora of the healthy individuals [25]. Two researchers Feuerpfeil and Stelzer [26] found that 98% of the fecal samples contained antibiotic-resistant *E. coli*. The presence of antibiotic resistant bacteria strains in the sewage, soil, surface water and ground water is of increasing health concern. The gastro intestinal tract of animals is found to contain abundant bacteria that carry beta-lactamases and hence a potential chance of pathogens in humans to take up theses resistance genes [27].

Beta Lactam Resistance in *E. coli*:

Beta lactam antibiotics are bactericidal agents that inhibit the bacterial cell wall synthesis. Resistance to the β-lactam antimicrobials is primarily mediated by the beta lactamases, which either bind or hydrolyze the beta-lactam ring and diminish it before they reach the target [28]. Initially it was discovered that the presence of beta lactamase enzymes in the gram negative bacteria can metabolize the antibiotics such as ampicillin, cephalothin, carbencillin and other related drugs [29]. Since then a large number of Beta lactam antibiotics have been developed against these resistant bacteria through molecular modifications. Based upon several functional and structural characteristic of the enzymes, over 200 beta-lactamases have been widely divided into four groups and eight different sub-groups [30]. The production of beta-lactamases in bacteria is an important mechanism of resistance to beta-lactam antibiotics [31]. Since a very long time, most common beta lactamase genes such as CTX-M, TEM, OXA, SHV, CMY have

been isolated from a majority of *E. coli* isolates [32]. TEM, SHV and CTX-M type beta lactamases belong to the group of extended spectrum of beta lactamases (ESBL) that hydrolyze and become resistant to new beta lactam antibiotics including the expanded spectrum antibiotics [33].

CTX Type Beta Lactamases:

The CTX-M type beta lactamase are classified into 5 subgroups namely CTX-M-1, CTX-M-3, CTX-M-8, CTX-M-9, and CTX-M-25. These groups confer the resistance to cephalosporins such as ceftazidime, ceftriaxone, and cefepime [34]. These enzymes are found primarily in strains of *Salmonella* Enterica and *E. coli* but have also been found in other species of Enterobacteriaceae. Since the early 1990's, Escherichia isolates harboring the CTX-M beta lactamase genes are found to cause many community acquired-infections [35]. The exact molecular mechanism behind these beta lactamases are not known as the molecular surveys were limited to the factors such as species type or specimen type or type of environment [36].

TEM Type Beta Lactamases:

The native TEM's show resistance to some of the most common antibiotic such as ampicillin, penicillin and first generation cephalosporins such as cephalothin. The ampicillin resistance in majority of the *E. coli* bacteria and penicillin resistance in species like *H. influenzae* and *Neisseria gonoorrheae* is attributed to this enzyme [37].

SHV Type Beta Lactamases:

The native SHV-1 beta lactamase enzyme primarily confers resistance to penicillin and first generation cephalosporins. It causes 20% of the plasmid mediated ampicillin resistance in

Klebsiella pneumoniae which acts as the primary reservoir of this enzyme [38]. Specific mutation occurring in this enzyme lead to an extended antibiotic hydrolyzing capability.

OXA Type Beta Lactamases:

These enzymes are able to degrade the oxacillin. In addition, they are able to confer resistance to penicillin, carpabenems, cephalosporins, cloxacillin. methicillin,. OXA enzymes are the rapidly growing group of enzymes due to their structural divergence [39]. These oxacillin-hydrolysing enzymes are frequently observed in *Pseudomonas aeruginosa*.

CMY Type Beta Lactamases:

CMY type beta lactamase have become the most common amp-C type beta lactamase [40]. It belongs to the Extended spectrum beta lactamases (ESBL) and the *E. coli* carrying this enzyme has become the most commonly acquired community pathogen by producing the CTX-M type ESBL's [41]. The infections such as bacterimia and urinary tract infections acquired by the *E. coli* carrying these CMY-type ESBL's are not prone to degradation by penicillins and cephalosporins [42].

This study focuses on the determination of antibiotic resistance genes in the bacteriophages specific to *E. coli* isolated from various meat samples as well as environmental samples. *E. coli* were isolated from chicken breast, ground turkey and pork chops from various states in the U.S. The environmental samples include the waste water samples and soil samples which were collected from the waste water treatment plants, Detroit river and Urban Gardens. Firstly, bacteriophages were isolated from the waste water samples, purified and preserved to obtain a high titer of the phage lysates. Secondly, bacteriophages were induced from *E. coli* isolated from meat sources, propagated in *E. coli* indicator strains and preserved to obtain a high titer of phage

lysate. Finally, phage DNA was isolated and Polymerase Chain Reaction (PCR) was performed to detect the presence of beta lactamase genes such as CTX-M1, TEM-1, CMY-2, OXA-2 and SHV-1 using specific primers. This study was carried out to identify the extent of phage serving as a reservoir of antibiotic resistance.

CHAPTER 2

MATERIALS AND METHODS

Specific Aim 1:

Isolation of bacteriophages specific to E. coli ATCC 13706 and ATCC 23631 from environmental samples

1. Bacterial host strains used:

The two *Escherichia coli* strains ATCC 13706 and ATCC 23631 were used for bacteriophage isolation and propagation [42]. The strain was grown in a Luria Bertani (LB) Broth (Merck). The solid media that the strain was propagated contained (1%w/v) of agar. The strain was then preserved in 40% glycerol-BHI stock in the -80°C.

2. Environmental samples:

A total of ten waste water samples were collected from waste water treatment plants, Belle Isle Yach club, Aquarium and Detroit river (Detroit, Michigan) at various times during the day. A total of 5 soil samples collected from Detroit urban gardens were used in the study.

3. Isolation of bacteriophages from the environmental samples:

Isolation of bacteriophages from water samples:

Bacteriophages were isolated from water samples according to Wall S.K *et al.*,2010 [43].

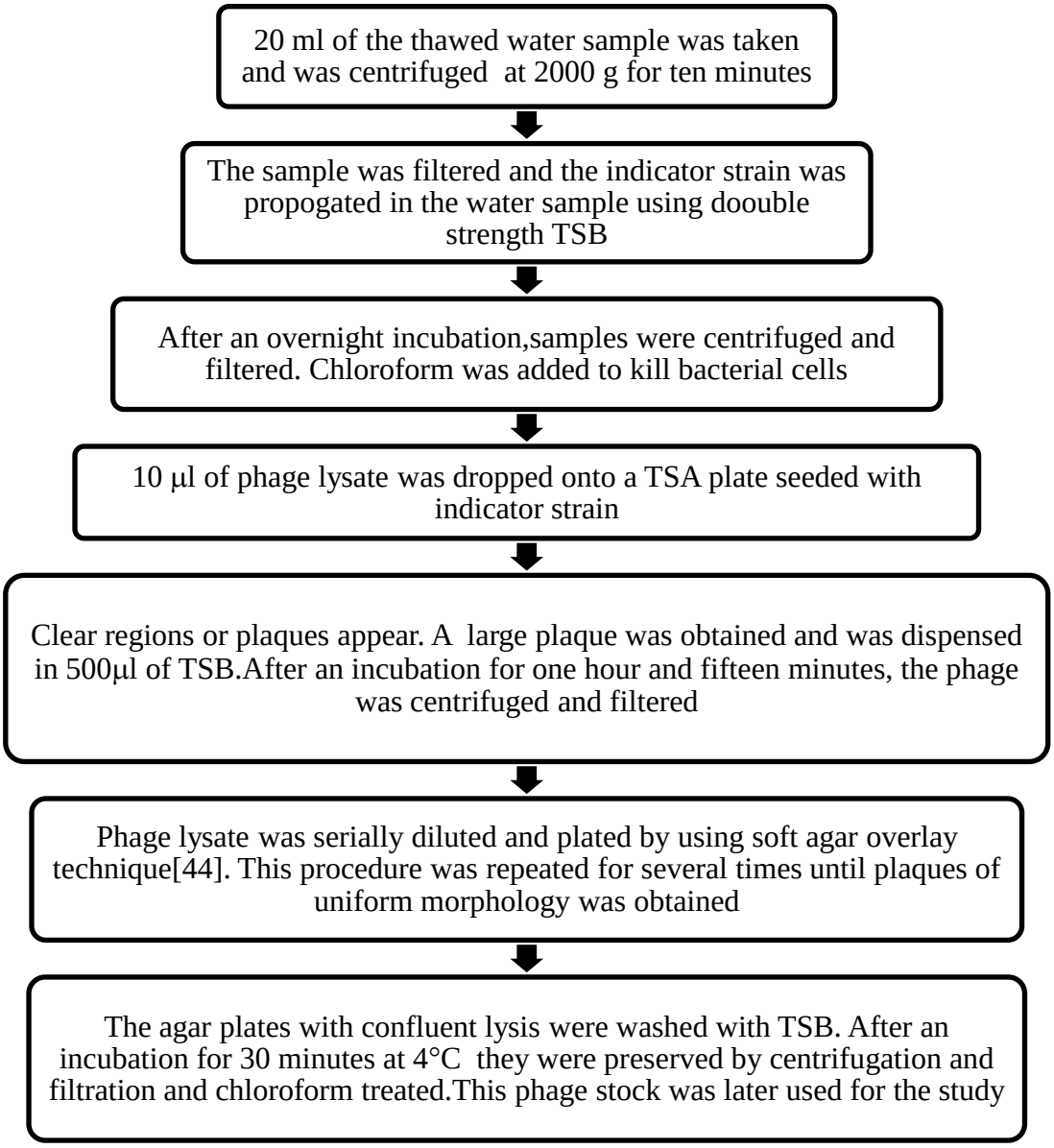

Figure 1: Isolation of bacteriophages from water sample

Isolation of bacteriophages from soil samples:

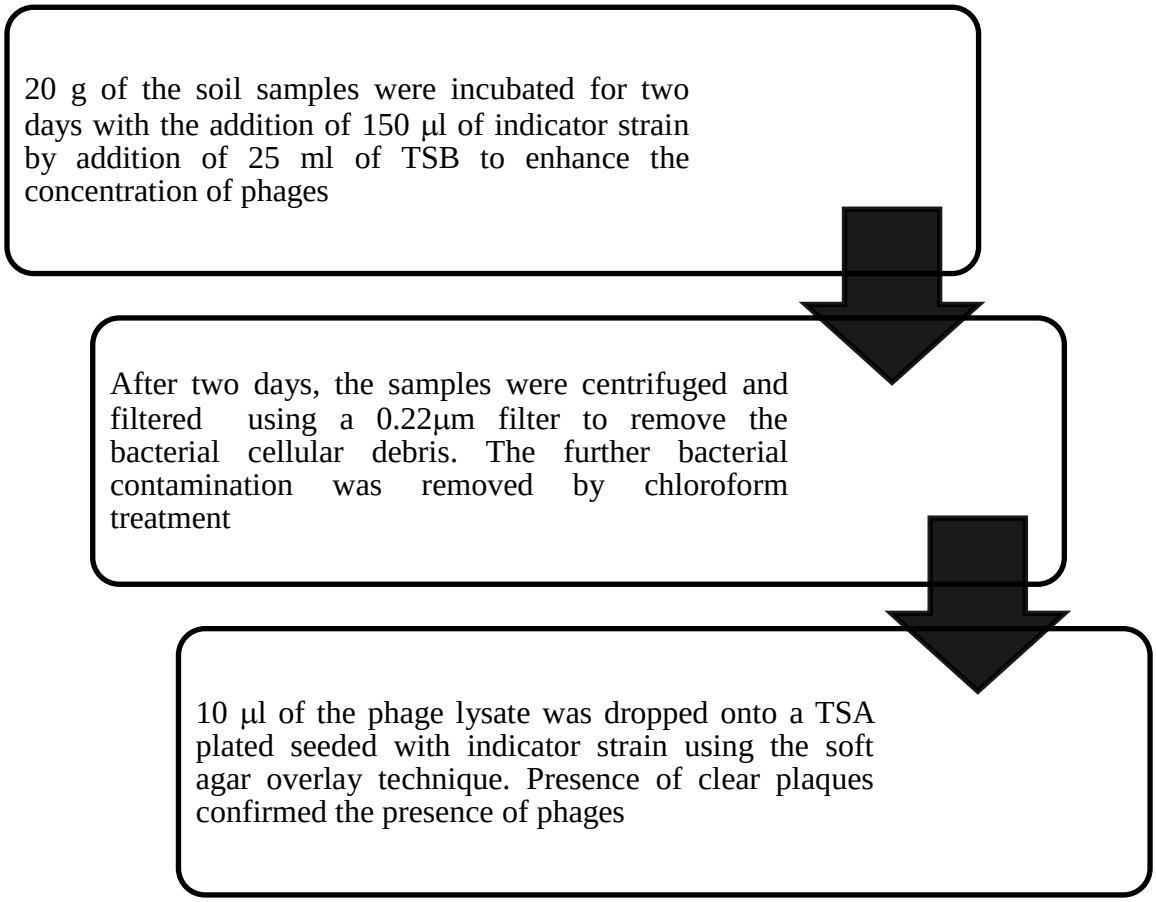

Figure 2: Isolation of bacteriophages from soil samples

Specific Aim 2:

Phage Induction from E. coli of meat origin

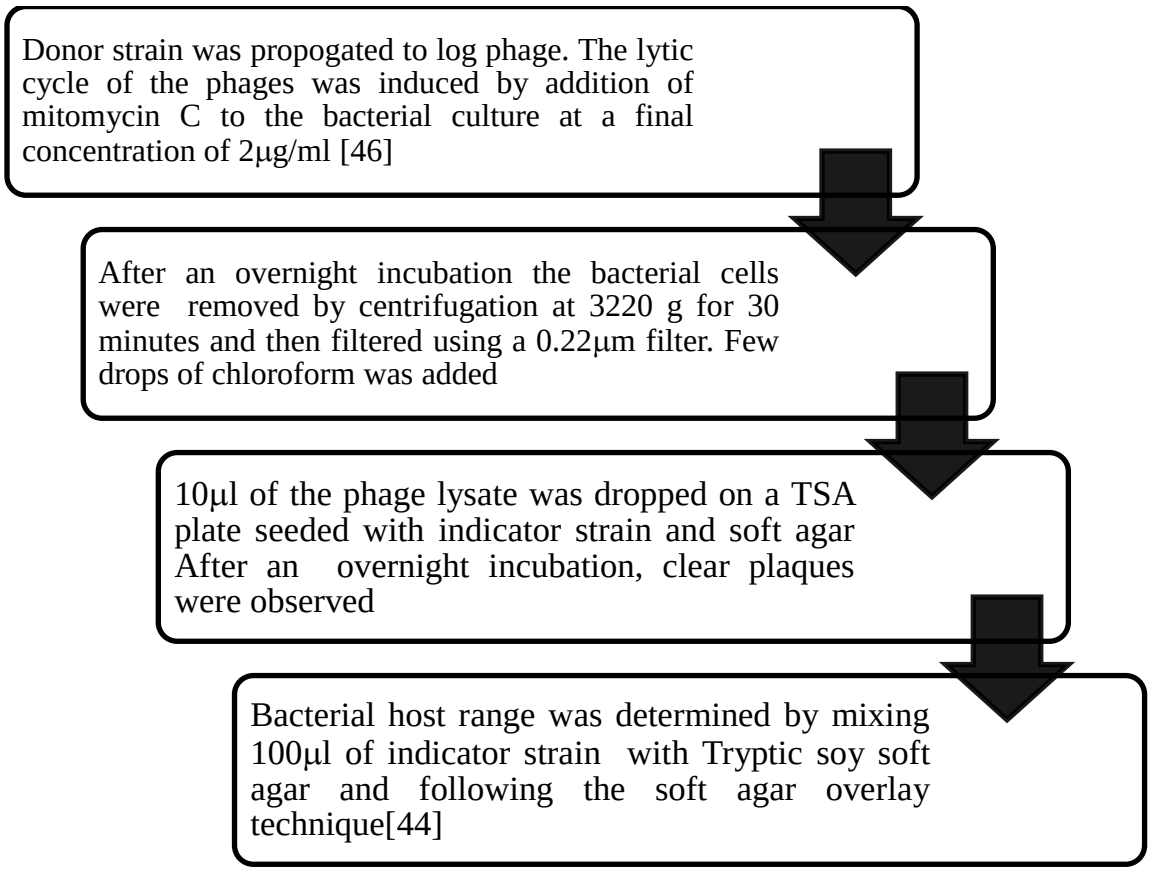

Figure 3: Phage Induction from *E. coli* of meat origin

Propagation of phages:

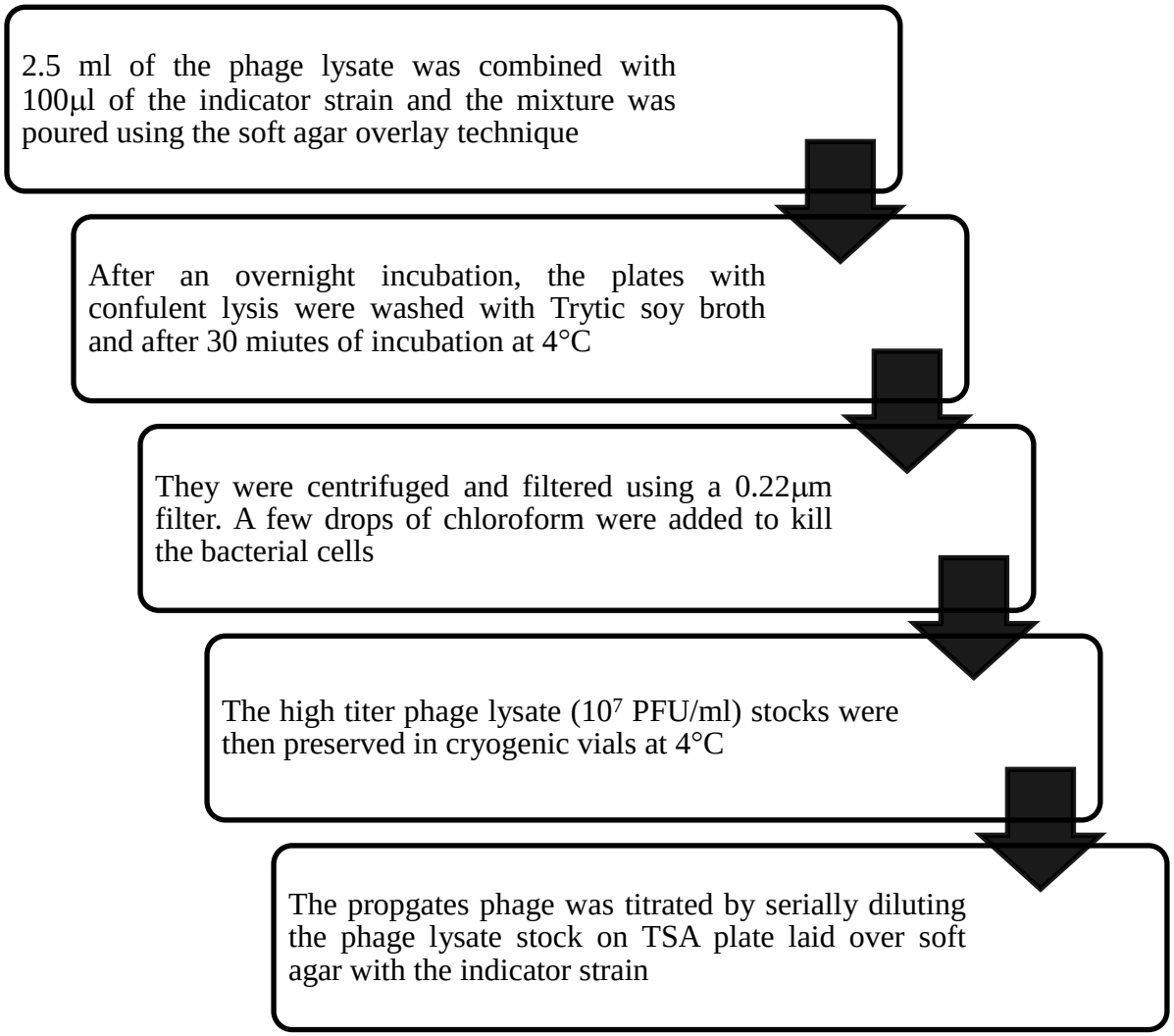

Figure 4: Propagation of Phages

Specific Aim 3:

Determination of antibiotic resistance genes

The presence of antibiotic resistance genes in the chromosomal, plasmid DNA of *E. coli* along with the phages induced from *E. coli* isolates and those isolated from environmental samples was determined by PCR (Polymerase chain reaction).

1. Determination of beta-lactamase genes in the chromosomal, plasmid DNA of *E. coli* isolates

The bacterial chromosomal DNA was isolated by the boiling an overnight culture of *E. coli* for 10 minutes and subsequent centrifugation to remove the cell debris. The supernatant was used as a template for the PCR reaction. The plasmid DNA from the *E. coli* were isolated using a High Pure Plasmid Isolation kit (Version 8.0, Roche). The plasmid DNA was then analyzed for the beta-lactamase genes mentioned above using PCR.

2. Determination of beta-lactamase genes in the bacteriophages induced from *E. coli*

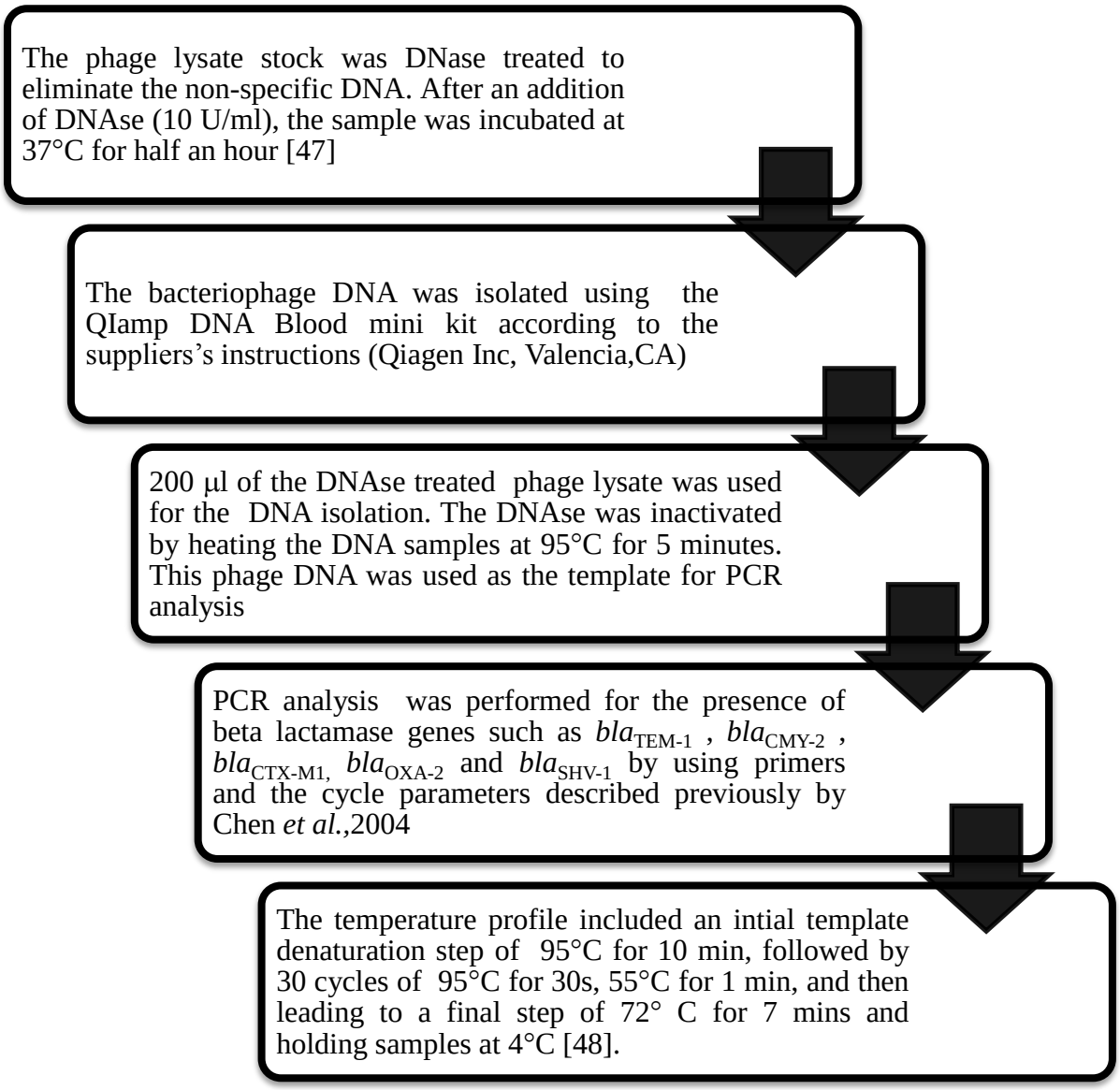

Figure 5: Determination of beta-lactamase genes in the bacteriophages induced from *E. coli*

Table 1: Primer sequences of the beta-lactam genes tested

Beta-lactam gene	**Oligonucleotide primer sequences**		**Size(bp)**
	Forward primer(5'-3')	**Reverse primer (3'-5')**	
bla$_{CMY-2}$	TGG CCG TTG CCG TTA TCT AC	CCC GTT TTA TGC ACC CAT GA	870
bla$_{SHV-1}$	GGC CGC GTA GGC ATG ATA GA	CCC GGC GAT TTG CTG ATT TC	714
bla$_{TEM-1}$	CAG CGG TAA GAT CCT TGA GA	ACT CCC CGT CGT GTA GAT AA	643
bla$_{CTX-M1}$	AAC GCT CAC GCT GTT GTT AG	TTG AGG CTG GGT GAA GTA AG	766
bla$_{OXA-2}$	CAA GCC AAA GGC ACG ATA GT	ACG ATT GCC TCC CTC TTG AA	644

3. Determination of beta-lactamase genes in the bacteriophages isolated from environmental samples

The isolated and purified phage lysate stocks were used to isolate the DNA and PCR was performed in the same way as the above using the parameters stated previously for the various antibiotic resistance genes such as $bla_{\text{CMY-2}}$, $bla_{\text{TEM-1}}$, $bla_{\text{CTX-M1}}$, $bla_{\text{SHV-1}}$ and $bla_{\text{OXA-2}}$.

CHAPTER 3

RESULTS

Specific Aim 1:

Isolation of bacteriophages from the environmental samples:

Waste water samples and soil samples were collected in Detroit and the surrounding regions. Out of the ten water samples collected, bacteriophages were isolated from five samples. From the 5 soil samples collected, bacteriophages were isolated from only one sample. The indicator strains ATCC 13706 and ATCC 23631 were found to be very potent indicator strains of the bacteriophages.

Table 2: Isolation of Bacteriophages specific to two *E. coli* indicator strains

Sample Source	**Indicator Strains**			
	ATCC 13706(A)	**Phage ID**	**ATCC 23631(B)**	**Phage ID**
Water samples				
Waste water treatment plant	+	PA	+	PB
Wastewater treatment plant	+	QA	+	QB
Detroit river	+	C9A	-	
Belle Isle (Detroit river)	+	RA	-	
Belle Isle Yach Club	+	UA	-	
Soil samples				
Soil from Urban Garden	+	SA	-	

Specific Aim 2:

Induction of bacteriophages from E. coli of retail meat origin

Inducible phages were recovered from 9 of the 30 *E. coli* isolates by spot lysis assay using the 2 indicator strains. Twenty five percent of the *E. coli* isolates released phages which could propagate in the indicator strains. Four of the thirteen *E. coli* isolates from Ground Turkey released bacteriophages. Four of the fourteen isolates from chicken breast and 1 out of 2 isolates from Pork chops were able to release bacteriophages. The bacterial host range profiles of the induced phages are given in the Table 4. Only the phages 5P, 8P, 9P, 11P were able to infect the two indicator strains ATCC 13706 and ATCC 23631. The other phages were able to propagate in only one of the two indicator strains. All the phages were very prone to multiply in the indicator strains ATCC 13706 except for one of them, indicating that ATCC 13706 is a very potent *E. coli* indicator strain which can host a wide range of bacteriophages.

Table 3: Phage Profiles from 30 *E. coli* Isolates

Strain ID	**Inducible Phage**	**Phage ID[a]**	**Source**
E1,E3,E7,E10,E12,E16,E17,E23,E26	-		Ground Turkey
E5	+	P5	Ground Turkey
E8	+	P8	Ground Turkey
E20	+	P20	Ground Turkey
E30	+	P30	Ground Turkey
E2,E4,E6,E13,E18,E19,E22,E24,E25,E27,E29	-		Chicken Breast
E11	+	P11	Chicken Breast
E14	+	P14	Chicken Breast
E15	+	P15	Chicken Breast
E21	+	P21	Chicken Breast
E9	+	P9	Pork Chop
E28	-		Pork Chop

[a] Phage was named by the prefix P followed by the ID of the *E. coli* isolate that released the phage

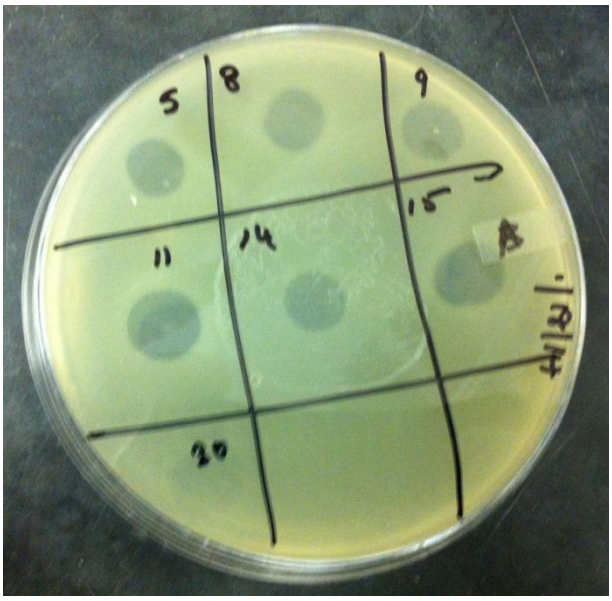

Figure 6: Plaques indicating successful bacteriophages induction from the *E. coli* isolate

Table 4: Induced *E. coli* phages propagated in two indicator strains

Phage ID	**Standard Indicator Strain**	
	ATCC 13706(A)	**ATCC 23631(B)**
5P	+	+
8P	+	+
9P	+	+
11P	+	+
14P	-	+
15P	+	-
20P	+	-
21P	+	-
30P	+	-

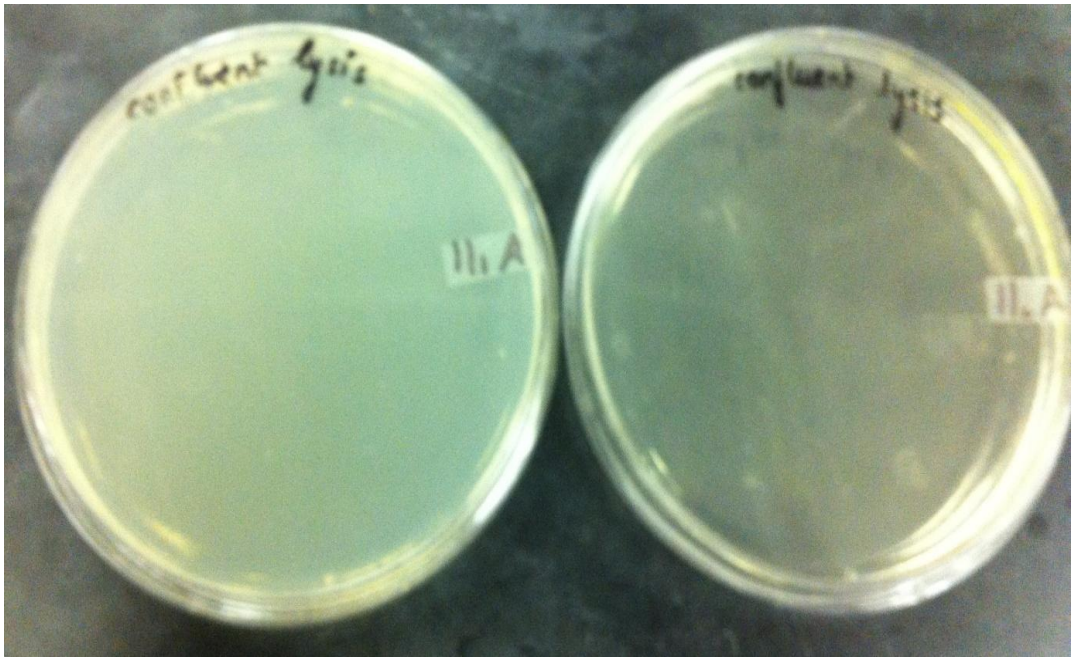

Figure 7: Plates showing complete lysis of bacteria by bacteriophages

Specific Aim 3:

Determination of Beta-lactamase gene

1. Determination of Beta-lactamase genes in the Chromosomal and Plasmid DNA

Ninety five percent of the *E. coli* isolates carried the antibiotic resistance genes such as $bla_{\text{TEM-1}}$, $bla_{\text{CMY-2}}$ and $bla_{\text{CTX-M1}}$ genes on their genomic DNA as well as plasmid DNA. Considering the genomic DNA, 25 of the 30 *E. coli* isolates carried $bla_{\text{CMY-2}}$ genes and 8 of the 30 *E. coli* isolates carried $bla_{\text{TEM-1}}$ genes. $bla_{\text{CTX-M1}}$ genes were carried on *E. coli*.

In the plasmid DNA, 95% of the 30 isolates exhibited the $bla_{\text{CMY-2}}$ genes at a higher frequency compared to the $bla_{\text{TEM-1}}$ genes which were present only in 10 of the *E. coli* isolates. Only 2 of the 30 isolates exhibited the $bla_{\text{CTX-M1}}$ gene.

Figure 8: Beta lactamase genes present in the chromosomal DNA

Figure 9: Beta-lactamase genes in the plasmid DNA

1. Determination of Beta-lactamase genes in the Induced Phage DNA and Phages isolated from Environmental samples

The antibiotic resistance genes were identified by PCR analysis. All the 13 induced phages carried the *bla*$_{\text{TEM-1}}$ gene and 20% of the induced phages carried the *bla*$_{\text{CMY-2}}$ gene indicating that there could be a successful gene transfer of the genes from the donor strain to the indicator strains through the bacteriophages as vectors. The other genes such as *bla*$_{\text{SHV-1}}$, *bla*$_{\text{OXA-2}}$ were not found on the phage genome. The *bla*$_{\text{TEM-1}}$ genes were carried on the phage DNA at a higher frequency as compared to the *bla*$_{\text{CMY-2}}$ gene. Ninety percent of *E. coli* from which the bacteriophages were induced were primarily isolated from Ground Turkey and Chicken Breast, while 10% of them were isolated from Pork chop. These E.coli isolates were found resistant to a wide range of antibiotics, with third generations cephalosporins and ampicillin having the highest MIC (Minimum inhibitory concentrations) [56].

The bacteriophages isolated from water samples were also analyzed by PCR for the detection of antibiotic resistance genes. Sixty percent of the isolated bacteriophages carried the *bla*$_{\text{CMY-2}}$ genes on their genome. Forty percent of the phages carried the *bla*$_{\text{TEM-1}}$ genes on them. Hence all the bacteriophages isolated from either the waste water treatment plants or the soil samples had either the *bla*$_{\text{TEM-1}}$ genes or the *bla*$_{\text{CMY-2}}$ genes on their genome, thereby indicating that phages can behave as very potent reservoirs of antibiotic resistance genes.

Table 5: Antibiotic resistance genes present in the genomic DNA of the donor strain, plasmid DNA of the donor strain and induced phage DNA

Donor strain	**β-lactamase genes isolated from different DNA templates**		
	Genomic DNA	**Plasmid DNA**	**Phage DNA**
E1	*bla*$_{\text{CMY-2}}$	*bla*$_{\text{CMY-2}}$	
E2	*bla*$_{\text{CMY-2}}$	*bla*$_{\text{CMY-2}}$	
E3	*bla*$_{\text{CMY-2}}$	*bla*$_{\text{CMY-2}}$	
E4	*bla*$_{\text{CMY-2,}}$ *bla*$_{\text{TEM-1}}$	*bla*$_{\text{CMY-2}}$	
E5	*bla*$_{\text{CMY-2,}}$ *bla*$_{\text{TEM-1}}$	*bla*$_{\text{CMY-2,}}$ *bla*$_{\text{TEM-1}}$	*bla*$_{\text{TEM-1}}$
E6	*bla*$_{\text{CMY-2}}$	*bla*$_{\text{CMY-2}}$	
E7	*None*		
E8	*bla*$_{\text{CMY-2}}$	*bla*$_{\text{CMY-2}}$	*bla*$_{\text{CMY-2}}$, *bla*$_{\text{TEM-1}}$
E9	*bla*$_{\text{CMY-2}}$	*bla*$_{\text{CMY-2}}$	*bla*$_{\text{CMY-2}}$, *bla*$_{\text{TEM-1}}$
E10	*bla*$_{\text{CMY-2}}$, *bla*$_{\text{TEM-1}}$	*bla*$_{\text{CMY-2}}$, *bla*$_{\text{TEM-1}}$	
E11	*bla*$_{\text{CMY-2}}$	*bla*$_{\text{CMY-2}}$	*bla*$_{\text{TEM-1}}$
E12	*bla*$_{\text{CMY-2}}$		
E13	*bla*$_{\text{CMY-2}}$	*bla*$_{\text{CMY-2}}$, *bla*$_{\text{TEM-1}}$	
E14			*bla*$_{\text{TEM-1}}$
E15	*bla*$_{\text{CMY-2,}}$ *bla*$_{\text{TEM-1}}$	*bla*$_{\text{CMY-2}}$	*bla*$_{\text{TEM-1}}$
E16	*bla*$_{\text{CMY-2}}$	*bla*$_{\text{CMY-2}}$, *bla*$_{\text{TEM-1}}$	
E17	*None*		
E18	*bla*$_{\text{CMY-2}}$, *bla*$_{\text{CTX-M1}}$		
E19	*bla*$_{\text{CMY-2}}$	*bla*$_{\text{CMY-2}}$	
E20	*bla*$_{\text{CMY-2}}$, *bla*$_{\text{TEM-1}}$	*bla*$_{\text{CMY-2}}$, *bla*$_{\text{TEM-1}}$	*bla*$_{\text{TEM-1,}}$ *bla*$_{\text{CTX-M1}}$

E21	*bla*$_{\text{CTX-M1}}$	*bla*$_{\text{CTX-M1}}$	*bla*$_{\text{TEM-1}}$
E22	*bla*$_{\text{CTX-M1}}$		
E23	*bla*$_{\text{CMY-2}}$	*bla*$_{\text{CMY-2}}$, *bla*$_{\text{TEM-1}}$	
E24	*None*		
E25	*bla*$_{\text{CMY-2}}$	*bla*$_{\text{CMY-2}}$	
E26	*bla*$_{\text{CMY-2}}$, *bla*$_{\text{TEM-1}}$	*bla*$_{\text{CMY-2}}$, *bla*$_{\text{TEM-1}}$	
E27	*bla*$_{\text{CMY-2}}$	*bla*$_{\text{CMY-2}}$	
E28	*bla*$_{\text{CMY-2}}$, *bla*$_{\text{TEM-1}}$	*bla*$_{\text{CMY-2}}$, *bla*$_{\text{TEM-1}}$	
E29	*bla*$_{\text{CTX-M1}}$	*bla*$_{\text{CTX-M1}}$	
E30	*bla*$_{\text{CMY-2}}$, *bla*$_{\text{TEM-1}}$	*bla*$_{\text{CMY-2}}$, *bla*$_{\text{TEM-1}}$	*bla*$_{\text{TEM-1}}$

Table 6: Antibiotic resistance genes present in the phage DNA of the environmental samples

Phage ID	**Beta-lactamase genes**
PA	*bla*$_{\text{TEM-1}}$
PB	*bla*$_{\text{CMY-2}}$, *bla*$_{\text{TEM-1}}$
QA	*bla*$_{\text{TEM-1}}$
QB	*bla*$_{\text{CMY-2}}$
RA	*bla*$_{\text{CMY-2}}$
UA	*bla*$_{\text{CMY-2}}$
SA	*bla*$_{\text{CMY-2}}$
C9A	*bla*$_{\text{CMY-2}}$

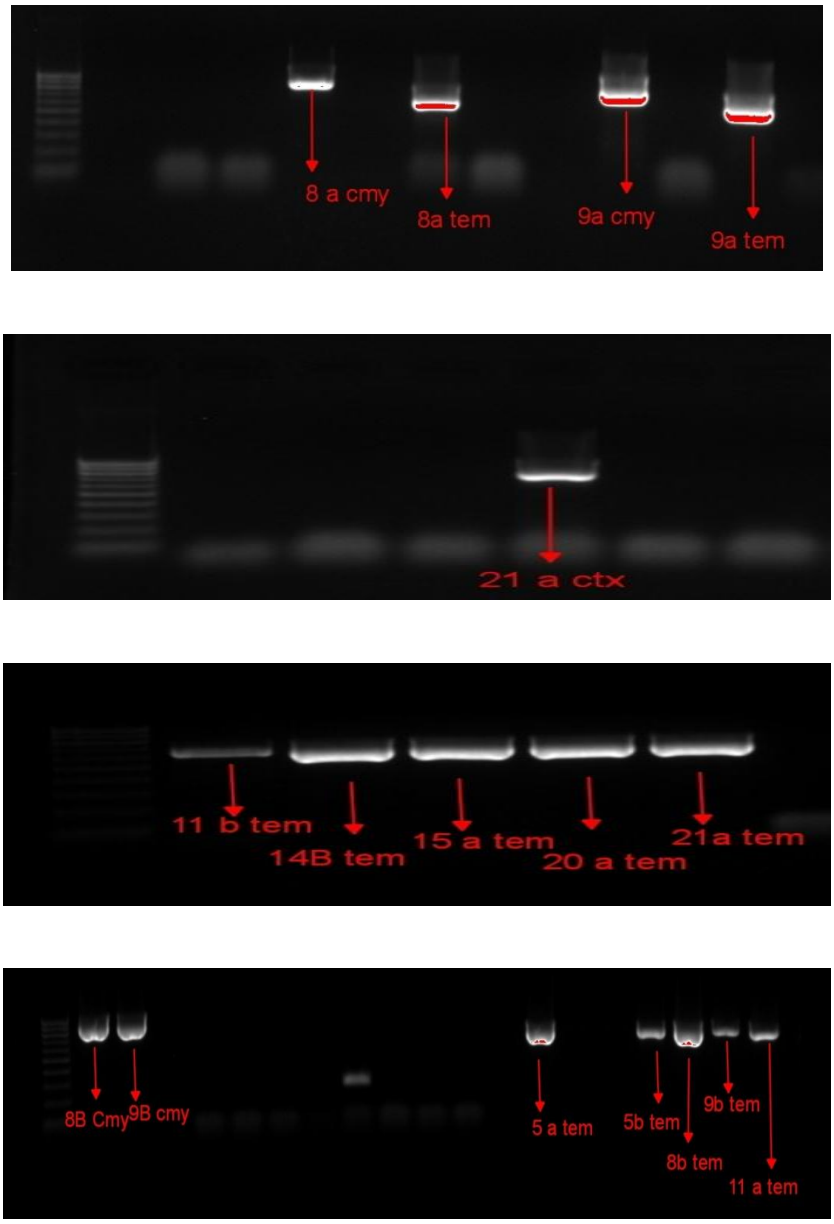

Figure 10: Beta-lactamase genes in Phage DNA

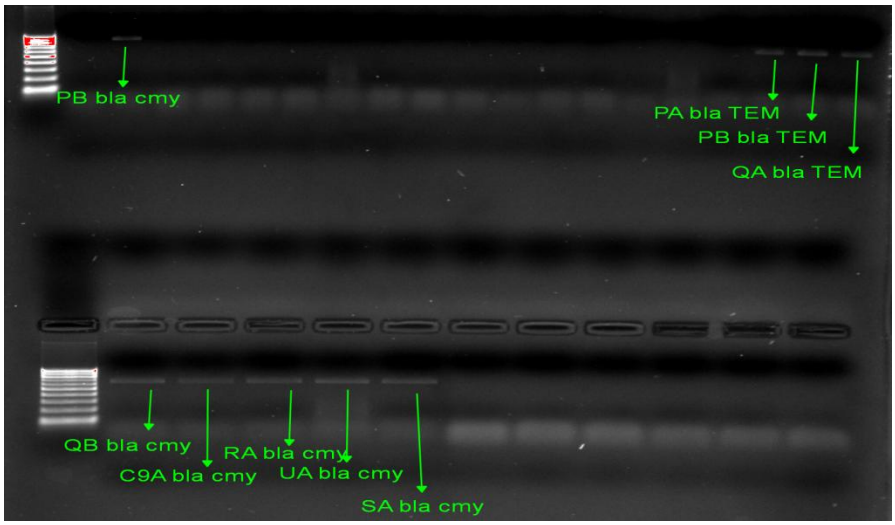

Figure 11: Beta-lactamase genes present in the phages isolated from environmental samples

CHAPTER-4

DISCUSSION

The role of bacteriophages as reservoirs of antibiotic resistance is not well studied. Recent study by Marta *et al.*,[42] establishes that bacteriophages from water sources can act as a potent reservoir for antibiotic resistance genes. Several authors have indicated the possibility of environmental origin of these genes. The genes examined in our study are the most widely spread in our environment. The $bla_{\text{TEM-1}}$ gene is the most prevalent antibiotic resistance gene in the environment [49]. In our study, bacteriophages were isolated from the environmental samples such as waste water, soil samples and investigated for the carriage of antibiotic resistance genes. Phages from both water and soil samples carried the $bla_{\text{TEM-1}}$ genes, with the majority of them carrying the $bla_{\text{CMY-2}}$ genes. The reason behind this can be attributed to the increased usage of antibiotics in human and veterinary medicine. Several studies relate the antibiotic resistance and waste water [50], [51]. There are several characteristics of waste water that make it highly susceptible for the presence of these antibiotic resistance genes such as the presence of many antibacterial substances from the antibacterial detergents and soaps used in household products, or the antibiotics from human waste, or even high bacterial load down the drain. Eighty to ninety percent of the ingested antibiotics used in human and animal medicine are not broken down, but they pass through the body and are dispersed in the environments as waste. The presence of antibiotic resistance genes in the bacteriophage fraction of environmental samples provides an understanding to which extent the ecosystem proves to be a pool for these antibiotic resistance genes [52]. The antibiotic resistance is picked up by the pathogenic bacteria and transferred to the clinical settings through the Horizontal gene transfer, which is the most crucial way of dispersing antibiotic resistance genes, thereby posing a serious threat in treating

these antibiotic resistant strains [53]. However, our experiments do not prove that fact that these genes confer resistance in a given bacterial host.

This study was conducted to investigate the presence of antibiotic resistance genes in the temperate phages in *E. coli* isolates from animal foods. As studied earlier, bacteriophages are very common entities and highly specific to the bacterial hosts they infect but they reproduce very rapidly when the ecosystem permits [54]. An overall 64% (19 of the 30) *E. coli* isolates released phages on a plaque assay. This showed a high percentage of inducible phages on the chromosome of *E. coli* isolates true to the fact that two-thirds of all gamma-proteobacteria harbour prophages [55]. Since this study shows that a very high percentage (%) of antibiotic resistance genes are carried on bacteriophages, the bacteriophages may act as a potential reservoir of the antibiotic resistance genes such as $bla_{\text{CMY-2}}$, $bla_{\text{TEM-1}}$ and $bla_{\text{CTX-M1}}$.

$bla_{\text{TEM-1}}$ is one of the most clinically significant resistance gene among the family of beta-lactamases. It is very active against the third generation broad spectrum cephalosporins. These beta-lactamses pose a serious health threat in the clinical environment. The genomic DNA and the plasmid DNA of the *E. coli* isolates was screened for the antibiotic resistance gene pattern. It was seen that 90% of the *E. coli* isolates carried $bla_{\text{CMY-2}}$ genes on their genomic DNA, whereas only 60% of them carried $bla_{\text{TEM-1}}$ genes. The same pattern was observed in the plasmid DNA of the E.coli isolates. However, on the contrary, there was a very high prevalence of the occurrence of $bla_{\text{TEM-1}}$ genes on the phage DNA. The E. coli isolates were resistant to the third generation cephalosporins such as ceftriaxone, cefoxitin, ceftiofur at a high MIC level of 32μg/ml, 8μg/ml, 16μg/ml respectively [56]. The high prevalence of TEM enzymes in the phage DNA goes along with the previous statement that they degrade the third generation cephalosporins and carry resistance thereby serving to be a potential reservoir of resistance genes. The *E. coli* isolates also

show resistance to ampicillin. As mentioned previously, these antibiotics are susceptible to degradation by CMY beta-lactamases. Twenty two percent of the phages carry *bla*$_{\text{CMY-2}}$ genes which shows that these phages can act as a vehicle of the antibiotic resistance genes which eventually leads to the emergence of antibiotic resistant pathogens.

bla$_{\text{CMY-2}}$ were observed on genomic and plasmid DNA as well as induced phage DNA from *E. coli*, suggesting both plasmid and phage can carry this gene, although the possibility of trace genomic DNA contamination also exists. Phage transduction can be designed to confirm the role of phage in transferring *bla*$_{\text{CMY-2}}$. All the induced phages carried *bla*$_{\text{TEM-1}}$ genes. It was observed that 30% of the phage DNA, carried the bla$_{\text{TEM-1}}$ genes which were not carried on the genomic or plasmid DNA of the *E. coli*. This may be attributed to the fact that the *bla*$_{TEM-1}$ gene present only on the phage DNA could have possibly been transferred from other bacteria in the environment. This explains the fact that the bacteriophages can receive genes from the environment and due to the abundance of antibiotic resistant strains of bacterial pathogens in the surroundings, there are high chances of phages being a reservoir of these genes.

The structural characteristics of phages make them better competitiors in surviving the action of nucleases, temperature, radiation in comparison to the free DNA (either as linear fragments or plasmid). Thus, due to the different structural characteristics, their higher chance of survival in the extra intestinal environment makes them potent vectors for gene transfer than plasmids or transposons [42].

In the human gastrointestinal tract the bacteria population is very high. The colon is estimated to contain about 10^{11} bacteria/ml [8]. With such a high microbial count, it is highly possible to have great number of bacteriophages and highly likely that these phages host many

antibiotic resistant genes. Thereby these genes could be transferred to species of different biomes.

Although the number of phages carrying these genes could not be determined in this study, a rough estimation that these bacteriophages carried the genes could be established through PCR. Purifying these phages, obtaining a single phage and screening those for the presence of antibiotic resistant genes would confirm the presence of these genes in these phages. Further, screening of the transductants for the presence of these beta-lactamase genes would affirm that these bacteriophages could act as a potent reservoir of antibiotic gene.

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ABSTRACT

BACTERIOPHAGE ISOLATED FROM THE ENVIRONMENT AND *E. COLI* OF MEAT ORIGIN AS A RESERVOIR OF ANTIBIOTIC RESISTANCE

by

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Major: Nutrition and Food Science

Degree: Master of Science

This study was aimed to determine the extent of antibiotic resistance reservoir in bacteriophage. Bacteriophages were isolated and purified from waste water treatment plants, Detroit river, Belle Isle, Urban Garden soils and examined for the presence of beta-lactam antibiotic resistance genes. Thirty *E. coli* isolates from retail meat were analyzed for the presence of beta-lactam antibiotic resistance genes. 95% of *E. coli* them carried at least one antibiotic resistance gene on their genome. Bacteriophages were induced from the *E. coli* isolates by subjecting them to mitomycin C, thereby creating a stressful environment resulting in the release of prophages. Phages were induced from 40% of *E. coli* and isolated from 70% of the environmental samples by spot lysis assay by using two *E. coli* indicator strains. Phage DNA was analyzed for several beta-lactamase genes such as $bla_{\text{TEM-1}}$, $bla_{\text{CMY-2}}$, $bla_{\text{CTX-M1}}$, $bla_{\text{OXA-2}}$ and $bla_{\text{SHV-1}}$. Among the bacteriophages induced from the *E. coli* isolates, all of them carried the $bla_{\text{TEM-1}}$ genes on their DNA and 20% of them carried the $bla_{\text{CMY-2}}$ genes. Forty percent of phages isolated from environmental samples carried $bla_{\text{TEM-1}}$ and 60% of them carried $bla_{\text{CMY-2}}$.

This study suggests that phage from the environment and bacteria can be an important reservoir of antibiotic resistance.

AUTOBIOGRAPHICAL STATEMENT

In Dr. Zhang's lab I have been able to understand the molecular concepts of the microbes involved in Food. This study has really made an impact in me by creating an awareness of how important is it to eliminate the risks associated with microbial contamination of foods. I strongly believe that skills and knowledge that I gained through my Master of Science at Wayne State University would help me achieve my career goals.